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Chemical structure and anticoagulant activity of highly pyruvylated sulfated galactans from tropical green seaweeds of the order Bryopsidales

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ABSTRACT

Sulfated and pyruvylated galactans were isolated from three tropical species of the Bryopsidales, *Penicillus capitatus*, *Udotea flabellum*, and *Halimeda opuntia.* They represent the only important sulfated polysaccharides present in the cell walls of these highly calcified seaweeds of the suborder Halimedineae. Their structural features were studied by chemical analyses and NMR spectroscopy. Their backbone comprises 3-, 6-, and 3,6-linkages, constituted by major amounts of 3-linked 4,6-*O*-(1′-carboxy)ethylidene-D-galactopyranose units in part sulfated on C-2. Sulfation on C-2 was not found in galactans from other seaweeds of this order. In addition, a complex sulfation pattern, comprising also 4-, 6-, and 4,6-disulfated galactose units was found. A fraction from *P. capitatus*, F1, showed a moderate anticoagulant activity, evaluated by general coagulation tests and also kinetics of fibrin formation was assayed. Besides, preliminary results suggest that one of the possible mechanisms involved is direct thrombin inhibition.

1. Introduction

There is still scarce information about structures of sulfated polysaccharides biosynthesized by green seaweeds belonging to the Bryopsidales (Chlorophyta), however, it is known that in most of them galactans predominate (Bilan, Vinogradova, Shashkov, & Usov, 2007; Chattopadhyay, Adhikari, Lerouge, & Ray, 2007; Ciancia et al., 2012).

Only the sulfated polysaccharides from some species of the genus *Codium* (Bilan et al., 2007; Ciancia et al., 2007; Estevez, Fernández, Kasulin, Dupree, & Ciancia, 2009; Farias et al., 2008; Fernández, Ciancia, Miravalles, & Estevez, 2010; Fernández, Estevez, Cerezo, & Ciancia, 2012; Fernández et al., 2013; Love & Percival, 1964; Ohta, Lee, Hayashi, & Hayashi, 2009; Fernández, Arata & Ciancia, 2014) have been studied in detail. They biosynthesize sulfated galactans constituted by 3-linked β-D-galactopyranose residues partially sulfated on C-4 and/or C-6, with ramifications on C-6 and important amounts of pyruvate forming mainly five-membered cyclic ketals (*S* configuration) with *O*-3 and *O*-4 of non-reducing terminal β-D-galactose residues. A minor part of pyruvate forms six-membered cyclic acetals with *O*-4 and *O*-6 (*R* configuration). On the other hand, the major sulfated polysaccharides from *Bryopsis plumosa* are linear 3-linked β-D-galactans highly pyruvylated and also partially sulfated mainly on C-6 of some

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of the galactose units. In this galactan, pyruvic acid was forming a ketal linked to *O*-4 and *O*-6 (*R* isomer) of some 3-linked units (Ciancia et al., 2012).

The order Bryopsidales has been divided into two suborders (Lam & Zechman, 2006). Codium and Bryopsis, belong to the suborder Bryopsidineae, whereas, the species studied in this paper, *Penicillus capitatus*, *Udotea flabellum*, and *Halimeda opuntia*, belong to the suborder Halimedineae which comprises partially calcified species, present in tropical and subtropical habitats. From this suborder, only some structural features of heteroglycan sulfates from *Caulerpa* species were previously reported (Chattopadhyay et al., 2007; Mackie & Percival, 1961). That from *Caulerpa racemosa* was described as a branched polymer containing, 3-linked galactose, terminal- and 4-linked xylose, and 4- and 3,4-linked arabinose residues. Sulfate groups, when present, were linked to C-3 of 4-linked arabinose and C-6 of 3-linked galactose units. No pyruvic acid was informed (Chattopadhyay et al., 2007). Polysaccharides from species of this genus were found to have different biological activities, however, in most of the reports, only minor characterization of the active compounds was carried out (Ghosh et al., 2004; Gurgel Rodrigues, de Sousa Oliveira Vanderlei, et al., 2011; Gurgel Rodrigues, de Queiroz, et al., 2011; Ji, Shao, Zhang, Hong, & Xiong, 2008; Santos Pereira Costa et al., 2012).

Antithrombotic agents have been extensively used as a systemic therapy in cardiovascular or tromboembolic diseases and heparin is the initial choice, nevertheless it can induce several side effects, such as development of thrombocytopenia, arterial embolism (Kelton & Warkentin, 2008), bleeding complications (Anand, Yusuf, Pogue, Ginsberg, & Hirsh, 2003; Kelton & Hirsh, 1980), and so on. Furthermore, the incidence of prion-related diseases in mammals and the increasing requirements of anticoagulant treatments indicate the need to look for alternative sources of anticoagulant and antithrombotic compounds.

Many different sulfated polysaccharides have been thoroughly studied proving to have anticoagulant effects (Ciancia, Quintana, & Cerezo, 2010). The important differences in their mechanisms of action could be attributed to the diversity of structures and to the fact that one compound may have more than one target protease. These differences denote the importance of the knowledge of specific structural characteristics of these products and their interaction with the different proteins involved in coagulation cascade, contributing for the development of new antithrombotic agents. Some sulfated polysaccharides even though bearing significant levels of sulfation, have scarce effects toward the coagulation system; while others, even carrying lower sulfation content, can show surprising levels of anticoagulant activity (Mourão, 2004; Pomin, 2009). This observation has clearly proved the concept that sulfation, and therefore electronegative-charge densities, in marine carbohydrates are not the solely structural determinants for the anticoagulant activities of these molecules.

In this work we isolated and characterized highly pyruvylated galactan sulfates from three tropical species of the Halimedineae, *P. capitatus*, *U. flabellum*, and *H. opuntia*. In particular, a detailed structural study was carried out on the water soluble polysaccharides from *P. capitatus* and their anticoagulant activity was investigated.

2. Experimental

2.1. Algal sample

Specimens of the green macroalgae studied here were collected in the coast of Venezuela and identified by Dr. Santiago Gómez from Instituto de Biologia Experimental, Universidad Central de Venezuela and Dr. Mayra Garcia from Fundación Instituto Botánico de Venezuela, Universidad Central de Venezuela: according to Taylor (1960), *P. capitatus*, in Chichiriviche, state of Falcón (10°24′24″ N, 68°15′1″ O) in June 2007; *H. opuntia* in Tucacas, state of Falcón (10°51′26″ N, 68°18′42″ O) in May 2006; *U. flabellum* in La Ciénaga, Ocumare de la Costa, state of Aragua (10°28′21″ N, 67°48′39″ O) in July 2008. The samples used in this work were in the vegetative state. Thalli of the seaweeds were washed with filtered seawater and analyzed for epiphytic and epizoic contaminants in a Nikon AFX-II macroscope (Nikon, Japan). Voucher specimens were deposited in the National Herbarium of Venezuela (Collection Code 200706003, 200606024 and VEN 405855, respectively). Each algal sample, previously milled, was hydrolyzed in conditions suitable for fibrillar polysaccharides, a first step was carried out in 100% TFA for 1 h at 37 °C, followed by dilution of the acid to 80%, heating at 100 °C for 1 h, and further dilution to 2 M to achieve the regular hydrolysis conditions for soluble polysaccharides (Morrison, 1988); the sugar mixture was derivatized to the corresponding alditol acetates (see below).

2.2. Extraction of the polysaccharides

Sterile plants of *P. capitatus* were freeze dried. The dry material was first extracted with methanol at room temperature and the residue from the alcohol extraction was sequentially and exhaustively extracted with water (20 g/L) at room temperature and hot-water. Briefly, the residue of the first room temperature extraction was removed by centrifugation and the supernatant was concentrated, dialyzed and freeze-dried. The residue from the first water extraction was extracted one more time in similar conditions. The residue from the second room temperature water extraction was extracted twice for 3 h with water at 90 °C, giving two hot water extracts, which gave similar characteristic by chemical analysis (see later) so they were studied together as one sample, which was treated with α-amylase (Knutsen & Grasdalen, 1987). Thalli from *U. flabellum* were extracted in a similar way, but only once at each temperature. On the other hand, the yield from the room temperature water extract from *H. opuntia* was very low, so extraction was carried out in controlled acid conditions as described by Cases, Stortz, & Cerezo, 1992, for red seaweed *Corallina officinalis*.

2.3. Ion exchange chromatography (IEC)

PA1 was chromatographed on DEAE-Sephadex A-25. The sample (100 mg) was dissolved in water, centrifuged and the supernatant was applied to a column (90 × 1.5 cm id), previously stabilized in H_2O. The first elution solvent was water and then NaCl solutions of increasing concentration up to 4 M. Fractions of 4 mL were collected. Finally, the phase was boiled in 4 M NaCl solution. The presence of carbohydrates in the samples was detected by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956); after obtaining blank readings, the eluant was replaced by another with higher concentration of NaCl. Seven fractions were obtained, dialyzed (molecular weight cut off 3500) and freeze dried (F1–F7).

2.4. Chemical analyses

The total sugars content was analyzed by the phenol–sulfuric acid method (Dubois et al., 1956), in some cases, modification adapted for insoluble material (Ahmed & Labavitch, 1977) was used. Sulfate was determined turbidimetrically (Dodgson & Price, 1962), while the percentage of pyruvic acid was determined according to Koepsell and Sharpe (1952). The protein content was determined by the method of Lowry, Rosenbrough, and Farr, (1951). To determine the monosaccharide composition, samples were derivatized to the alditol acetates (Stevenson & Furneaux, 1991).

2.5. Desulfation of F1 and F6

The reaction was carried out by the microwave-assisted method described by Navarro, Flores, and Stortz (2007). The sample (40 mg) was converted to the pyridinium salt and dissolved in 10 mL of DMSO containing 2% of pyridine. The mixture was heated for 10 s intervals and cooled to 50 °C (×6). It was dialyzed 3 days against tap water and then 24 h against distilled water (MWCO 3500) and lyofilized. An aliquot was methylated as described below without previous isolation of the product.

2.6. Removal of pyruvic acid residues from F1

The reaction was carried out according to Bilan et al. (2007). The sample (50 mg) was heated in 1% CH_3COOH (10 mL) for 4 h at 100 °C, the solution was neutralized with $NaHCO_3$, dialyzed, and lyophilized to give depyruvylated product (21.5 mg). An aliquot of desulfated F1 was treated in the same way to give F1desulf–depyr.

2.7. Methylation analysis

The polysaccharide (10–20 mg) was converted into the corresponding triethylammonium salt (Stevenson & Furneaux, 1991) and methylated according to Ciucanu and Kerek (1984). The sample was dissolved in dimethylsulfoxide; finely powdered NaOH was used as base. For F1, different times between addition of the base and of CH_3I were assayed and also two sequential methylation steps were carried out. However, no substantial changes in the pattern of partially methylated derivatives obtained after hydrolysis of the polysaccharide were observed. The methylated samples were submitted to reductive hydrolysis and acetylation to give the alditol acetates in the same way as the parent polysaccharides (Stevenson & Furneaux, 1991). In some cases, methylated samples were also hydrolyzed with 2 M TFA for 2 h at 120 °C and the partially methylated sugars were converted into the corresponding aldononitrile acetates (Stortz, Matulewicz, & Cerezo, 1982).

2.8. Gas chromatography

GC of the alditol acetates were carried out on a Agilent 7890A gas–liquid chromatograph (Avondale, PA, USA) equipped with a flame ionization detector and fitted with a fused silica column (0.25 mm i.d. × 30 m) WCOT-coated with a 0.20 μm film of SP-2330 (Supelco, Bellefonte, PA, USA). Chromatography was performed: from 200 °C to 230 °C at 1 °C min^{-1}, followed by a 30-min hold for alditol acetates. For the partially methylated alditol acetates, the initial temperature was 160 °C, which was increased at 1 °C min^{-1} to 210 °C and then at 2 °C min^{-1} to 230 °C. N_2 was used as the carrier gas at a flow rate of 1 mL min^{-1} and the split ratio was 80:1. The injector and detector temperature was 240 °C.

2.9. GC–MS

GC–MS of the methylated alditol acetates was performed on a Shimadzu GC-17A gas–liquid chromatograph equipped the SP-2330 interfaced to a GCMSQP 5050A mass spectrometer (Kyoto, Japan) working at 70 eV. He total flow rate was 7 mL min^{-1}, the injector temperature was 240 °C. Mass spectra were recorded over a mass range of 30–500 amu.

2.10. NMR spectroscopy

500 MHz ^1H NMR, proton decoupled 125 MHz ^{13}C NMR spectra, and two-dimensional NMR experiments (HMQC and COSY) were recorded on a Bruker AM500 at room temperature, with external reference of TMS. The samples (20 mg) were exchanged in 99.9% D_2O (0.5 mL) four times. Chemical shifts were referenced to internal acetone (δ_H 2.175, δ_{CH3} 31.1). Parameters for ^{13}C NMR spectra were as follows: pulse angle 51.4°, acquisition time 0.56 s, relaxation delay 0.6 s, spectral width 29.4 kHz, and scans 25,000. For ^1H NMR spectra: pulse angle 76°, acquisition time 3 s, relaxation delay 3 s, spectral width 6250 Hz and scans 32. 2D spectra were obtained using standard Bruker software.

2.11. Treatment with pronase E

F3 (10 mg) was dissolved in a phosphate buffer 0.2 M at pH 7.2 (1 mL) and 3 mg of protease Type XIV from *Streptomyces griseus* (Sigma P 5147) were added. The mixture was agitated for 24 h at 37 °C and then dialyzed (MWCO 6–8 kDa) against distilled H_2O (1 L) and freeze dried.

2.12. General coagulation assays

The tests were performed with a coagulometer ST4 (Diagnostica Stago, Asnieres sur Seine, France). Determinations of prothrombin time (PT), activated partial thromboplastin time (APTT), and thrombin time (TT) were assayed according to established methods (Laffan & Bradshaw, 1995). Reagents were supplied by Diagnostica Stago, except tromboplastin reagent. Since polybrene, a known neutralizer of heparin, is added to the majority of the PT commercial reagents tromboplastin was extracted from rabbit brain according to the method described by Quick (1935). Normal platelet depleted citrated plasma (900 μL) was mixed with 100 μL of each polysaccharide samples, in different concentrations (*test solution*), and incubated for 1 min at 37 °C. Heparin (Sigma, St. Louis, MO, USA) and dermatan sulfate (Syntex, Buenos Aires, Argentina) were used for the comparison of anticoagulant activity of the fractions. Saline solution (0.9% NaCl) was used as control. TT-like assays were also performed with purified fibrinogen (Sigma, St. Louis, MO, USA) (3 mg/mL) instead of human plasma. All clotting assays were performed in quadruplicate. Results were expressed as ratios between clotting time of a solution of the anticoagulant and clotting time of the control.

2.13. Fibrin formation

In order to perform fibrin formation studies, clots were generated by addition of thrombin (Wiener, Rosario, Argentina) (0.5 IU/mL) to the preincubated plasma (*test solution*). During the coagulation process, optical density (OD) was recorded at 405 nm with 1 min intervals up to constant values (ELx808, BioTeck Instruments Inc., Winooski, VT, USA) (Weisel, Veklich, & Gorkun, 1993). Assays were carried out in polystyrene strips, in the presence of different concentrations (5–50 μg/mL) of F1, heparin, dermatan sulfate or saline solution as control. The curves obtained (OD versus time) were characterized by three parameters: the lag phase, that reflects the time required for initial protofibril formation; the slope, that corresponds to the maximum velocity achieved (V_{Max}) and the final network OD (OD_{Max}) at the plateau phase, which is influenced by the number of protofibrils per fiber. All assays were performed in quadruplicate.

2.14. Statistical analysis

Data were analyzed using the statistical software Statistix 8 (Analytical Software, Tallahassee, FL, USA). Results were expressed as mean ± standard deviation (SD). F1-treated and control samples were compared using Student's *t* test, and p values < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Extraction and characterization of the sulfated polysaccharides

Monosaccharide composition of milled seaweed material for each species, in condition suitable to hydrolyze the fibrillar components (Morrison, 1988), showed xylose as major sugar (50–55%), in agreement with previous findings that indicated that 3-linked β-D-xylans replace cellulose in seaweeds of these genera (Percival & McDowell, 1981). The other important monosaccharides were glucose and galactose.

Yields in water soluble polysaccharides were extremely low (Table 1). Low yields were also obtained for water extracts of other Bryopsidales (Ciancia et al., 2007, 2012), but in these cases, the even lower yields are due at least in part to the important amount of calcium carbonate deposited (aragonite). For *H. opuntia* a maximum degree of 90% calcification was previously reported (Böhm, 1973). Consequently, extraction of soluble polysaccharides in this case was carried out at controlled acid pH (Cases et al., 1992), and, in these conditions yield of the room temperature water extract increased from 0.04% to 0.22%, still very low. Besides, for *P. capitatus* and *U. flabellum* a maximum degree of calcification (56% and 38%, respectively), not so high, but still important, was informed (Böhm, 1973).

P. capitatus was extracted with water sequentially twice at room temperature and at 90 °C (Table 1). In the first extract (PA1), galactose was the major monosaccharide component, although small amounts of other sugars were also present, mainly glucose which could arise from contaminant polymers, as reserve α-glucans. The percentage of uronic acids was negligible. On the other hand, proteins were present in important amounts (20.7%). The ratio galactose:sulfate:pyruvic acid was 1.00:0.62:0.56 for this extract. The second extraction at room temperature gave a product (PA2) with only 50% galactose, but 31% of glucose. Moreover, extraction with hot water gave two extracts (PC1 and PC2) constituted mostly by glucose (Table 1), which were further analyzed together as one fraction. NMR spectra of this fraction, confirmed a low molecular weight 4-linked α-glucan structure, as signals at δ 100.3/5.34, 96.8/4.59, and 93.0/5.16, corresponding to 4-linked and terminal β- and α-reducing units were present in the anomeric region, and peaks at δ 72.4/3.54, 73.63.68, 77.6/3.59, 70.9/3.90, and 61.5/3.88,3.76, which were assigned to C-2/H-2–C-6/H-6,6′ of the 4-linked units (McIntyre & Vogel, 1993; Synytsya & Novak, 2013). Moreover, treatment of this sample with α-amylase gave PCa with only 36% of glucose. Thus, these extracts were not investigated further.

The room temperature water extract from *U. flabellum* (UA) is mostly constituted by galactose, important amounts of sulfate and pyruvic acid were also present in a ratio galactose:sulfate:pyruvic acid of 1.00:0.51:0.56, just a small amount of protein (4.5%) was found in this extract. The residue was then extracted with hot water, obtaining UC, which has important amounts of galactose, but also glucose, so only UA was selected for further analyses. Besides, the extract obtained from *H. opuntia* by extraction at room temperature at low and controlled pH (HA) contained also galactose as major monosaccharide component (77.2%) and a ratio galactose:sulfate:pyruvic acid of 1.00:0.78:0.26. Hot water extraction of the residue gave only trace amounts of polysaccharides, which were not analyzed.

3.2. Structural studies of sulfated galactans

PA1 from *P. capitatus* was fractionated by anion exchange chromatography on Sephadex A-25. The sample was eluted with water and then with increasing NaCl concentrations and seven fractions were isolated (Table 2). The major fraction, F1, eluted with water, in spite of the fact that it has a significant percentage of sulfate and pyruvic acid, giving a ratio galactose:sulfate:pyruvic acid of 1.00:0.84:0.49. Only a minimum quantity of protein was still present (1.1%). Most of the protein appeared in F3 and F4 (32.0 and 46.6%, respectively). By treatment of F3 with pronase E, the amount of protein decreased to 22.3%. Taking into account the small percentages of these fractions, they were not studied further. F5 and F6 showed important amounts of carbohydrates, mainly galactose, and sulfate, but small amounts of protein (4.3 and 1.4%, respectively).

Structural analysis was carried out on F1 and F6 by chemical methods and NMR spectroscopy; the latter fraction was selected due to the high yield and sulfate content. F1 was submitted to methylation, desulfation–methylation, and depyruvylation–methylation procedures. The desulfated and depyruvylated derivatives obtained, F1desulf and F1depyr, were analyzed (Table 3), showing a similar monosaccharide composition to that of the parent sample, and that the expected reaction was achieved. In the case of the depyruvylation procedure, only slight desulfation occurred. However, for the modified derivatives of F1, the methylation reaction gave a certain degree of degradation, therefore, these results should be taken only qualitatively (Table 4). Moreover, a sequential desulfation–depyruvylation procedure was carried out on F1, and methylation analysis (Table 4) showed an important amount of terminal units for this sample, indicating partial depolymerization. Nevertheless, it was possible to confirm that this galactan has 3-, 6-, and 3,6-linked units.

An important amount of non-methylated galactose was detected by methylation of F1 in different conditions, but only small percentages were found in the modified derivatives, consequently, it was attributed to completely substituted galactose units in F1. The first structural evidence from the NMR spectra is that pyruvic acid is forming a 6-membered ring *R*-configuration linked to *O*-4 and *O*-6 of some of the galactose units (signals at δ 177.0, 101.7, and 26.0/1.41 correspond to C-1 C-2 and C-3/H-3 of this substituent, Table 5, Fig. 1) (Bilan, Vinogradova, Shashkov, & Usov, 2006). Methylation analysis showed that this substituent, present in half of the whole structural units, could be linked to 3-linked galactose (2-sulfate), and, in minor amounts, to terminal galactose residues. These results were confirmed by the presence of important quantities of 3-linked β-D-galactose and, mainly, β-D-galactose 2-sulfate in F1depyr, detected by methylation analysis, which were confirmed by analysis of the NMR spectra of this derivative (Tables 4 and 5, Fig. 2). On the other hand, spectra of the desulfated sample showed the absence of anomeric signals at 103.6/4.79 and 103.5/4.85 ppm, which show displacement to higher fields of the carbon signals, consequence of the presence of a sulfate group on C-2 (Table 5, Figs. 1 and 2).

The remaining units comprise 3-linked 6-sulfated and 4,6-disulfated galactose, the latter, more important in F6 (see later), as well as 6- and 3,6-linked, possibly in part 4-sulfated galactose units. 6-Sulfation was very clear in the spectra of F1 and F1depyr by the presence of a signal at δ 68.0/4.30 which was assigned to C-6 of 3-linked galactose 6-sulfate units. The signal corresponding to C-5/H-5 was deduced by proton correlation in the COSY spectrum. On the other hand, the signal at δ 75.9/4.99 was attributed to C-4/H-4 of 6-linked galactose 4-sulfate units. Spectroscopic assignment is in agreement with previous data (Bilan et al., 2007; Canelón, Ciancia, Suárez, Compagnone, & Matulewicz, 2014; Ciancia et al., 2012; Ferreira et al., 2012; Stortz, Bacon, Cherniak, & Cerezo, 1994).

Some of the structural units of F1 are also part of the structure of F6 but in different quantities, with higher degree of sulfation

Table 1
Yields and analyses of extracts obtained from *Penicillus capitatus* (PA, PC), *Udotea flabellum* (UA, UC), and *Halimeda opuntia* (HA) by extraction with water.

Extract[a]	Yield[b] %	Carbohydrates %, anh[c]	Sulfate as SO_3Na %	Monosaccharide composition (moles %)						
				Rha	Fuc	Ara	Xyl	Man	Gal	Glc
PA1[e]	0.3	34.2	10.0	2.0	4.6	–	3.2	5.6	77.0	7.5
PA2	0.05	28.2	10.4	1.5	4.9	1.4	5.3	5.9	49.9	30.6
PC1	1.3	73.4	2.4	Tr.[d]	–	–	Tr.	–	5.9	93.1
PC2	0.5	72.2	2.9	Tr.	–	Tr.	Tr.	Tr.	6.9	93.8
PCa	33.7	n.d.	n.d.	1.9	2.1	Tr.	3.5	3.8	52.6	36.0
UA[e]	1.4	55.1	16.8	1.9	–	1.8	1.6	–	94.7	Tr.4
UC	0.6	44.6	21.2	2.4	Tr.	2.9	4.8	2.7	56.4	30.7
HA[e]	0.22	44.3	21.7	1.0	1.3	2.3	2.1	9.8	77.2	6.1

[a] Analysis of PC1 and PC2 were similar, so they were worked out together. After treatment with α-amylase, PCa was obtained.
[b] For 100 g of the residue from the methanolic extraction. For PCa, yield of the enzymatic treatment of PC.
[c] Values obtained by method for insoluble material.
[d] Tr = traces.
[e] 8.0, 16.3, and 6.2% of pyruvic acid for PA1, UA and HA, respectively.

Table 2
Yields and analyses of the fractions obtained by anion exchange chromatography of the room temperature water extract from *Penicillus capitatus* (PA1).

Fraction	Eluant NaCl, M	Yield[a] %	Carbohydrates %, anh	Sulfate as SO_3Na %	Protein %	Monosaccharide composition (moles %)						
						Rha	Fuc	Ara	Xyl	Man	Gal	Glc
F1[b]	–	60.9	29.3	12.9	1.1	2.4	3.5	0.4	1.6	4.0	82.0	6.2
F3[c]	0.5	5.3	25.1	9.7	32.0	3.0	4.0	2.2	5.1	7.9	67.7	10.0
F4	0.75	11.9	24.0	8.7	46.6	1.3	1.5	0.5	2.6	4.3	86.5	3.3
F5	1.0	5.3	54.8	23.1	4.3	1.6	1.9	0.9	2.6	3.6	87.3	2.2
F6[b]	1.5	15.6	45.9	35.3	1.4	0.3	0.7	0.1	1.2	0.7	95.7	1.2

[a] 70.0% of PA1 was recovered. Fractions F2, which eluted with 0.25 M NaCl solution, and F7, which was obtained after boiling the phase in 4 M NaCl, comprised less than 1% of PA1, so they were not included in the table.
[b] 6.4 and 6.5% of pyruvic acid for F1 and F6, respectively.
[c] After treatment with protease, the carbohydrate content was 34.9% and the percentage of protein, 22.3%.

Table 3
Yields and analyses of the products obtained by depyruvylation and desulfation of F1.

Fraction	Gal:Sulf:Pyr molar ratio	Monosaccharide composition (moles %)						
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc
F1[a]	1:0.8:0.5	2.4	3.5	0.4	1.6	4.0	82.0	6.2
F1depyr	1:0.6:0.0	4.4	–	2.1	3.8	3.9	80.3	5.5
F1desulf	1:0.1:0.5	5.4	3.0	3.8	2.4	2.1	79.3	4.1

[a] Included for comparison.

and lower amounts of pyruvylated units. Accordingly, non-sulfated pyruvylated 3-linked galactose units were not detected by NMR spectroscopy (absence of the signal at 79.5/4.16 ppm, due to C-3 of these units), while signals corresponding to 3-linked galactose 4,6-disulfate units were clear. Besides, 6-linked galactose 4-sulfate was not detected in F6 (peak at δ 76.0/4.99, corresponding to C-4/H-4 of these units was not found), while it is present in the spectra of F1 and F1depyr.

Results from methylation analysis and NMR spectra of the room temperature aqueous extracts from *U. flabellum* and *H. opuntia*, UA and HA (Table 4, Fig. 3), show a similar pattern, similar also to those of F1 and F6. Both have important amounts of pyruvylated 3-linked β-D-galactose units partially sulfated on C-2, the latter substitution being more important for HA. Methylation analysis of UA and HA showed 2,4-di-*O*-methylgalactose as major monosaccharide derivative, which could correspond to 3-linked galactose 6-sulfate or 3,6-linked galactose, or to a mixture of these units. The presence of a signal at δ 68.0/4.30, which could correspond to C-6/H-6 would indicate 6-sulfation. However, the signal at 70.4/4.04,3.83, which was previously assigned to C-6/H6,6′ of

Table 4
Methylation analysis of fractions F1, F6 and of their modified derivatives, and of UA and HA.[a]

Monosaccharide	F1	F1depyr	F1desulf	F1desulf, depyr	F6	F6desulf	UA	HA
2,3,4,6-Gal	Tr.	15	5	27	–	12	3	–
2,4,6-Gal	7	18	11	20	10	7	9	9
2,3,4-Gal	–	3	3	5	–	7	–	–
4,6-Gal	4	12	12	4	3	6	4	5
6-Gal	1	Tr.	–	–	–	–	2	1
2,3-Gal	9	4	15	4	24	12	3	7
2,4-Gal	26	33	47	36	30	42	38	31
2-Gal	21	10	4	4	20	7	25	21
3/4-Gal	–	2	2	Tr.	–	3	6	2
Gal	32	2	1	Tr.	13	4	10	21

[a] Small percentages of derivatives of glucose, mannose, rhamnose, and xylose were detected in some of the samples, but they were considered to arise from contaminant polysaccharides and they were not included in the table.

Table 5
NMR signal assignments (ppm) of substituted galactose units found in galactans from *Penicullus capitatus*, *Udotea flabellum* and *Halimeda opuntia* and their modified derivatives.[a]

Structural unit[b]	Chemical shifts, ppm						Detected clearly in the spectrum of[b]
	C-1/H-1	C-2/H-2	C-3/H-3	C-4/H-4	C-5/H-5	C-6/H6,6′	
3G2S	103.6/4.79	79.0/4.40	81.0/4.27	70.1/3.70[c]	76.0/3.62	62.0/3.70	F1depyr
3G	104.9/4.66	70.3/3.67[c]	83.0/3.81	69.3/4.13	76.0/3.62	62.0/3.70	F1depyr
3G6S	104.9/4.66	70.3/3.67[c]	84.8/3.73	69.3/4.13	74.2/3.84	68.0/4.30[c]	F1depyr
3,6G	104.9/4.66	71.1/3.71	83.0/3.81	69.3/4.22	74.2/3.98	70.4/4.04,3.83	F1desulf
3G4,6S	105.4/4.53	71.8/3.54	77.2/4.01	78.2/4.79	74.2/3.95	67.8/4.23[c]	F6
3GP,2S	103.5/4.85	76.2/4.48	77.2/4.39	71.8/4.11	66.9/3.54	65.9/3.85,3.97	F1
3GP	104.2/4.46	71.9/3.54	79.5/4.16	71.8/4.11	66.9/3.54	66.0/3.85,3.97	F1desulf
tG	105.4/4.53	71.8/3.53	73.7/3.57	69.5/3.92	76.0/3.62	62.0/3.70	F1depyr
tGP	104.2/4.46	72.4/3.64[c]	72.1/3.62[c]	71.9/4.05	66.9/3.54	66.0/3.85,3.97	F1desulf

[a] Signals at δ 177.0, 101.7, and 26.0/1.41 were assigned to C-1, C-2, and C-3/H-3 of pyruvic acid forming a 6-membered ring *R*-configuration. They were present in spectra of all the samples, with the exception of F1depyr.
[b] Fraction where the unit was present in important amounts, bearing full assignment, diagnostic peaks were present in several spectra for each unit.
[c] Assignments could be interchanged.

3,6-linked galactose units was not detected, in the HMQC of UA or in that of HA, indicating that these units are not important. This fact would indicate a low degree of ramification. Also, 3-linked non-sulfated β-D-galactose units are present in significant amounts. In the ^{13}C NMR spectrum of HA there are minor signals in the anomeric region at δ 102.7 and 99.1, which were not assigned. The fact that small amounts of mannose and glucose are present in this extract suggests that they could derive from contaminating structures.

3.3. Sulfated galactans from the Halimedineae

As far as we know, this is the first report about structural features of water soluble sulfated polysaccharides from these three different genera from the Bryopsidales, particularly only very scarce information was previously published about sulfated polysaccharides from the suborder Halimedineae (Chattopadhyay et al., 2007; Mackie & Percival, 1961).

These results allow to make some generalizations, namely, the Bryopsidales biosynthesize galactans as major soluble sulfated polysaccharides with 3-, 6-, and 3,6-linkages; these polysaccharides are also substituted by important quantities of pyruvic acid.

For the seaweeds studied in this paper, galactans represent the only important sulfated polysaccharides, as the only other polymers found in the water extracts in significant amounts are 4-linked α-glucans, which are neutral reserve polysaccharides, not part of the cell wall. These galactans are obtained in extremely low yields calculated considering the milled seaweed dry weight. However, taking into account the amount of calcium carbonate reported previously for these calcified seaweeds (Böhm, 1973), yields would be still low, but of the same order as those reported for other Bryopsidales (Ciancia et al., 2007, 2012). If any of these polymers were suitable for application as biologically active compounds, yield would not be a major hindrance due to the availability of row material.

All the sulfated pyruvylated galactans studied here have common structural characteristics, namely, (i) major amounts of 3-linked 4,6-*O*-(1′-carboxy)ethylidene-D-galactopyranose units in part sulfated on C-2, and also possibly terminal 4,6-*O*-(1′-carboxy)ethylidene-D-galactopyranose residues, but in much lower amounts, (ii) a complex sulfation pattern, comprising additionally 4-, 6-, and 4,6-disulfated galactose units. Until now, it is not know wether both kind of substitution patterns coexist in the same molecule or if they are present in different molecules that

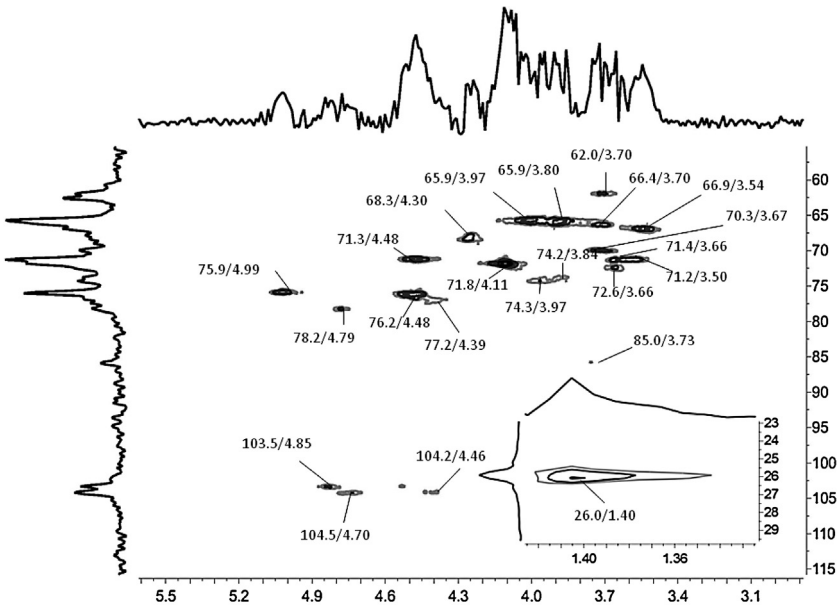

Fig. 1. HMQC spectrum of F1, showing signals corresponding to the major units. A detail with the peak of C3/H3 of pyruvic acid ketal is included.

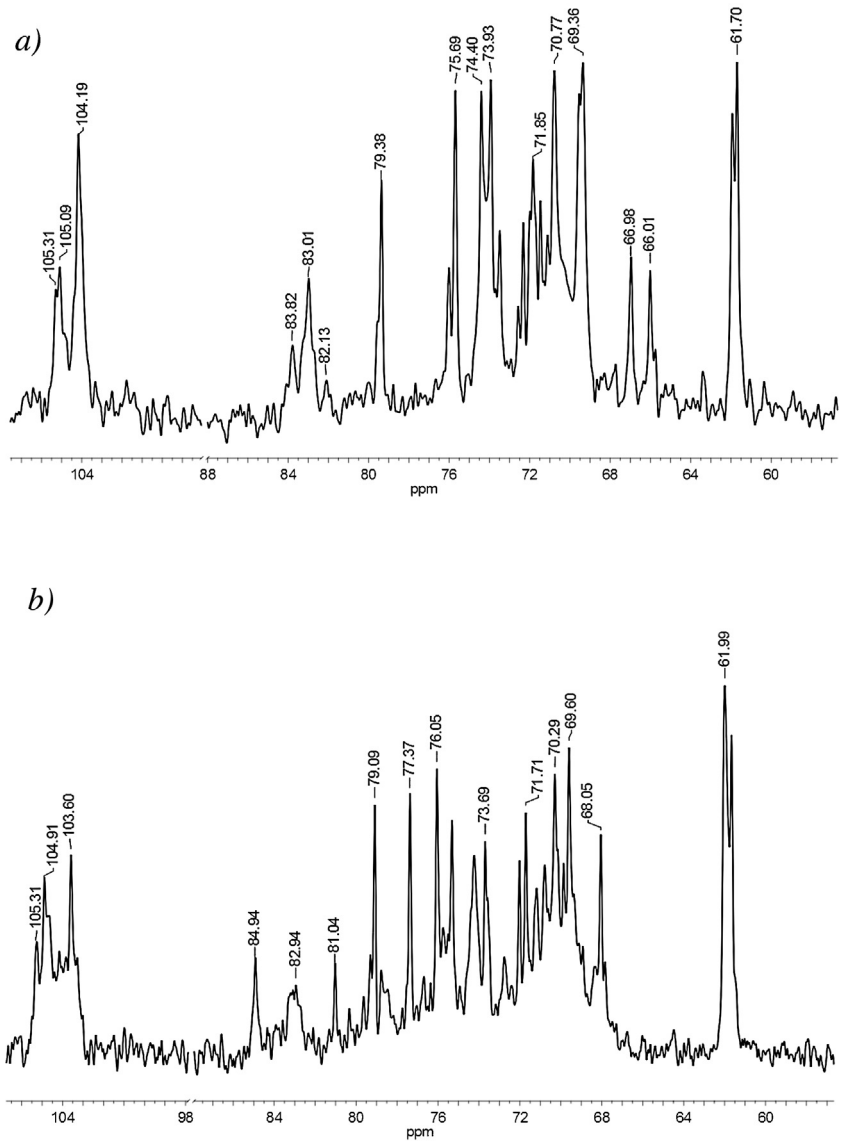

Fig. 2. ^{13}C NMR spectra of F1desulf (a) and F1depyr (b).

are obtained together in spite of the efforts carried out to separate them.

There are important differences between these galactans and the structure previously reported for sulfated polysaccharides from *Caulerpa racemosa*, which belongs to the same suborder (Chattopadhyay et al., 2007). In the latter, xylose and arabinose were detected in important quantities and were attributed to single stubs or short side chains, sulfate was found at C-6 of the galactose units, as found for galactans from *B. plumosa* (Ciancia et al., 2012) and no reference to the presence of pyruvic acid ketals was made. The authors studied the oligosaccharides obtained by acid hydrolysis of this polymer by MALDI-TOF-mass spectrometry, but in conditions necessary to achieve partial hydrolysis of glycosidic linkages, pyruvic acid would be lost.

3.4. Anticoagulant activity

3.4.1. Global coagulation tests

Anticoagulant activity of polysaccharides from all the seaweeds was assessed by measuring the prothrombin time (PT), the activated partial thromboplastin time (APTT), and thrombin time (TT). All the samples proved to have similar anticoagulant effect (Supplementary Table S1). Since F1 from *P. capitatus*, was available in higher quantities, all the studies were focused only on this fraction. The activity of the sample was compared with model anticoagulants, heparin and dermatan sulfate (Fig. 4).

Supplementary Table S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.10.030.

No clotting inhibition was observed in PT test at the concentrations assayed. In order to avoid possible neutralization effects of polybrene, added in the majority of commercial reagents a homemade thromboplastin was used. Polybrene is a polycation that can form complexes with polyanions, such as heparin and possibly the sulfated polysaccharide assessed here, blocking their action (Carroll, 1999). Even with the homemade thromboplastin, no effect was observed.

On the contrary, APTT and TT were statistically prolonged, in a concentration dependent manner, regards to the control (Table 6, Fig. 4). TT increases were higher than those observed in APTT. For TT, increases were up to 705 ± 9% and for APTT 197 ± 10%,

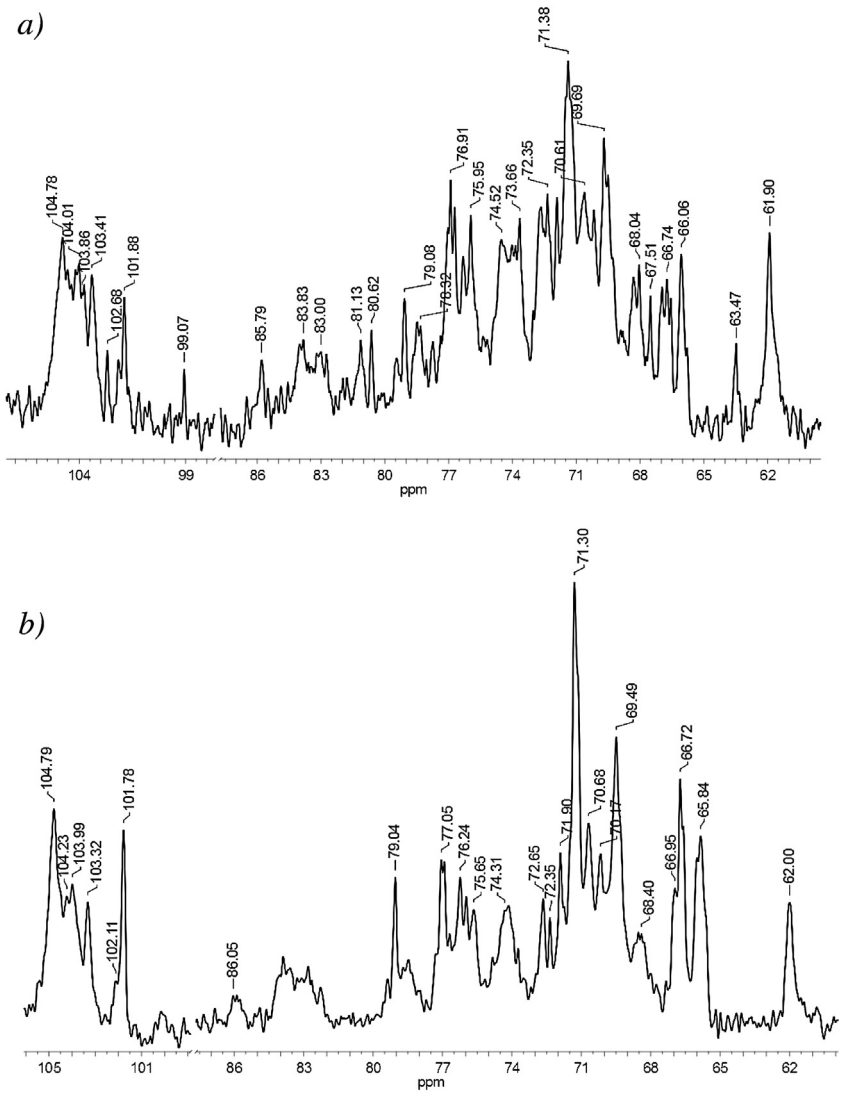

Fig. 3. ^{13}C NMR spectra of UA (a) and HA (b).

were achieved at 100 μg/mL. Prolongation of the APTT suggests inhibition of the intrinsic and/or common pathway of coagulation process, meanwhile the increase of TT indicates either thrombin inhibition (direct or mediated by AT and/or HCII) or impaired fibrin polymerization. Since these results showed anticoagulant activity, the lack of prolongation in the PT may be explained by the fast kinetics of the assay, which could not allow detecting slight differences. Moreover, prolongation of the TT-like assay, using fibrinogen instead of plasma, was also observed (ratio 4.8 ±0.1). This is a first signal of a possible direct inhibition of F1 on thrombin activity, taking into account that AT and HCII were absent in the test. However, more assays need to be done to confirm this hypothesis.

Our results demonstrate that F1 exerts anticoagulant effect, but, when compared to model anticoagulants such as heparin and dermatan sulfate, F1 proved to be less potent in preventing in vitro clot formation.

Table 6
APTT and TT ratios for F1, dermatan sulfate and heparin.

Sample[a]	Concentration (μg/mL)[b]				
	5	10	25	50	100
APTT					
F1	1.0 ± 0.0	1.0 ± 0.0	1.3 ± 0.0*	1.9 ± 0.1*	3 ± 0.1*
Dermatan sulfate	1.0 ± 0.0	1.5 ± 0.0*	3.6 ± 0.0*	4.0 ± 0.1*	6.3 ± 0.0*
Heparin	7.9 ± 0.1*	>10*	>10*	>10*	>10*
TT					
F1	1.1 ± 0.0	1.3 ± 0.0*	2.4 ± 0.1*	4.9 ± 0.1*	8.1 ± 0.1*
Dermatan sulfate	4.2 ± 0.0*	6,7 ± 0.1*	>10*	>10*	>10*
Heparin	>10*	>10*	>10*	>10*	>10*

[a] Results were expressed as ratios between clotting time of a solution of the anticoagulant and clotting time of the control; they are the mean ± standard deviation ($n = 4$).
[b] Concentration corresponds to the samples in the *test solution*.
* Statistically significant differences regards to the control ($p < 0.05$). Student's *t* test was used to compare anticoagulant and control samples.

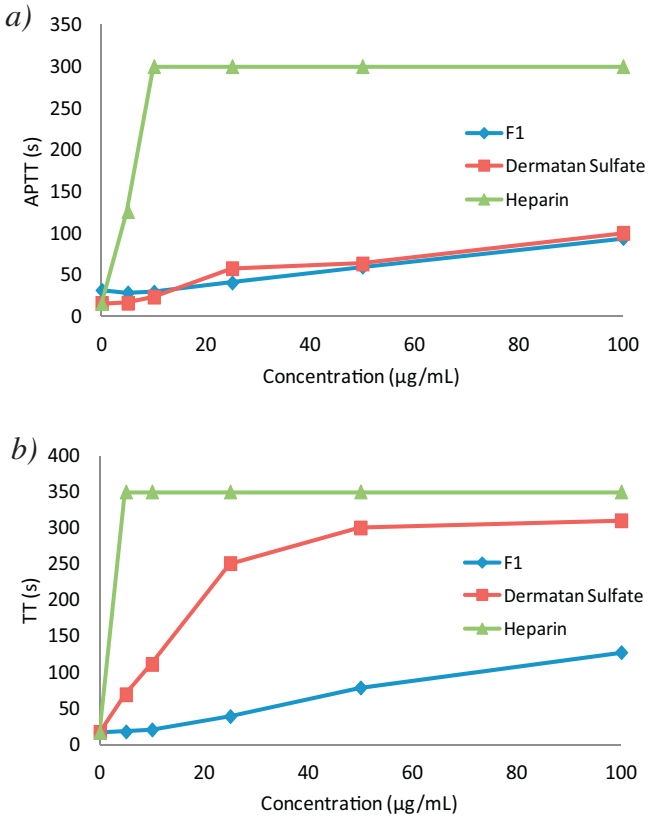

Fig. 4. Activated partial thromboplastin time (APTT) (a) and thrombin time (TT) (b) of the fraction F1 from *P. capitatus*, heparin and dermatan sulfate.

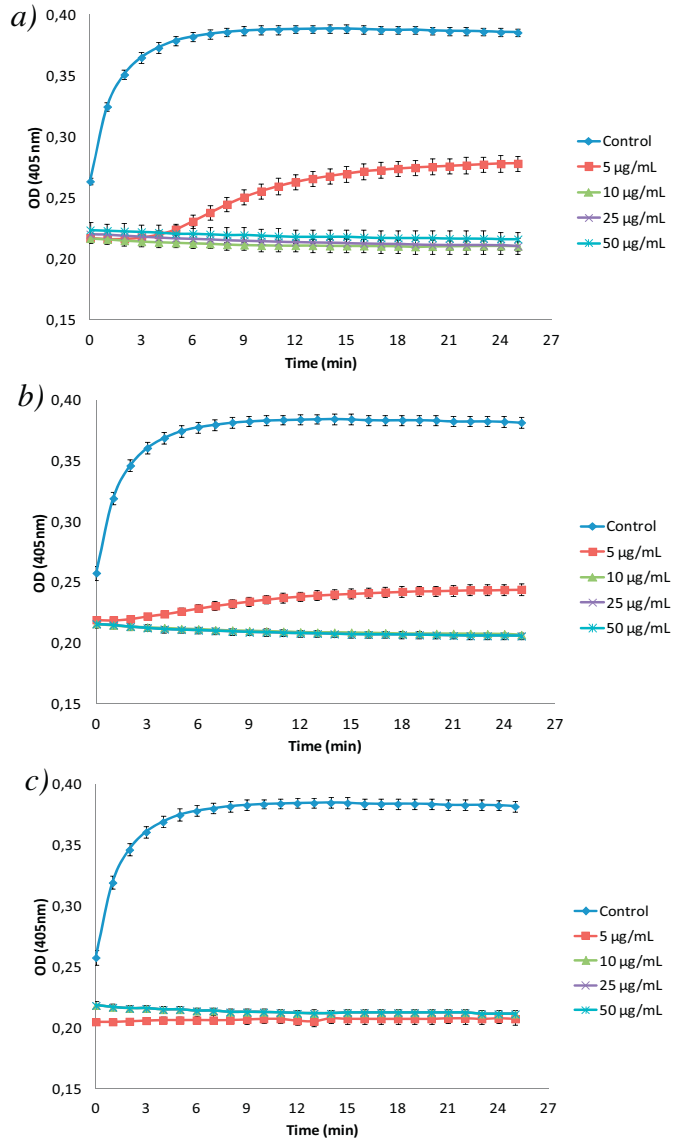

Fig. 5. Effects of F1 from *P. capitatus* (a), dermatan sulfate (b) and heparin (c) on the kinetics of fibrin formation. Curves represent the average of OD (405 nm) versus time ($n = 4$) and bars indicate the SD.

Only a few sulfated polysaccharides from green seaweeds have been thoroughly studied from both the structural point of view and their biological behavior, particularly in haemostatic system. From Bryopsidales, there are few reports about their anticoagulant activities, only the species *Caulerpa okamurai* and *Bryopsis maxima* and several species from the genus *Codium*, such as, *Codium fragile*, *Codium istmocladum*, *Codium divaricatum*, *Codium latum*, *Codium vermilara* among others were described (Ciancia et al., 2010). However, for species of *Codium*, the most active compounds would be sulfated arabinans (Fernández et al., 2013).

3.4.2. Fibrin formation kinetics assays

Clot plays a crucial role in the hemostatic system, and formation, structure, and lysis of fibrin networks are important features to be studied. In the present work the effects of sulfated galactans from *P. capitatus* on fibrin formation process were studied. Table 7 and Fig. 5 show the kinetics of plasma fibrin formation in the presence of F1, heparin and dermatan sulfate. All the assayed concentrations of F1 produced statistically significant changes in kinetics parameters, in a concentration-dependent way. No coagulation was detected with F1 (10 µg/mL), which was considered as a positive control of the anticoagulant effect. Similar effects were detected when dermatan sulfate in the same concentration and a 5 µg/mL solution of heparin were used.

Moreover, F1 caused increased lag phase and decreased slope and OD_{Max} regards to control. Since the lag phase (which shows the time required for initial protofibril formation) is increased, and the slope (that corresponds to the fibrin formation rate) is diminished, an impaired assembly of fibrin monomers into the fibrin polymer would be involved. In the first polymerization step non-covalent interactions, in particular, electrostatic bindings are involved. Therefore, the presence of F1, a highly negatively charged molecule, could affect fibrin assembly showing an anticoagulant activity. In general, all the results are in accordance with those reported for dermatan sulfate, a well known anticoagulant compound (Lauricella, Castañon, Kordich, & Quintana, 2013).

On the other hand, the diminished final optical density suggests that the fibrin fibers resulted thinner than those from control networks. Although mass/length ratio (μ) is a quantitative measure of the fiber structure, the final turbidity (OD) can be used to study the qualitative features of the fibrin network (Wolberg, Gabriel, & Hoffman, 2002).

Kinetics of fibrin formation gives a different insight into the study of the coagulation process, contributing in this case to the understanding of anticoagulant activity of F1. To the best of our knowledge, this is the first study where this experimental strategy was evaluated using sulfated polysaccharides extracted from seaweeds.

Table 7
Parameters of the kinetics of fibrin formation in the presence of F1, heparin and dermatan sulfate.[a]

	Lag phase (min)	Slope (min^{-1})	OD_{max} [b]
Control	0.0 ± 0.0	61.5 ± 2.1	0.382 ± 0.004
F1 (μg/mL)			
5	4.1 ± 0.9*	7.3 ± 0.1*	0.278 ± 0.006*
10	n.c.[c]	n.c.	0.210 ± 0.007*
25	n.c.	n.c.	0.210 ± 0.004*
50	n.c.	n.c.	0.200 ± 0.000*
Dermatan sulfate (μg/mL)			
5	2.4 ± 1.0*	3.0 ± 0.8*	0.244 ± 0.005*
10	n.c.	n.c.	0.207 ± 0.001*
25	n.c.	n.c.	0.211 ± 0.001*
50	n.c.	n.c.	0.213 ± 0.003*
Heparin (μg/mL)			
5	n.c.	n.c.	0.208 ± 0.005*
10	n.c.	n.c.	0.207 ± 0.002*
25	n.c.	n.c.	0.211 ± 0.001*
50	n.c.	n.c.	0.219 ± 0.001*

[a] Results were expressed as mean ± standard deviation ($n = 4$).
[b] Maximum optical density.
[c] n.c.: no clotting detected.
* Statistically significant difference ($p < 0.05$). Student's *t* test was used to compare the curves parameters of the anticoagulant and control samples.

4. Conclusions

The structural characteristics of sulfated and pyruvylated galactans from some tropical Bryopsidales have been determined, showing 3-, 6-, and 3,6-linked units with a very complex patterns of substitution. Particularly, galactans from *P. capitatus* were studied in detail.

Taking into account results from global coagulation assays and fibrin formation assays, it was demonstrated that these galactans exert anticoagulant effects. Moreover, one of the possible mechanisms involved would be direct thrombin inhibition.

Recently a highly sulfated pyranosic arabinan from *Codium vermilara* was found to have important anticoagulant activity by a mechanism involving direct thrombin inhibition. It was shown that this effect is mostly due to the presence of a sulfate group on C-2 of the β-L-arabinopyranose units (Fernández et al., 2013). The fact that related galactan structures from *Codium* species, that are not sulfated on C-2, do not have important anticoagulant action, induces us to speculate that the active structure in the galactans studied here could be related to sulfation on C-2 of the galactan chain, which has similar configuration to that of the latter arabinan. If it were so, then it would be interesting to obtain galactan fractions where this sulfation pattern is yet more predominant, giving an important increase in the activity. In addition, data presented here could be useful in planning modification of other polymers with related structures to increase the activity, or even for the synthesis of new anticoagulant agents, more research is still needed. In general, knowledge of specific structural characteristics of seaweed polysaccharides and their relationship with the anticoagulant activity could contribute to the understanding of the regulation of haemostatic processes and to the development of new antithrombotic therapeutic agents.

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