ANTICOAGULANT SCREENING OF MARINE ALGAE FROM MEXICO, AND PARTIAL CHARACTERIZATION OF THE ACTIVE SULFATED POLYSACCHARIDE FROM *Eisenia arborea*

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ABSTRACT. The *in vitro* anticoagulant activity of 41 water extracts of various seaweeds from Baja California Sur, Mexico was evaluated. In this study, nine extracts exhibited anticoagulant activity in the prothrombin time assay and 29 extracts were positive in the activated partial thromboplastin time assay. The water extract obtained at 25 °C from the brown seaweed *Eisenia arborea* was the most active in both assays, increasing the normal blood clotting-time over 300 s at 100 g mL^{-1}. The fractionation of this extract by anion exchange chromatography yielded 3 fractions. Fraction 2 eluted with 1.0 M sodium chloride increased the clotting-time over 300 s in the activated partial-thromboplastin time assay at 5 g mL^{-1}, being more active than sodium heparin. Chemical and spectroscopic analysis of fraction 2 showed it to be a sulfated heterofucan composed of 56.2 % ± 0.1% of total sugars and 45 % of sulfates.The neutral sugar constituents of the active heterofucan was determined to be 47.6 % fucose, 35.5 % xylose and 16.9 % rhamnose, with substitutions of sulfate groups at C-4 (axial), and minor substitutions at C-2 and-or C-3.

Keywords: Seaweed anticoagulants, Baja California Sur, activated partial thromboplastin time, heparin, heterofucan, prothrombin time, sulfated polysaccharides.

Monitoreo de anticoagulantes en algas marinas de México y caracterización parcial de polisacáridos sufatados activos de *Eisenia arborea*.

RESUMEN. Se evaluó la actividad anticoagulante *in vitro* de 41 extractos acuosos de diversas algas de Baja California Sur. Nueve extractos exhibieron actividad anticoagulante en el ensayo de tiempo de protrombina y 29 extractos fueron activos en el ensayo de tiempo de tromboplastina parcial activada. El extracto acuoso de *Eisenia arborea* obtenido a 25 °C fue el más activo en ambos ensayos, incrementando el tiempo normal de coagulación a más de 300 s, a una concentración de 100 g mL^{-1}. El fraccionamiento de este extracto por cromatografía de intercambio iónico resultó en 3 fracciones. La fracción 2 eluída con cloruro de sodio 1.0 M incrementó el tiempo de coagulación a más de 300 s en el ensayo de tiempo de la tromboplastina parcial activada a una concentración de 5 g mL^{-1}; resultando más activa que el control positivo (heparina de sodio). Los análisis químicos y espectroscópicos mostraron que la fracción 2 era un heterofucano sulfatado, compuesto por 56.2 % ± 0.1 % de azúcares totales y 45% de sulfatos. Los azúcares neutros constituyentes del heterofucano activo fueron determinados como 47.6 % fucosa, 35.5 % xylosa y 16.9 % ramnosa, con sustituciones de los grupos sulfato en C-4 (axial) y sustituciones menores en C-2 y/o C-3.

Palabras clave: Anticoagulantes de algas, tiempo de tromboplastina parcial activada, Baja California Sur, heparina, heterofucano, tiempo de protombina, polisacáridos sulfatados.

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INTRODUCTION

The side effects of the current anticoagulant drugs, such as hemorrhage and thrombocytopenia (Chong, 2003), together with the expensive monitoring during the antithrombotic treatment and risks for viral or prional infections, have prompted the development of new anticoagulants for prophylaxis and therapy of thromboembolic events (Weitz & Bates, 2005).

The algal polysaccharides have long been recognized to have several biological activities (Teixeira & Hellewell, 1997; Schaeffer & Krylov, 2000; Haround-Bouhedja *et al*., 2002), although their potent anticoagulant activity has been the most studied (Shanmugam & Mody, 2000; Melo *et al*., 2004). The marine algae are a rich source of anticoagulant compounds because they biosynthesize a wide variety of sulfated fucans (Pereira *et al*., 1999; Berteau & Mulloy, 2003), sulfated galactans (Sem *et al*., 1994; Farias *et al*., 2000), and proteoglycans (Jurd *et al*., 1995; Matsubara *et al*., 2000) that are potent thrombin inhibitors (Pereira *et al*., 2002; Melo *et al*., 2004).

The purpose of our work was to gain some insight into the previously unstudied anticoagulant potential of seaweeds collected off the coast of Baja California Sur, Mexico. We first screened forty one water extracts from twenty one algae for anticoagulant activity *in vitro*. Prothrombin time and activated partial thromboplastin time assays were used to select the most active extract. Then the selected extract was fractionated by ion exchange chromatography and the structure and anticoagulant activity of the fractions were partially characterized.

MATERIALS AND METHODS

Seaweeds sampling, extraction, and anticoagulant screening

The examined seaweeds were gathered between February and August 2004 at different offshore locations, in Baja California Sur (Mexico). Sampling was done by hand in the intertidal zone or by diving for deeper samples. *Gelidium robustum* was collected at Isla Natividad (27° 52' 27" N; 115° 09' 49" W), *Eisenia arborea* was collected at Punta Eugenia (27°50'01" N; 115°04'03" W), and *Gracilaria vermiculophylla* was collected at Laguna de San Ignacio (26°47'25" N; 113°14'50" W). The other seaweeds listed were recovered from Bahía de La Paz (between 24°12'50" N; 110°33'38" W and 24°21'48" N; 110°16'27" W). The collected algae were washed with tap water to clean them of conspicuous epiphytes and sediment. The algae were then dried in the shade and ground with a manual miller. Ten grams of each alga were separately extracted with 200 mL of distilled water at 25 °C under continuous agitation for 4 h. After the extraction, the liquid was decanted and the algal tissue was set aside. The supernatant was clarified by filtration under vacuum. Each clarified solution was mixed with three volumes of distilled ethanol and left overnight under refrigeration to promote the precipitation of macromolecules. Precipitates were recovered by centrifugation (1700 × *g*, 15 minutes). The supernatant was disposed of and the pellet was dried at 50 °C for 20 h. The algal tissue previously extracted was extracted a second time with distilled water at 80 °C for 2 h. The hot water extract was processed in the same way as the first extract. The extraction time and temperature were experimentally optimized *a priori* to get the highest yield.

The anticoagulant activity of each extract was evaluated by the increase of the prothrombin time (PT) and the activated partial thromboplastin time (aPTT). The PT and the aPTT were measured using human plasma treated with sodium citrate and a stock solution of 10 mg mL^{-1} of each algal extract. Each assay was done by mixing 90 L of human plasma with 10 L of the extract in distilled water and the mixture incubated at 37 °C for 2 minutes. After incubation, 200 L of reagent was added to the mixture following the manufacturer's instructions (Biopool® by Trinity Biotech, plc. Ireland). The time for clot formation was determined by visual inspection and reported in seconds. Distilled water was used as a negative control and sodium heparin (Sigma Chemical Co., USA) was used as a positive control of anticoagulant activity. All assays were made in duplicate and the data were subjected to mean-standard deviation analysis. The Tukey test was used to find statistical differences ($P < 0.05$) by comparison of the clotting time of each extract and the negative control clotting time. The extract with the highest PT and aPTT was selected for further fractionation.

Fractionation of the selected extract from the *Eisenia arborea*

The extract of *Eisenia arborea* obtained with distilled water at 25 °C was selected for

fractionation and characterization of the active polysaccharide fraction because it had the highest clotting time in both anticoagulant assays. One hundred grams of dried *Eisenia arborea* was extracted again with 1 L of distilled water at 25 °C with continuous agitation for 4 h. After filtering, the filtrate was precipitated by adding 1 L ethanol. The precipitate was recovered by centrifugation (1700 × *g*, 15 minutes) and dried at 55 °C to yield 2.5 g of crude extract. The extract (1.85 g) was dispersed in distilled water at room temperature with continuous agitation. The dispersion was centrifuged (1700 × *g*, 15 minutes) to remove insoluble material and the supernatant was diluted with 600 mL ethanol to cause precipitation. The precipitate was recovered by centrifugation and dried to leave 1.24 g of a brownish powder. One gram of the powder was dispersed again in 20 mL of distilled water by sonication and added to a column (25 mm × 200 mm, internal diameter × length) packed with 40 g of diethyl amino ethyl-cellulose (DEAE-cellulose; anion exchanger DE52, Whatman® by Whatman International Ltd., UK). The anion exchanger was equilibrated with 20 mM TRIS·HCl buffer (pH 8.3). The column was eluted with 0.25 L of 0.5 M sodium chloride, followed by same amount of 1.0 M and 2.0 M NaCl. The flow rate was set at 0.5 mL min^{-1}. Fifteen 50 mL eluates were collected. The relative hexose content in the eluates was determined by the phenol-sulfuric acid reaction (Dubois *et al*., 1956). Fractions were dialyzed extensively against distilled water through a cellulose membrane (MW cutoff 12000-14000, Spectra/Por® by Spectrum Laboratories, Inc. USA), lyophilized, and tested by the PT and aPTT assays.

Characterization of fractions from *Eisenia arborea*

The total sugar content of the fractions was estimated colorimetrically by the phenol-sulfuric acid method (Dubois *et al*., 1956) using L-fucose as the standard. The sulfate ester content was measured by infrared (IR) spectroscopy (Lijour *et al*., 1994). Uronic acid content was measured by the 3-phenyl-phenol reagent as described by Blumenkrantz & Asboe-Hansen (1973) using galacturonic acid as the standard. The neutral sugar constituents were determined by GC-MS analysis of the alditol acetate derivatives. For this, 20 mg of active polysaccharide was hydrolyzed by adding 0.18 mL of concentrated sulfuric acid at room temperature for 20 h. The hydrolyzate was neutralized with $BaCO_3$. The mixture was reduced with $NaBH_4$ (pH 11) for 4 h, afterward the product was neutralized with some drops of 50 % acetic acid (v/v). The alditols obtained were then acetylated by dissolving it in pyridine and acetic anhydride (1 mL/1mL) under reflux for 2 h. The residue was re-dissolved in 1 mL of chloroform and injected to a gas chromatograph (Varian, Saturn 3) equipped with a Varian column DB5-Factor 4 (30 m × 0.25 mm i.d.). Helium at 1 mL min^{-1} was used as carrier gas. The oven temperature was set at 195 °C increasing 2.5 °C by minute until 225 °C was reached. The injector and detector temperature were 260 °C and 280 °C respectively. The sugar content was determined by comparison of the chromatographic peaks and the fragmentation patterns of authenticated monosaccharide.

In addition to the compositional data, the fractions were characterized by IR and ^1H nuclear magnetic resonance (NMR) spectroscopy. The IR spectra were recorded in transmission with a Perkin Elmer Spectrum 200 Fourier-transformed Infrared spectrometer using sodium chloride as the support for samples. All spectra were acquired in a spectral range of 4000 cm^{-1} - 600 cm^{-1} with 128 repetitive scan at a resolution of 4 cm^{-1}. For nuclear magnetic resonance of protons (^1H-NMR) spectroscopy, samples were dissolved in D_2O (*ca.* 30 mg mL^{-1}). The spectra were acquired at 25 °C with a Varian Unity NMR spectrometer at 300 MHz. The chemical shifts were measured in parts per million relative to internal 3-(trimethyl)-silyl propionic acid-d_4-sodium salt.

RESULTS

Anticoagulant activity of seaweeds extracts

Table 1 shows the PT and the aPTT values in seconds of all the 41 algal extracts tested. Nine extracts (22 %) significantly increased ($P < 0.05$) the control PT at the tested concentration. The most active was the extract of *Eisenia arborea* obtained at 25 °C, followed by the extracts of *Codium cuneatum*, *Drudresnaya colombiana*, *Cladophora sericea*, *Gracilaria subsecundata*, and *Hydroclathrus clathratus*. To the best of our knowledge, this is the first report of anticoagulant activity from those species. In contrast, 29 (71 %) from all the 41 extracts showed a significant increase ($P < 0.05$) on the aPTT control. Extracts from *Codium brandegeii*, *Codium cuneatum*, *Cladophora*

Table 1. Anticoagulant activity of Mexican seaweeds aqueous extracts evaluated by the prothrombin time and the activated partial thromboplastin time assays at the concentration of 100 g mL^{-1}.

Order Family *Species name* Author, year	Prothrombin Time [a] mean (in seconds) ± s.d., n = 2		activated Partial Thromboplastin Time [b] mean (in seconds) ± s.d., n = 2	
	Extracted at 25 °C	Extracted at 80 °C	Extracted at 25 °C	Extracted at 80 °C
Caulerpales				
Codiaceae				
Codium amplivesiculatum Setchell & Gardner, 1924	13.5 ± 0.2	13.2 ± 0.2	55.0 ± 6.3	85.8 ± 1.8 [c]
Codium brandegeeii Setchell & Gardner, 1924	11.1 ± 0.2	14.5 ± 0.3	51.9 ± 1.0	>300 [c]
Codium cuneatum Setchell & Gardner, 1924	35.5 ± 0.7 [c]	72.0 ± 2.8 [c]	>300 [c]	>300 [c]
Ceramiales				
Ceramiaceae				
Spyridia filamentosa (Wulfen) Harvey, 1833	13.0 ± 0.0	16.0 ± 1.4	44.0 ± 7.0	52.5 ± 3.5
Rhodomelaceae				
Laurencia johnstonii Setchell & Gardner, 1924	12.7 ± 0.4	12.9 ± 0.1	40.3 ± 7.0	31.9 ± 1.6
Cladophorales				
Cladophoraceae				
Cladophora sericea (Hudson) Kützing, 1843	48.5 ± 0.7 [c]	Not extracted	>300 [c]	Not extracted
Cryptonemiales				
Dumontiaceae				
Dudresnaya colombiana Taylor, 1945	44.3 ± 3.9 [c]	50.4 ± 10.7 [c]	>300 [c]	>300 [c]
Dictyotales				
Dictyotaceae				
Padina mexicana Dawson, 1944	11.2 ± 0.3	13.4 ± 0.1	100.1 ± 7.6 [c]	>300 [c]
Fucales				
Sargassaceae				
Sargassum horridum Setchell & Gardner, 1924	13.9 ± 0.1	13.6 ± 0.1	>300 [c]	>300 [c]
Gelidiales				
Gelidiaceae				
Gelidium robustum (Gardner) Hollenberg & Abbott, 1965	13.5 ± 0.3	13.4 ± 0.1	176.1 ± 9.5 [c]	76.1 ± 5.2 [c]
Gigartinales				
Hypnaceae				
Hypnea valentiae (Turner) Montagne, 1841	12.6 ± 0.3	13.6 ± 0.2	85.5 ± 9.3 [c]	279.4 ± 7.9 [c]

[a] Control prothrombin time = 13.4 s ± 2.1
[b] Control activated partial thromboplastin time = 30.2 s ± 1.5
[c] Clotting time significantly higher ($P < 0.05$) when compared with the control time

sericea, *Dudresnaya colombiana*, *Padina mexicana*, *Sargassum horridum*, *Gracilaria subsecundata*, *E. arborea*, *Ganonema farinosum*, *Hydroclathrus clathratus*, *Chnoospora implexa,* and *Rosenvingia intricata* increased the aPTT over 300 s. Markedly, the extract from *Eisenia arborea* obtained at 25 °C was the only one having a clotting time > 300 s in both assays, thus rendering it as the most active.

Activity of polysaccharide fractions from *Eisenia arborea*

Ion exchange column chromatography of the selected extract from *E. arborea* (2.5 % on

Table 1. Continued.

Order Family Species name Author, year	Prothrombin Time [a] mean (in seconds) ± s.d., *n* = 2		activated Partial Thromboplastin Time [b] mean (in seconds) ± s.d., *n* = 2	
	Extracted at 25 °C	Extracted at 80 °C	Extracted at 25 °C	Extracted at 80 °C
Soleriaceae				
Sarcodiotheca dichotoma (Howe) Dawson, 1944	13.3 ± 0.1	15.4 ± 1.1	44.2 ± 5.7	266.0 ± 43.1 [c]
Gracilariales				
Gracilariaceae				
Gracilaria veleroae Dawson, 1944	12.8 ± 0.1	12.9 ± 0.1	44.5 ± 1.8	34.5 ± 0.8
Gracilaria vermiculophylla (Ohmi) Papenfuss, 1967	13.3 ± 0.3	13.3 ± 0.3	27.5 ± 0.7	33.7 ± 2.8
Gracilaria subsecundata Setchell & Gardner, 1924	27.5 ± 0.7 [c]	27.0 ± 0.0 [c]	>300 [c]	>300 [c]
Laminariales				
Lessoniaceae				
Eisenia arborea Areschoug, 1876	>300 [c]	16.8 ±1.6	>300 [c]	>300 [c]
Nemaliales				
Liagoraceae				
Ganonema farinosum (Lamouroux) Fan & Wang, 1974	17.0 ± 0.0	16.0 ±1.4	112.0 ± 4.2 [c]	>300 [c]
Syctosiphonales				
Syctosiphonaceae				
Hydroclathrus clathratus (Agardh) Howe, 1920	16.5 ± 1.6	26.0 ± 0.3 [c]	>300 [c]	>300 [c]
Chnoospora implexa Agardh, 1848	11.8 ± 0.2	17.0 ± 0.1	114.8 ± 4.7 [c]	>300 [c]
Rosenvingia intricata (Agardh) Børgesen, 1914	11.5 ± 0.2	17.5 ± 0.6	111.9 ± 9.4 [c]	>300 [c]
Ulvales				
Ulvacea				
Ulva rigida Agardh, 1823	12.7 ± 0.1	12.9 ± 0.6	28.6 ± 0.8	72.2 ± 1.2 [c]

[a] Control prothrombin time = 13.4 s ± 2.1
[b] Control activated partial thromboplastin time = 30.2 s ± 1.5
[c] Clotting time significantly higher ($P < 0.05$) when compared with the control time

a dry basis) yielded three fractions named CC2F1 (0.34 %), CC2F2 (0.35 %), and CC2F3 (0.005 %). Figure 1 shows the elution profile determined by measuring the relative content of total sugars in all of the 15 eluates. The fractions were completely eluted with 0.5 M NaCl, 1.0 M NaCl, and 2.0 M NaCl. Table 2 shows the blood clotting time for the three polysaccharide fractions. CC2F2 was the most active with the clotting-time increasing to > 300 s in the PT and aPTT assays at 25 g mL^{-1} and 5 g mL^{-1}. Fraction CC2F3 showed just marginal activity at the maximum test concentration (323 g mL^{-1}) and fraction CC2F1 had no activity in the PT assay, although it showed significant activity in the aPTT assay (266 s ± 5.7 s) at the same concentration. The positive con-

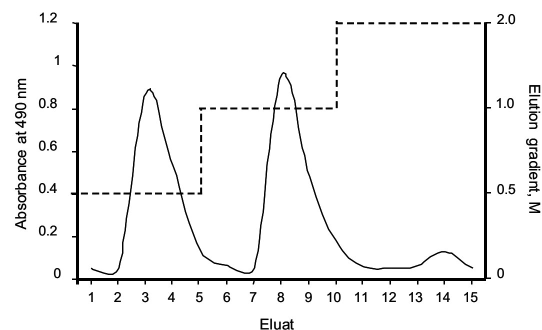

Figure 1. Elution profile of the extract from *Eisenia arborea* on DEAE-cellulose column (—) eluted with NaCl (- - -).

Table 2. Relationship between the anticoagulant activity and the concentration of polysaccharide from *E. arborea*, assayed by the prothrombin time (PT) and the activated partial-thromboplastin time (aPTT). Distilled water was used as negative control and sodium heparin was used as positive control of blood coagulation.

Sample	Concentration (g mL^{-1})	Clotting time average (s) ± s.d., *n* = 2	
		PT	aPTT
Fraction CC2F1	100	16.0 ± 0.0	266.0 ± 5.7
	50	14.5 ± 0.1	84.4 ± 9.4
	25	14.0 ± 0.1	60.5 ± 2.2
	5	14.3 ± 0.0	39.9 ± 1.3
	0.5	13.7 ± 0.6	31.3 ± 0.6
	0.05	12.6 ± 0.5	30.8 ± 0.2
Fraction CC2F2	100	>300.0	>300.0
	50	>300.0	>300.0
	25	>300.0	>300.0
	5	21.2 ± 3.4	>300.0
	0.5	13.9 ± 0.4	45.0 ± 1.4
	0.05	12.9 ± 1.2	30.0 ± 0.6
Fraction CC2F3	100	36.0 ± 1.4	>300.0
	50	14.9 ± 0.2	Not tested
	25	13.6 ± 1.4	Not tested
	5	13.0 ± 0.6	Not tested
	0.5	12.8 ± 0.4	Not tested
	0.05	13.1 ± 0.4	Not tested
Sodium heparin	100	>300.0	>300.0
	50	>300.0	>300.0
	25	>300.0	>300.0
	5	>300.0	107.0 ± 4.2
	0.5	12.7 ± 0.0	38.0 ± 2.3
	0.05	12.0 ± 1.5	29.9 ± 0.3
Distilled water		13.4 ± 2.1	30.2 ± 1.5

trol, sodium heparin, was slightly more active than fraction CC2F2 in the PT assay, but in the aPTT assay fraction CC2F2 was more active than heparin.

Characterization of fractions from *Eisenia arborea*

The compositional data, in Table 3, shows fraction CC2F2 to be composed of 56.2 % sugars and *ca.* 45 % sulfate groups. In this fraction just 1.3 % ± 0.8 % were uronic acids. The neutral sugar constituents of CC2F2 were fucose (47.6 %), xylose (35.5 %) and rhamnose (16.9 %). The IR spectrum of CC2F2 (Figure 2) showed typical absorption bands for a sulfated heterofucan, which agrees with the chemical analysis. That assignment was supported by the ^{1}H-NMR data that helped us to establish the glycosidic bond to be, mainly, (1 3) with sulfate group substitutions at C-4, and less frequently substitutions of sulfate groups at C-2 and C-3.

In contrast, the fraction CC2F1 was composed of a higher content of uronic acids (35 % ± 0.2 %), and just 2.8 % of sulfates. In agreement with that composition, the IR spectrum of CC2F1 showed typical bands for a polyuronic derivative which resemble the alginic acid, the universal polysaccharide from brown seaweeds. Unfortunately, the fraction CC2F3 could not be identified because the amount recovered was not sufficient for additional analysis.

DISCUSSION

Anticoagulant screening

From the five algal chlorophytes assayed for anticoagulant activity, *Cladophora sericea* and the three species of *Codium* (*C. cuneatum, C. amplivesiculatum,* and *C. brandegeeii*) were actives. The extract obtained at 25 °C from *Cladophora sericea* had 4 to 10 times the normal PT and aPTT. No citations were found in the literature on the anticoagulant activity of *C. sericea.* Extracts (25 °C and 80 °C) of *C. cuneatum* showed anticoagulant activity in both assays, whereas the extracts from *C. amplivesiculatum* and *C. brandegeeii* were active just for the aPTT assay. The anticoagulant activity found in the algae of the *Codium* genus is consistent with other studies, where it has been demonstrated than *C. dwarkense, C. indicum, C. tomentosum, C. geppi* (Shanmugam *et al*., 2002), *C. fragile* (Shanmugan & Mody, 2000), *C. pugniformis* (Matsubara *et al*., 2000), *C. giraffa* (De Lara & Álvarez, 1994), and *C. latum* (Uehara *et al*., 1992) are sources of substances with anticoagulant activity.

Of the red algae studied, only the extracts from *Dudresnaya colombiana* and *Gracilaria subsecundata* (25 °C and 80 °C) showed anticoagulant activity in the PT assay. In the aPTT assay the extracts from *Hypnea valentiae*, *Gelidium robustum,* and *Sarcodiotheca dichotoma* proved to be active. It is well documented than algae belonging to the Gelidiaceae, Gracilariaceae, and Gigartinaceae families contain various amounts of galactans such as agar or carragenans and the activity of extracts from red algae might be caused by the presence of those polysaccharides (Shanmugam & Mody, 2000). Because the extraction

Table 3. Chemical composition of the polysaccharide fractions CC2F1, CC2F2 and CC2F3 obtained from *E. arborea*.

Polysaccharide fraction	Percentage composition in dry base (average ± s.d., *n* = 2)					
	Total sugars	Fuc [a]	Xyl [a]	Rham [a]	Uronic acids	Sulfate [a]
CC2F1	77.2 ± 0.1	n.d.	n.d.	n.d.	35.4 ± 0.2	2.8
CC2F2	56.2 ± 0.1	47.6	35.5	16.9	1.3 ± 0.8	45.0
CC2F3	8.6 ± 0.5	n.d.	n.d.	n.d.	n.d.	25.9

[a] *n* = 1, [b] n.d. = not determined, Fuc = fucose, Xyl = xylose, Rham = rhamnose

method used in this study is not selective, we assume that the extracts from *Gracilaria subsecundata*, *Hypnea valentiae*, *Gelidium robustum,* and *Sarcodiotheca dichotoma* might contain small amounts of sulfated galactans as agar or carragenans. This would explain the activity observed. However, a the study on *Gelidium crinale* from Brazil demonstrated that the anticoagulant activity was caused by sulfated galactans structurally different from agar or carragenans (Pereira *et al*., 2005).

Of the 7 brown algae studied, just the extract obtained at 25 °C from *E. arborea* and that obtained at 80 °C from *H. clathratus* showed activity in the PT assay. The extract from *E. arborea* increases the PT (normal control 13 s) more than 22 times, but the extract from *H. clathratus* just doubles the normal PT. In the aPTT assay the extracts obtained at 25 °C from *Rosenvingea intricada*, *Chnoospora implexa*, *Ganonema farinosum,* and *Padina mexicana* had similar activity, increasing the normal aPTT more than three times. Extracts from *Eisenia arborea*, *Hydroclathrus clathratus,* and *Sargassum horridum* increase the aPTT more than 10 times. The extracts obtained at 80 °C from all these algae were active.

No reports of the anticoagulant activity from *E. arborea* and *H. clathratus* were found. However, it is known that the anticoagulant activity of the brown algae is related to the presence of fucoids (Shanmugam & Mody, 2000).

Characterization and activity of fractions from *Eisenia arborea*

From the compositional analysis, the predominant monomers were uronic acids in CC2F1 (35.4 %) and fucose in CC2F2 (47.6 %). In fraction CC2F3 it was not possible to detect any monomer because the sugar content was too low (8.6 %). Fraction CC2F1 had

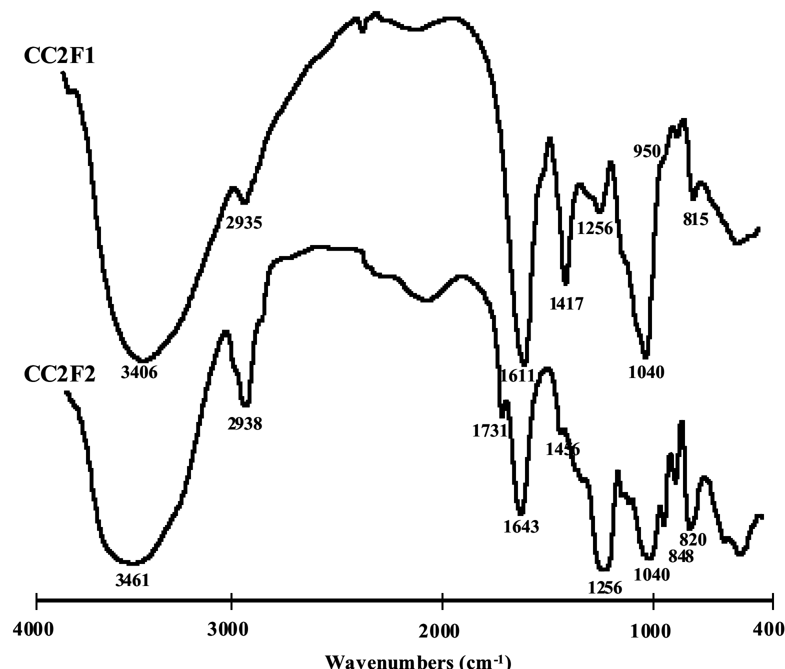

Figure 2. Infrared spectra of the polysaccharide fractions CC2F1 and CC2F2 isolated by ion exchange chromatography from *Eisenia arborea*.

the lowest degree of sulfation containing just 2.8 % of sulfate groups. Fraction CC2F2 had 45 % of sulfate content (Table 3).

The infrared spectrum of fraction CC2F1 (Figure 2) showed absorption bands at 3406 cm^{-1} attributable to vibration of the hydroxyl bond. The band at 2935 cm^{-1} was assigned to the stretching of the C–H bonds. Absorptions at 1611 cm^{-1} and 1417 cm^{-1} were interpreted as the vibration of a carboxyl function. The spectrum also showed bands at 1070 cm^{-1} and 1040 cm^{-1} corresponding to the vibrations of hemiacetal links of the sugar ring. The band at 1256 cm^{-1} is typically assigned to the vibration of the sulfate ester group. All these absorption bands in addition to those at 890 cm^{-1}, 815 cm^{-1}, and the little shoulder at 950 cm^{-1} agree with those reported for alginic acid salts (Sartori *et al*., 1997; Ronghua *et al*., 2003). The comparison of infrared spectra from CC2F1 and sodium alginate from *E. arborea* allowed us to make the matches (Murillo-Álvarez & Hernández-Carmona 2007). The polyuronic nature of CC2F1 was additionally supported by NMR data. The nuclear magnetic resonance of nuclide ^{13}C (^{13}C-NMR) spectrum showed a signal at   176.11 ppm typical of carboxyl groups. The signal of the anomeric carbon (C-1) was observed at   100.77 ppm. In addition, carbons of the sugar ring appeared between 60 ppm and 80 ppm. A peak at high field (17.65 ppm) was assigned to the methyl group of a deoxysugar, such as fucose (Patankar *et al*., 1993). All the analytical data strongly suggest that the composition of fraction CC2F1 is consistent with a polyuronic acid derived compound containing small amounts of sulfated fucose and other sugars (inferred by signals at   62.09 ppm and 60.35 ppm). Fraction CC2F1 was inactive in the PT assay, but it duplicated the normal control clotting time at *ca.* 25 g mL^{-1} in the aPTT assay. The moderate activity observed for fraction CC2F1 can be explained by its sulfate and fucose concentration, because the fucose and sulfate content and the substitution patterns have been recognized as important factors for the anticoagulant effect (Nishino & Nagumo, 1991; Nishino & Nagumo, 1992; Chizhov *et al*., 1999; Shanmugam & Mody, 2000).

The infrared spectrum of fraction CC2F2 shows typical absorption bands of fucoids. The band at 3461 cm^{-1} is attributed to the vibration of the hydroxyl function. The band at 2938 cm^{-1} corresponds to the stretching of the C-H bond. The band at 1731 cm^{-1} was assigned to vibration of an ester carbonyl group (Silva *et al*., 2005). The IR spectrum of CC2F2 also showed bands at 1070 and 1040 cm^{-1} corresponding to the vibrations of the hemiacetal of the sugar ring. The band at 848 cm^{-1} was attributed to the torsion of the ester group. The total sulfates in CC2F2 were inferred by the presence of the absorption band at 1250 cm^{-1}. The CC2F2 IR spectrum also showed sulfate bands at 840-845 and 830-820 cm^{-1}. According to previously reported data the band at 840-845 cm^{-1} is typical of axial sulfate groups (C4-SO_4) of fucose, and the shoulder at 830-820 cm^{-1} is an indication of equatorial sulfate groups (C2- SO_4 and-or C3-SO_4, Patankar *et al*., 1993; Chizhov *et al*., 1999; Marais & Joseleau, 2001), which suggests the substitution pattern of fucose to be mainly at C-4, with minor substitutions at C-2 and-or C-3. These findings are consistent with those reported in the literature for the fucoidan structure in *Cladosiphon okamuranus* (Tako *et al*., 2000), *Ascophyllum nodosum* (Chevolot *et al*., 1999), *Fucus vesiculosus* (Chevolot *et al*., 2001), and *Fucus evanescens* (Bilan *et al*., 2002). Fraction CC2F2 was active in the anticoagulant assays. In the aPTT assay fraction CC2F2 was more active than sodium heparin with a clotting time > 300 s at 5 g mL^{-1}, whereas sodium heparin gave a clotting time in the aPTT assay of 107 s at the same concentration. In comparison with fucans isolated from different sources, CC2F2 was more active than all the heterofucans isolated from *Dictyota menstrualis* (Albuquerque *et al*., 2004) and *Padina gymnospora* (Silva *et al*., 2005). The notable difference of anticoagulant activity between fractions CC2F1 and CC2F2 might be explained by the compositional relationship of sulfate:total-sugar-residue ratio. In previous works the sulfate:total-sugar-residue ratio approaching unity was related to the enhancement of the anticoagulant activity (Nishino & Nagumo, 1992). This can be observed in fraction CC2F2 with a sulfate:total-sugar-residue ratio near unity (45/56.2 = 0.8), whereas fraction CC2F1 has a ratio of 0.03.

The study of the potential of the algal resources found in Baja California Sur, Mexico as a source of anticoagulant active compounds showed in general that of every 10 aqueous extracts tested, 7 were active in the aPTT assay and 2 were active in the PT assay. This appears to be the first time in which the majority of the species assayed were studied this way. The algal resource found in Baja Ca-

lifornia Sur constitutes a great reserve of substances with anticoagulant activity and it should be thoroughly explored.

The study on characterization and anticoagulant activity of the polysaccharide fractions obtained from *E. arborea* resulted in our obtaining the highly sulfated heterofucan that we named CC2F2. The structural analysis of CC2F2 demonstrated it was composed mainly of fucose with substitutions of sulfate groups at C-4 (axial), and minor substitutions at C-2 and-or C-3 (equatorial). The heterofucan CC2F2 showed anticoagulant activity comparable to that of heparin.

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