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A NEW MOLECULE OF WATER-SOLUBLE POLYSACCHARIDE ISOLATED FROM *EUCALYPTUS* GROWING IN LEBANON

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ABSTRACT

Objectives: To study and compare the chemical structure characteristics of the polysaccharides extracted from leaves of *Eucaluptus globulus* with chemical structure of fucoidans extracted from *Dictyopteris polypodioides* brown algae. **Materials and Methods:** The water-soluble polysaccharides were extracted and the structures were identified by Fourier transform infrared spectroscopy, proton nuclear magnetic resonance, carbon-13 nuclear magnetic resonance and mass spectrometric analysis. **Results:** The total yield of fucoidans was 2.1% and 3.5% for Eucalyptus and Algae respectively. Results showed the presence of the typical structural of fucoidans: α-L-fucopyranose and fucoidan-like sulfated residues structures in both samples, whereas D-galactopyranosyl residues were absent in Eucalyptus. The main monosaccharides identified were fucose, glucose, galactose, xylose and glucuronic acid. Infrared characteristic band of sulfated groups were detected and sulfate substitutions of polysaccharides were indicated. **Conclusion**: Several similarities and small disparities in the structure of these polysaccharides were identified. The polysaccharide extracted from Eucalyptus leaves could be a promising new source of extra-marine fucoidans.

KEYWORDS: Fucoidan, Eucalyptus, Brown Algae, Polysaccharide.

1. INTRODUCTION

Marine algae are among the richest sources of bioactive sulfated polysaccharides, which possess important in-vitro pharmacological activities such as anticoagulant, antioxidant, antiproliferative, antitumoral, anti-inflammatory, antiviral, antipeptic and antiadhesive activities.[1,2] The structure of algal sulfated polysaccharides varies according to the species of algae.[3] Fucoidan belongs to a large family of marine sulfated polysaccharides, the fucans, mainly constituted of sulfated L-fucose. It is found mainly in the fibrillar cell walls and intercellular spaces of brown algae of the class Phaeophyceae. The chemical composition of fucoidans is extremely variable depending on eco-physiological parameters.[4] Fucoidan designates a group of certain fucose-containing sulfated polysaccharides that have a backbone built of (1→3)-linked α-L-fucopyranosyl or of alternating (1→3)- and (1→4)-linked α-L-fucopyranosyl residues, but also include sulfated galactofucans with backbones built of (1→6)-β-D-galacto- and/or (1→2)-β-D-mannopyranosyl units with fucose or fuco-oligosaccharide branching and/or glucuronic acid, xylose or glucose substitutions.[5] Fucoidans exhibit several bioactivities against a wide spectrum of pathological situations including antitumor, immunomodulatory, anti-inflammatory, antiviral, antithrombotic, anticoagulant and antioxidant effects as well as specific activities against kidney, liver and urinary system disorders, with a remarkable absence of adverse effects. The levels of L-fucose and sulfate as well as the molecular weight are major structural parameters whose variation affect the biological properties.[4,5]

In the other hand, Eucalyptus is one of the world's important and most widely planted genera. Eucalyptus species are well known as medicinal plants because of their biological and pharmacological properties. The most important and represented species is *Eucalyptus globulus*.[6] Among its main uses is the production of essential oils, which are used in the treatment of pulmonary infections and they possess antimicrobial and anti-inflammatory activities.[7]

Recently, water soluble polysaccharides were extracted from dried leaves of *E. globulus* and they showed in vitro antibacterial, antiproliferative and antioxidative

activities.[7,8] In this work, we aim to study and compare the chemical structure characteristics of these novel polysaccharides extracted from leaves of *E. globulus* with chemical structure of fucoidans extracted from *Dictyopteris polypodioides* brown algae.

2. MATERIALS AND METHODS

2.1. Samples collection

Thirty grams of *E. globulus* leaves were collected from the Lebanese University campus at Hadath, Beirut. The samples were air dried at room temperature in the dark for a few weeks. Their final moisture content was 10.0%. Before use, the dried samples were ground in a blender so that the particle size varies between 0.8-0.9 mm.

Twenty grams of the marine brown algae *D. polypodioides* was collected from the coast of Batroun-Lebanon and identified as described by Oksana and Kanaan.[9] The algae were cleaned, washed in distilled water and set to dry at room temperature.

2.2. Polysaccharides (Fucoidans) extraction

Fucoidans were extracted as described by Imbs *et al.*[10] Dried algae and Eucalyptus leaves were extracted twice with ethanol 96% for 3 h at 40℃ (Ethanol/ Eucalyptus or Algae: 1/0.8 w/w) to remove low molecular weight compounds and for depigmentation. The samples were centrifuged at 4000 r/min for 20 min. The supernatant was discarded while the residual was dried for 3 h and extracted twice with 150 mL of hydrochloric acid (pH 2.0–2.3) at 60℃. Again, the supernatant containing anionic and cationic polysaccharides, called fucoidan and laminarin respectively, was centrifuged (4000 r/min for 20 min) and chromatographed on a column (polytetrafluoroethylene, 15 cm × 6.5 cm). Fractions were eluted with water to obtain the fucoidan and elution was continued until the test of the phenolsulfuric acid showed the absence of carbohydrates in the eluate. All The chemicals were purchased from Sigma Aldrich Lebanon.

2.3. Proton and carbon-13 nuclear magnetic resonance (NMR) spectra

In order to identify the structure of fucoidans, NMR (^{1}H NMR and ^{13}C NMR) was performed. Three milligrams of the water soluble polysaccharide were dissolved in 0.5 mL of 99% deuterium oxide (D_2O). NMR spectra of the samples were recorded using Ultrashield Broker 300 spectrometer at room temperature with a frequency of 300 MHz, an acquisition time of 5.29 s and pulse duration of 11 ms. Tetramethylsilane was used as an internal standard.

Further ^{1}H NMR spectra was recorded after acidic hydrolysis of samples in order to identify monosaccharide composition of the fucose-containing sulfated polysaccharides. The full hydrolysis was performed in aqueous solution (4.3 mg/mL) by adding trifluoroacetic acid up to 0.8 M and heating the sample at 95°C for 6-8 hours. After cooling, the solution was evaporated under the N_2 flux and the residue was solved in D_2O.

2.4. Fourier transform infrared spectroscopy (FTIR) analysis

FTIR spectrum of the fucoidans extracted from *E. globulus* and *D. polypodioides* were recorded on a JASCO FT/IR-6300 spectrometer in order to reveal the polysaccharide functional groups. The resolution was 4 cm^{-1}. Data were collected in the range of 4000–400 cm^{-1}. All samples were prepared for the measurement in the form of KBr pellets (sample/ KBr: 2%).

2.5. Mass spectrometric (MS) analysis

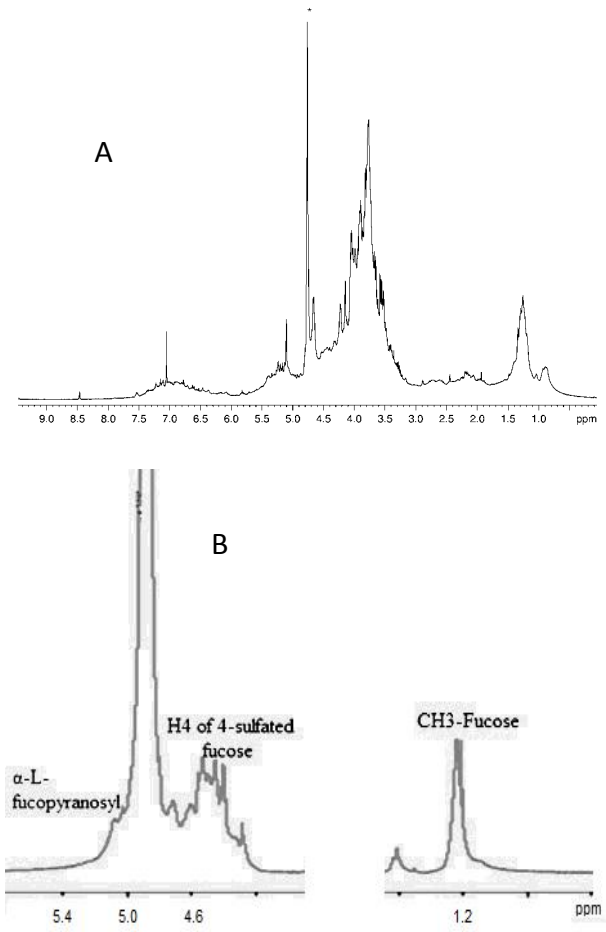
In order to investigate polysaccharides fragments, MS qualitative analysis was performed using LCMS-2020 Shimadzu instrument. Aqueous solutions (1mg/mL) of algae and Eucalyptus polysaccharides were prepared and 0.1 µL of solutions were directly injected. Instrument settings are as follows: positive and negative ionization; interface T: 350℃; nebulizing gas: N_2; gas flow rate: 0.2 mL/min; detector voltage: 1 KV; scan mode: 20<m/z<2000.

3. RESULS

The total yield of fucoidans extracted from *E. globulus* and *D. polypodioides* was 2.1% and 3.5% respectively.

3.1. Analysis of ^{1}H and ^{13}C NMR spectra

The ^{1}H NMR spectra of both *Eucalyptus* and brown algae samples, showed the presence of the anomeric signals at 5.0–5.4 ppm consistent with the presence of α-L-fucopyranosyl, as is the broad methyl signals at 1.2-1.3 ppm assigned to a C_6 methyl proton group of L-fucopyranose. A signal at 4.65 ppm can tentatively be assigned to H4 of 4-sulfated fucose (Figure 1A-B). The signals at 4.37 and 3.99 ppm were assigned to the presence of 4-linked 2-mono-O-sulfated L-fucopyranose residues, whereas the signals at 4.58 and 4.39 ppm were assigned to be due to disulfated residues (α3-linked 2,4-di-O-sulfated L-fucopyranose residues.[13]). The signal at 4.61 ppm in algal spectrum might be assigned to a 3-linked D-galactopyranosyl residue.[14]



*: The residual signal of water.

Figure 1 A: ^{1}H NMR spectrum of fucoidan isolated from the leaves of *E. globules.*

1B: ^{1}H NMR spectrum of fucoidan isolated from brown algae.

^{13}C NMR spectra of fucoidan is complex with major signals of L-fucane sulphates observed between 93.8 and 107.0 ppm (C1) and between 15.0 and 16.7 ppm (C6) (Figure 2A). The signal at 57.4 ppm in the ^{13}C spectra was tentatively assigned to C4. This signal is not present in the spectra of fucoidan extracted from brown algae (Figure 2B).

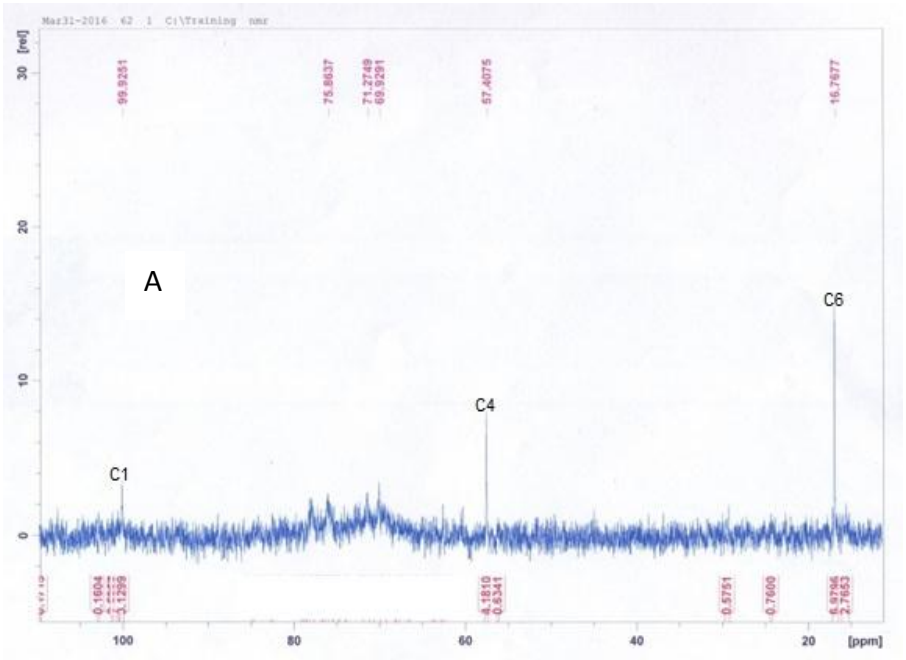


Figure 2A: ^{13}C RMN spectrum of fucoidan isolated from the leaves of *E. globules.*

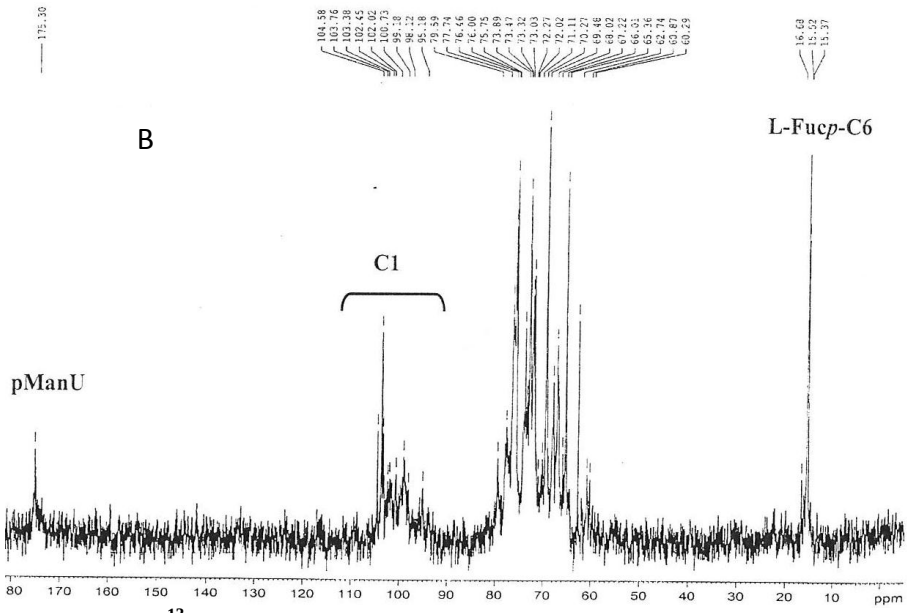


Figure 2B: ^{13}C RMN spectrum of fucoidan isolated from the brown algae.

3.2. Analysis of acidic hydrolysis products

The ^{1}H NMR analysis of hydrolysis products shows the presence of fucose, arabinose, galactose, glucose, xylose, rhamnose, and glucuronic acid (Figure 3). Monosaccharide composition of fucoidans from 2 samples was presented in table 1.

Table 1: Monsaccharides composition of fucans from Eucalytus leaves and brown algae.

	Monosaccharide Composition (Molar ratio with respect to fucose monomers set to 100)					
Samples	Fucose	Arabinose	Galactose	Glucose	Xylose	Rhamnose
E.globules	100	46.4	44.4	83.5	17.7	11.7
D.polypodioides	100	__	37.3	51.1	34.8	14.4

The anomeric signal of glucuronic acid at 4.70 ppm was partially overlapped with HDO signal in Eucalyptus sample and it was not possible to quantify it. Glucuronic acid and mannose are present in algae sample at lower levels. Not assigned hydrolysis products are also present giving additional ^{1}H NMR signals in the anomeric region (5.5 – 4.4).

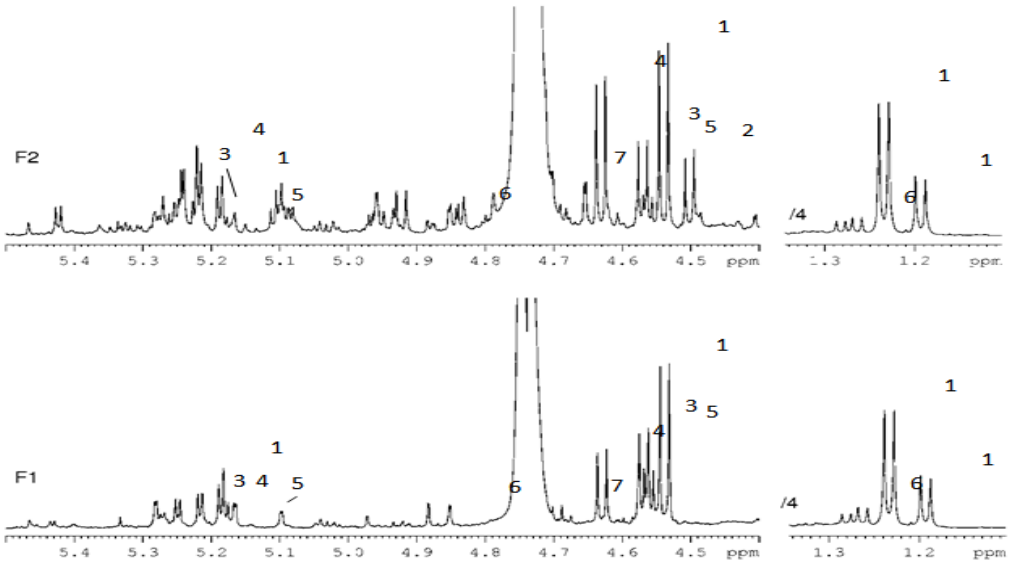


Figure 3: Characteristic regions of ^{1}H NMR spectra of F2 (Eucalytus) and F1 (Algae) acidic hydrolysis products. Assignments: 1, fucose; 2, arabinose; 3, galactose; 4, glucose; 5, xylose; 6, rhamnose; 7, glucuronic acid.

3.3. FTIR spectroscopic analysis

The FTIR spectra of fucoidan isolated from Eucalyptus leaves and brown algae are represented in figure 4A-B. The bands centered at around 3442 and 3448 cm^{-1} are assigned to the hydrogen bonded O-H stretching vibration, but Eucalyptus IR displayed wide absorption band. Weak bands at 2925 and 2928 cm^{-1} are assigned to a C-H stretching vibration. The bands centered at 1627 and 1634 cm^{-1} are assigned to the carbonyl group C=O (absorbance of uronic acid[11]) and the one at 1420 cm^{-1} is assigned to the C-O bond of the carboxylate group. The bands at 1235 and 1252 cm^{-1} are assigned to an S=O stretching vibration; Peaks at 1038 and 1043 cm^{-1} are assigned to the sulfate ester group. The small peak at 818.634 cm^{-1} is attributed to the C-O group of C-O-SO4. Sulfate groups at the equatorial C-2 and/or C-3 positions have been reported to give a small absorption at 820 cm^{-1}.[12] The peak between 510 and 560 cm^{-1} is assigned to the C-C=O bending. The band between 545 and 555 cm^{-1} is assigned to the -CH=CH2 vinyl compound, a residue of pollution of the environment.

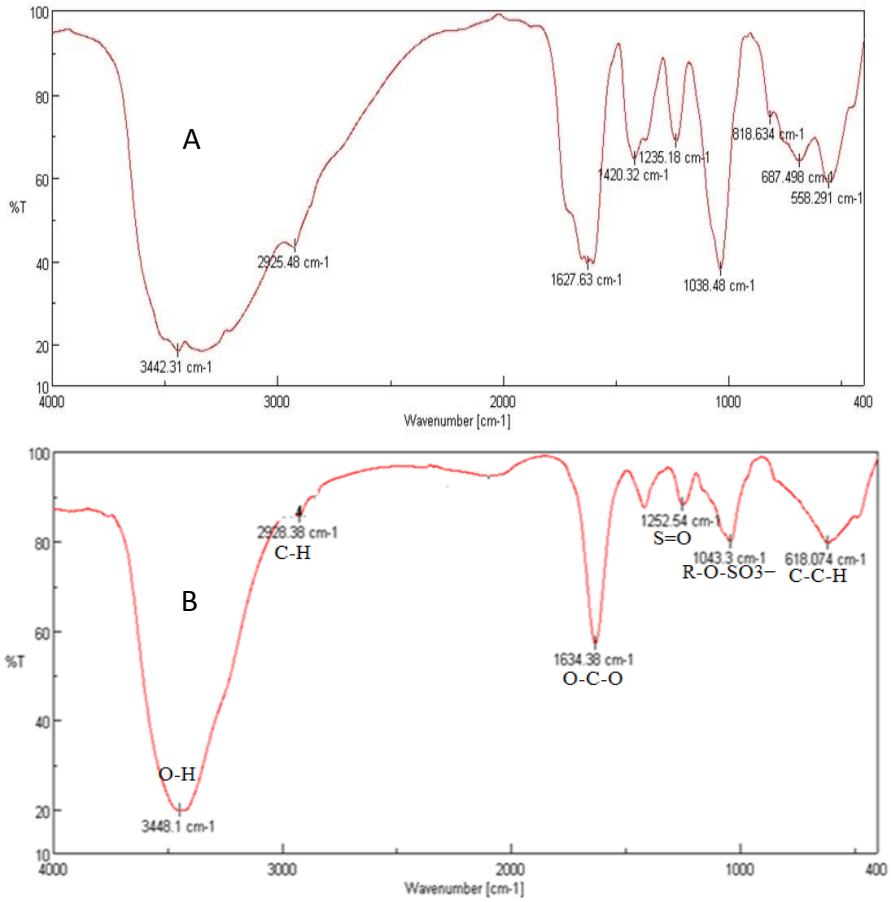


Figure 4A: Infrared spectrum of fucoidan isolated from the leaves of *E. globulus.*
4B: Infrared spectrum of fucoidan isolated from brown algae.

3.4. MS analysis

The spectrum of hydrolyzed fucoidans of both *E. globulus* and *D. polypodioides* were shown in figures 5 A and B respectively. The low intensity signals detected at m/z of 242 (figure 5A) and of 243 (figure 5B) were attributed to the sulfated fucose monomers. Signals detected at *m/z* 223 in high intensity in figure 5B and at 224 in low intensity in figure 5A might be assigned for dehydration of monosulfated fucose. Dehydrated galactose-containing residues might be marked with m/z of 177 in low intensity in *E. globules* sample but were absent in *D. polypodioides* sample. The signal at *m/z* 261 detected in brown algae in medium intensity was assigned to the presence of sulfated galactose. The signal at *m/z* 339 in both Eucalyptus and brown algae indicates the presence of glucuronic acid.[15,16] The spectra of both samples contained a set of intensive and moderate signals (*m/z* 111, 122, 142, 185, 205, 295, 311 and others); these signals might be due to disparities in polysaccharides composition.

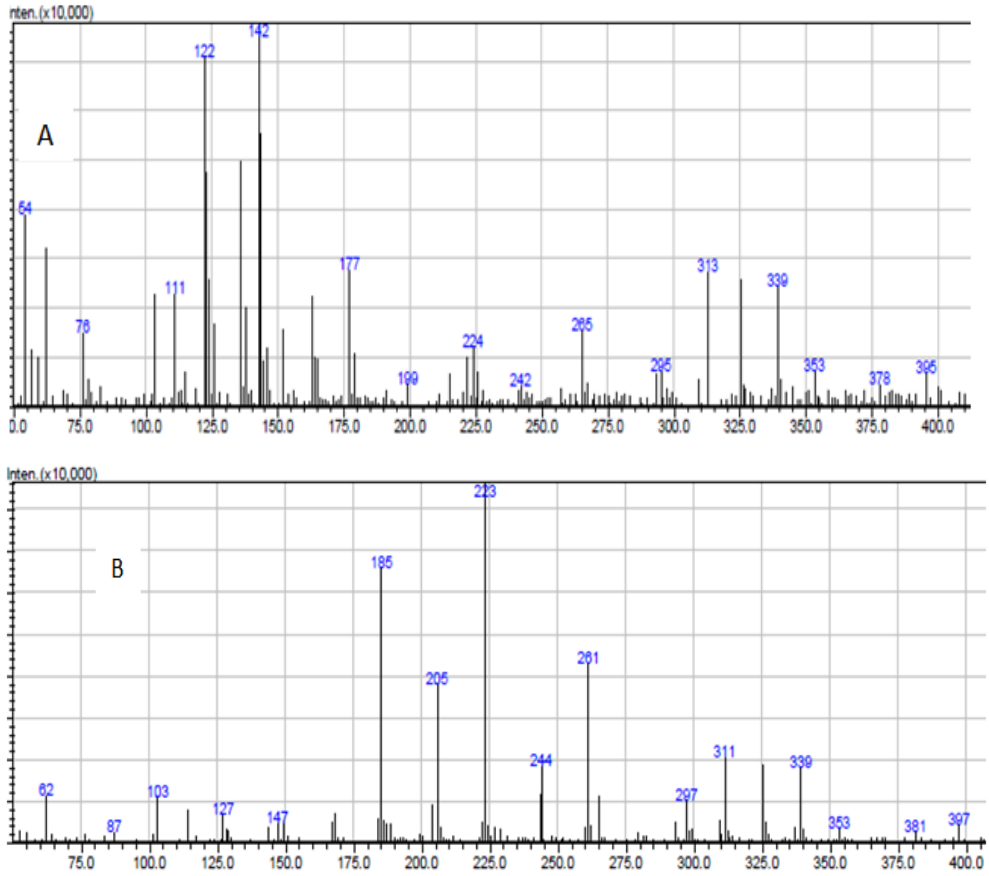


Figure 5 A: Spectrum of negative ion m/z fucoidan of *E. Globulus.*
B: Spectrum of negative ion m/z fucoidan of *D. polypodioides.*

4. DISCUSSION

In this study we aimed to investigate whether polysaccharides extracted from leaves of Eucalyptus and from brown algae contained fucoidan-like structures, composed of α-3-linked or/and α-3,4-linked L-fucopyranose residues, β-D-galactopyranose residues with fuco-oligosaccharide branching, and/or glucuronic acid, xylose or glucose substitutions.[5,17]

The yield of fucoidans extracted from *D. polypodioides* was higher than from *E. globules.* In agreement with reported chemical composition of the different order of brown seaweed species, the main monosaccharide components were fucose, glucose, xylose, galactose for algae and fucose, glucose, arabinose, galactose for Eucalyptus.[18]

The ^{1}H NMR spectrum of both samples showed signals consistent with the presence the typical structural of fucoidans in seaweeds: α-L-fucopyranose and fucoidan-like structures (α4-linked 2-mono-O-sulfated L-fucopyranose residues and α3-linked 2,4-di-O-sulfated L-fucopyranose residues).[17,18] The 3-linked D-galactopyranosyl residues, a typical structural component of fucoidan in brown algae, were not detected in Eucalyptus samples.

The ^{13}CNMR showed that L-fucane sulphates signals were observed at C1, C4 and C6 in Eucalyptus and not present at C4 in algae, indicating some structural differences between fucoidan extracted from Eucalyptus and from *D. polypodioides*. The sulfate groups on the fucans are typically located at C-4 of a (1→3)-linked unit or on C-2 of a (1→4)-linked residue.[19] The difference observed might be due to sample handling or extraction procedure.

With the exception of the peak at 818 cm^{-1}, the FT-IR spectra of fucoidan isolated from both species showed similarities in their infrared absorption properties. The IR spectra indicated that the sulfate substitutions of polysaccharides extracted from Eucalyptus were located in the equatorial C-2 and/or C-3 positions.[11]

Our results showed both several similarities and differences in the structure of these polysaccharides. Further studies are needed in order to resolve the repeating pattern of fucoidan oligomers.

Fucoidans are marine polysaccharides exhibiting a wide spectrum of biological activities with potential clinical applications. We proposed the polysaccharide extracted from Eucalyptus leaves as promising new source of extra-marine fucoidans. Further investigations are

required to elucidate structure, define the conditions of extraction and determine the reproducibility.

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