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# The effect of salinity on *Fucus ceranoides* (Ochrophyta, Phaeophyceae) in the Mondego River (Portugal)

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**Abstract** Algae (and their extracts) are increasingly important for pharmaceutical applications due to the diversity of useful compounds they contain. The genus *Fucus* contains one of the most studied species, *Fucus vesiculosus*. The species *F. ceranoides* differs from the others of the genus by presenting longitudinal air-vesicles and a capacity to survive at low salinities. It is an alga that inhabits the Mondego River estuary (Portugal), at the southern limit of its distribution, and can serve as a role model to understand the effect of a salt gradient on the production of bioactive compounds. We assessed the phenolic content and antioxidant activity of different *F. ceranoides* extracts (e.g. methanolic, aqueous and polysaccharide) prepared from samples harvested from two different zones to evaluate if the adaptation of *F. ceranoides* to different salinity levels influenced its chemical composition. The antioxidant activity of the extracts was determined using 1,2-diphenyl-picrylhydrazyl (DPPH) and 2.2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals. These assays demonstrated that the methanolic extract of lyophilized *F. ceranoides* that grew at low salinities was the most bioactive, i.e. DPPH ($IC_{50}$=50.39 μg/mL) and ABTS (TEAC=2.42). The total phenolic content (Folin-Ciocalteu method) and the methanolic extract of the lyophilized *F. ceranoides* collected from a low salinity habitat exhibited the highest phenolic content (PGE=49.48 μg/mg of lyophilized extract) amongst those sampled. Thin layer chromatography (TLC) and Fourier transform infrared spectroscopy (FTIR) were used for the identification of compounds in the extracts. This characterization allowed confirmation that the various extracts contained almost the same compounds but with notable quantitative differences. Based on these results, we conclude that there were differences in the quantity of the compounds due to the effect of salinity. The drying methods used were also found to have influenced the quality of the extracted compounds.

**Keyword**: *Fucus ceranoides*; salinity gradient; sequential extractions; phenolic content; dependent bioactivities; spectroscopic analysis

## 1 INTRODUCTION

The genus *Fucus* has been extensively studied in many cases and it is the dominant genus in coastal areas (especially in estuaries and port areas). It is considered to be at a low risk of over-exploitation since only small amounts are currently harvested to use as a soil fertilizer and animal feed in the north of Portugal. Sigma-Aldrich produces a fucoidan and alginic acid purified from *F. vesiculosus*.

*Fucus ceranoides* is currently the least studied species of the genus and it is characterized by being a greenish-brown alga with a dichotomously branched thallus. It has a prominent central vein, which is identical to that found in other species of the genus.

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*F. ceranoides* differs from other species of *Fucus* by the presence of a thallus with longitudinal vesicles, running parallel to the central vein, which can be expanded. Matured *F. ceranoides* can usually range in size of 30–60 cm, including terminal receptacles.

*Fucus ceranoides* is an endemic alga to the North Atlantic, and the Mondego River is the southern-most record of its European distribution. The occurrence of *F. ceranoides* in the Mondego River is constant in a range of about 13 km from the river mouth in Figueira da Foz (Portugal) to near Verride (Montemor-o-Velho, Portugal). This species is considered dominant in the estuarine tidal zones of the Mondego River, being subject to fresh water due to the tidal cycle. Towards the sea, it is replaced by *F. vesiculosus* and *F. spiralis*.

*Fucus ceranoides* can survive in salinities of 5–34 (the local salinity range is 34–36) throughout its life cycle (Khfaji and Norton, 1979). However, under laboratory conditions, the alga can survive 1.5–45 salinities during its first 10 weeks until it starts to show signs of tissue damage.

*Fucus ceranoides* can also survive a wide temperature range (7–17°C) without adversely influencing its growth (Bäck et al., 1992). Compared with other species such as *F. vesiculosus*, *F. ceranoides* does not grow quickly in a fully marine zone (e.g. salinities 30–34). It grows better in an intermediate salinity (24) environment. *F. ceranoides* is better adapted to lower salinity environments than other species of the same genus. Even its zygotes can form at a salinity of 8.5 and reproduce with success (Brawley and Johnson, 1992; Serrão et al., 1999).

The chemical composition and biological activity of extracts obtained from species of *Fucus* (in particular, *F. vesiculosus*) have been extensively studied. The most important bioactive compounds present were generally divided as follows: pigments (chlorophyll and fucoxanthin), polysaccharides (alginic acid and fucoidans), and phenolic compounds (mainly phlorotannins) (Pereira, 2016, 2018).

Among the pigments, fucoxanthin is the main carotenoid pigment with a chemical structure different from other carotenoids such as astaxanthin and *β*-carotene, but similar to neoxanthin, dinoxanthin, and peridinin (Peng et al., 2011). The fucoxanthin molecule has an unusual allenic bond that is responsible for most of the antioxidant power of the compound and a set of oxygen functional groups such as epoxy, hydroxyl, carbonyl, and carboxyl, amongst others (Peng et al., 2011). This pigment has known diverse bioactivities such as a high antioxidant activity (Xia et al., 2013), anti-inflammatory, and anti-diabetic properties, amongst others (Peng et al., 2011).

In terms of polysaccharides, species of *Fucus* contain alginic acid and fucoidans that provide both structural support and protection against mechanical damage. Fucoidans are characterized by having sulphated groups and a high content of fucose. Their basic structure is identical between sub-classes, the main difference being the conformation of the fucopyranose molecule, and how glucose, mannose, and xylose, amongst others, are linked. The chemical structures of these compounds have not been completely characterized (Cumashi et al., 2007). Currently, fucoidans (although with a relatively high degree of impurities) are used as dietary supplements and each sub-type may present different types of bioactivity (Cumashi et al., 2007; Imbs et al., 2013; Fitton et al., 2015).

The only bioactive compound from brown algae that is currently used by the pharmaceutical and health industries, is alginic acid as a drug carrier and healing gel (Pielesz et al., 2011). Alginic acid is an anionic, non-sulphated polymer, it is located extracellularly maintaining the physical integrity of the thallus, protecting it from mechanical damage.

In terms of polar compounds, phlorotannins which are phloroglucinol derivatives, with variable molecular weight and different conformations and connection types, act as active defense mechanisms, especially against UV exposure and herbivory (Singh and Bharate, 2006).

There is a general lack of information about the phytochemical composition and bioactivities of the components of *F. ceranoides*. The only study discussing the biological activity of extracts prepared from this species was written by Zubia et al. (2009), which reported that the crude extract of *F. ceranoides* had a higher antioxidant potential than *F. serratus*. However, the study did not specify details of the habitat from which the samples were collected. Several studies have suggested that environmental conditions, such as salinity, may have a significant influence on algal chemical composition. Bäck et al. (1992) stated that there were differences in the concentration of the sugar alcohol mannitol due to salinity and Beauchamp (2012) reported an increased concentration of chlorophyll and pigments in *Ulva lactuca* (Chlorophyta) and *Palmaria palmata* (Rhodophyta), correlated with the decreases in salinity. Note to the seasonal differences that may cause modifications on the chemical composition and

**Table 1 Physico-chemical parameters of water in algae collection areas (September 2014) (mean±SE)**

| Zone | pH | Temp. (°C) | Salinity | Conductivity (μS/cm) | Transparency (m) | Oxygen (%) | Oxygen (mg/L) |
|---|---|---|---|---|---|---|---|
| A | 7.73±0.01 | 21.3±0.1 | 34.93±0.01 | 48 812.0±0.1 | 3.50±0.25 | 145.0±0.1 | 10.63±0.01 |
| B | 7.65±0.01 | 22.3±0.1 | 25.17±0.01 | 37 440.0±0.1 | 1.80±0.25 | 102.7±0.1 | 7.72±0.01 |

**Table 2 Levels of nutrients in the water taken from the collection areas (September 2014) (mean±SE)**

| Zone | Silica ($\times10^{-6}$ Si) | Ammonia ($\times10^{-6}$ N) | Nitrogen nitrate $NO_3$+$NO_2$ ($\times10^{-6}$ N) | Nitrites ($\times10^{-6}$ N) | Phosphate ($\times10^{-6}$ P) |
|---|---|---|---|---|---|
| A | 1.173±0.015 | <0.02* | 0.072±0.025 | <0.066** | 0.092±0.02 |
| B | 1.368±0.015 | <0.02* | 0.303±0.025 | <0.066** | 0.056±0.02 |

*: limit of detection; **: limit of quantification.

bioactivity of the algae, such as physiological responses to abiotic factors (changes of temperature, salinity, nutrients, luminosity, etc.) or in the seasonal development of the algae (Stengel et al., 2011).

Compounds of commercial interest which have been previously identified in various species of *Fucus*, such fucoxanthin, phlorotannins, alginic acid and fucoidans, justify the study of other less known species such as *F. ceranoides* and also the need to take into consideration the environmental and raw material processing conditions which may impact the recovery of these bioactive compounds. The aim of this study was to perform a comparative analysis of an alga which is able to withstand prolonged periods of low salinity. To do this, samples were collected from two zones with different salinity conditions and which were also treated with different drying methods after harvest. The total phenolic content of the algal extracts was determined, and the chemical composition assessed using TLC and FTIR-ATR spectroscopy. Furthermore, the antioxidant activities of the extracts were characterized by two methods (i.e. 1,2-diphenyl-picrylhydrazyl (DPPH) radical-scavenging and 2.2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical-scavenging) to provide an indication of the effect of salinity on *F. ceranoides*.

## 2 MATERIAL AND METHOD

### 2.1 Chemical

Methanol, *n*-hexane, acetone, DPPH, Folin-Ciocalteu reagent, ABTS, hydrogen peroxide, petroleum ether, sodium carbonate, trolox, and phloroglucinol were purchased from Sigma-Aldrich, Quimica S.A., Portugal. Ethanol was obtained from Applichem Panreac, and potassium persulphate from Merck, Darmstadt, Germany.

### 2.2 Seaweed collection

Samples of *Fucus ceranoides* were collected from two different sites, 13 km apart, along the Mondego River. The collection spots were: Zone A-Figueira da Foz (Mondego River mouth) (coordinates: 40°8′45.81″N; 8°52′25.53″W); Zone B-Verride (upstream part of the estuary, about 13 km from the Mondego river mouth) (coordinates: 40°7′31.39″N, 8°46′15.76″W). According to data received from MARE (Marine and Environmental Research Center, University of Coimbra) the two collecting zones (A and B) exhibited a difference of about 10 in the salinity gradient, demonstrating an increased freshwater flow in zone B, during the month of the harvest (September 2014; Table 1). MARE also provided data for nutrient analysis for that month (Table 2).

Sampling was conducted in September 2014; the sampling was from the sites with well-established *F. ceranoides* patches and without epiphytes or degradation. Once harvested, the samples were stored in plastic bags for transport to the laboratory, in a cool box. All samples were washed thoroughly with distilled water to remove salts, sand, and epiphytes. The samples were dried using two methods: sun drying and lyophilization. The algae were dried in the sun during daytime and at night in an oven (at 30°C, a box with silica gel was added to reduce air humidity) for two consecutive days before they were completely dried. Lyophilization of the thalli was carried out for 24 h in a freeze drier (FTS EZDRY EX1501, USA, $T$=-50°C and $P$= 60 mTorr). The dried alga was finely ground with a commercial mill (≤1 mm) in order to render the samples uniform, and then, stored in a dark room, in a box with silica gel to reduce the humidity, at ambient temperature (±24°C). The sun-dried samples were referred to using the designated collection zone (either A or B), and the lyophilized samples were labeled LA and LB, respectively.

### 2.3 Sequential extraction method

A sequential extraction technique was used to separate non-polar, polar and polysaccharide compounds for further study. For the extraction of non-polar compounds, the powdered algal sample was immersed in *n*-hexane (1:20 w:v) in constant agitation for 20 min. The recovery of the *n*-hexane extract was made with a Gooch filter system (porosity G3) under vacuum. The second step consisted of the extraction of polar compounds using methanol and the algal residue (1:20 initial weight of algal powder:v) recovered from the first extraction. The mix of algal residue and methanol was constantly agitated for 20 min, the methanolic extract was recovered with Gooch funnels (porosity G3) under vacuum. The methanol residue was subsequently transferred to water after evaporation of the solvent using a rotary evaporator (Bucchi R-3000, SUI) which was then lyophilized. For the final extraction, the algal residue recovered from the methanolic extraction was immersed in 100 mL (1:100 initial weight of algal powder:v) of distilled water and heated 100°C for 2 h, on a heating plate. The solution was filtered through Gooch funnels (porosity G2 and G3). The dissolved polysaccharides in the filtered aqueous solution were precipitated by adding ethanol (1:1 v:v ratio). The solid polysaccharide was recovered with the aid of a metal spatula and the remaining solution, was rotary evaporated (in a water bath at 37°C) in order to recover the ethanol. After evaporation of the ethanol, this aqueous concentrate was lyophilized. All the extracts were stored at -20°C until use in the assays.

The polysaccharides recovered from the aqueous solution were purified by submerging in ethanol for 24 h (-4°C). After this time, the ethanol was discarded, by decantation. The extracted polysaccharide samples were oven dried under vacuum at 40°C.

### 2.4 Determination of total phenolic content (TPC)

The total phenolic content (TPC) of the *F. ceranoides* methanol extracts was assessed using the method of Tierney et al. (2013). Phloroglucinol was used as the standard for assessment of total phenol content. A methanolic phloroglucinol stock solution (100 µg/mL) was diluted with different volumes of methanol in order to make a serial dilution series (e.g. 5–40 µg/mL) for determination of a standard curve. For the analysis, 100 µL of MilliQ water, 100 µL of methanolic extract or standard solution, 100 µL of Folin-Ciocalteu reagent, 500 µL of 20% aqueous solution of sodium carbonate and 200 µL of MilliQ water were added to tubes. The samples were immediately vortexed for 30 seconds and incubated in the dark for 45 min at room temperature. The absorbance of the supernatant was measured at 750 nm using a Cintra 101 spectrophotometer. All measurements were carried out in triplicate. The measurements were compared to a calibration curve generated using the phloroglucinol standards and expressed as microgram phloroglucinol equivalents (PGE) per milligram of extract sample (µg PGE/mg).

### 2.5 Thin layer chromatography (TLC) for pigments

The method for detection of pigments in the algal samples was adapted from Mikami and Hosokawa (2013). Briefly, the lyophilized methanolic extracts were solubilized in methanol (5 mg/mL). After activation of the silica gel TLC plate (F254, Merck) (120°C for 5 min.), 20 µL of each extract were applied and developed in a chromatography chamber containing petroleum ether: acetone (7:3, v/v) until the front reaches a height of 10 cm. The plate was then removed and after complete evaporation of the solvent at room temperature, was observed under visible light. The identification of the constituents was obtained by calculating the retention factor (Rf) as follows: Rf=compound migration distance (cm)/ distance traveled by the eluent and comparing with a literature review (Jeffrey, 1972; Jeffrey and Humphrey, 1975; Mikami and Hosokawa, 2013).

### 2.6 Spectroscopic analysis

#### 2.6.1 UV/vis spectroscopy

The methanolic extracts were taken up in 70% acetone, the soluble fraction (70% acetone fraction) was separated from the insoluble fraction which was solubilized in methanol (Koivikko, 2008). The UV/ visible spectra of the methanolic fractions were obtained in a UV spectrophotometer (Hitachi U2000; V: GBC Scientific Equipment Ltd. Cintra 101) after appropriate dilution (between 4× and 30×).

#### 2.6.2 FTIR-ATR spectroscopy

FTIR-ATR spectra were recorded on an IFS 55 spectrometer, using a Golden Gate single reflection diamond ATR system, with no need for sample preparation, since these assays only required dried samples, according to Pereira et al. (2013). All spectra

are the average of two independent measurements with 128 scans, each at a resolution of 2 $cm^{-1}$.

### 2.7 Antioxidant assays

#### 2.7.1 DPPH radical assay

Free radical-scavenging activity was measured according to Blois (1958). Aliquots of methanolic extract (100 μL) were assessed by their reactivity with a methanolic solution of 500 μmol/L DPPH (500 μL) in the presence of 100 mmol/L acetate buffer, pH 6.0 (1 mL). Reaction mixtures (3 mL) were kept in the dark at room temperature for 30 min. The decrease in absorbance was measured at 517 nm. Different dilutions of each of the methanolic extracts were assayed and the results were calculated by interpolating the absorbance on a calibration curve obtained with Trolox (62.5–1 000 μmol/L).

The experiment was performed in triplicate for each of the sample extracts. Results were expressed as $IC_{50}$, defined as the concentration needed for neutralization of 50% of radicals and as Trolox equivalent antioxidant capacity (TEAC), defined as the concentration of the sample solution whose antioxidant capacity is equivalent to a 1.0 mmol/L solution of Trolox.

#### 2.7.2 ABTS (pH=7) radical assay

Free radical-scavenging activity was evaluated according to the method described by Re et al. (1999). The ABTS•+ radical was produced by the oxidation of 7 mmol/L ABTS with potassium persulphate (2.45 mmol/L, final concentration) in water. The mixture was allowed to stand in the dark at room temperature for 12–16 h before use, and then the ABTS•+ solution was diluted with MilliQ water at pH 7.4, in a proportion of 1:80 (v:v), to give an absorbance of 0.7±0.02 at 734 nm. Aliquots (50 μL) of 80% aqueous methanol of the methanolic extracts were mixed with 2 mL of the ABTS•+ preparation, vortexed for 10 s, and the absorbance was measured at 734 nm after 240 s. of reaction at room temperature. Different dilutions of each of the sample extracts were assayed and the results were obtained by interpolating the absorbance on a calibration curve obtained with Trolox (62.5–500 μmol/L). The results were expressed as TEAC values. The experiment in triplicate was performed for each of the sample extracts.

### 2.8 Statistical analysis

All of the statistical analyses were performed with Microsoft Excel and the IBM SPSS 22 in the case of Pearson's correlation test between TPC and antioxidants assays (DPPH and ABTS).

## 3 RESULT AND DISCUSSION

During the sampling procedure, it was observed that *F. ceranoides* exhibited different ecological characteristics in the sampling zones (A and B). In zone A, the alga was more common on the upper plateau of the intertidal zone in small patches, surrounded by *F. vesiculosus*, being the dominant alga of that zone. It was also noted that there were examples of *F. ceranoides*, mainly with *F. vesiculosus*, this was noted by the hybrids presenting dual characteristics from the two species. During a field visit on March 2015, to observe the patches and ecology of *F. ceranoides*, it was possible to verify an increase in the number of specimens and the patches occupied by this species, in the sampling area. The *F. ceranoides* colonized the upper level of the tide-high zone, while at the lower level the dominant algae were *F. vesiculosus*. In this zone, the specimens were very exposed to mechanical agitation by tides and currents and also adverse weather conditions. In zone B, the alga colonized the upper part of the zone influenced by the tides, being mostly protected by a species of terrestrial grass during low tide. The size of the area that *F. ceranoides* occupied in zone B was most recognizable during the high tide, due to a dense muddy substratum which camouflaged and protected the alga during the period between full tides. In this site, there were some specimens of *F. ceranoides* in the unprotected spots on the rocky substratum, but in a lower number than the *F. ceranoides* protected by the muddy substratum.

The physicochemical parameters (Tables 1, 2) of the sampling sites were provided by Dr. Ana Carla Garcia (Marefoz Lab-MARE UC). These data showed that the most notable differentiation between the selected zones was salinity, the difference of which was 9.76. The second most influential physicochemical parameter was oxygen as a result of more or less agitation of the water, this resulted in the agitation of the water in zone A, and because of that agitation, the algae produced more structural elements, such as alginic acid, to protect the thalli from the risk of damage.

Regarding nutrients, there were differences in the sampling zones, but the biggest difference was in the concentration of nitrate and nitrites which were of greatest concentration in zone B. These are essential nutrients for the metabolism of *F. ceranoides*.

**Table 3 Total phenolic content of *F. ceranoides* extracts (mean±SD; *n*=3)**

| Sample | Methanolic extract (μg PGE/mg of lyophilized extract) | Aqueous extract (μg PGE/mg of lyophilized extract) | Total phenolic content (μg PGE/g of dried algae) |
|---|---|---|---|
| A | 25.73±0.83 | 12.89±0.91 | 3 310.13±9.32 |
| B | 13.36±1.27 | 12.38±0.35 | 1 438.35±11.89 |
| LA | 19.37±0.27 | 42.38±0.37 | 3 581.59±4.94 |
| LB | 49.48±3.94 | 31.59±2.88 | 2 896.71±4.83 |

### 3.1 Determination of total phenolic content (TPC)

The results obtained for the quantification of total phenols are presented in Table 3. With the exception of the LA sample, all of the methanolic extracts exhibited a higher phenolic content than in their corresponding aqueous extracts. This could be due to the higher solubility of the polar compounds in methanol but might also because, in the sequential extraction, the methanol was used first and removed the polar compounds before the water was used. In relation to the aqueous extracts, the influence of the drying process on the two samples was evident; the lyophilized samples had the highest content of phenolic compounds: LA (42.38±0.37) μg PGE/mg and LB (31.59±2.88) μg PGE/mg, whereas those samples dried in the sun, much lower values were obtained: A (12.89±0.91) μg PGE/mg and B (12.38±0.35) μg PGE/mg.

On the estimated total phenolic content, the LA sample had more global phenolic content in one gram of dried alga (3581.59±4.94) μg PGE amongst the samples tested, these results were due to the aqueous extract from LA sample and not because the methanolic extract (LA: (19.37±0.27) μg PGE/mg; LB: (49.48±3.94) μg PGE/mg). The total phenolic content was estimated from the samples extract because we obtained the final biomass from methanolic extract, and that was sample A: 103.6±0.004 mg/g dried alga; sample B: 44±0.016 mg/g dried alga; sample LA: 33.5±0.007 mg/g dried alga; sample LB: 36.9±0.015 mg/g dried alga. In the aqueous extracts the biomass was: A sample: 50±0.024 mg/g dried alga; B sample: 68.7±0.029 mg/g dried alga; LA sample: 69.2±0.012 mg/g dried alga; LB sample: 33.9±0.008 7 mg/g dried alga.

The results suggested that there was a degradation of some bioactive phenolic compounds during sun drying, although in the case of the methanolic extract from sample A, the phenolic content in the sun-dried sample was higher than that of the freeze-dried sample from the same area (A: (25.73±0.83) μg PGE/mg; LA: (19.37±0.27) μg PGE/mg). Compared with the data obtained by Zubia et al. (2009), there was a higher concentration of phenolic compounds in the LB samples. In general, the results obtained in this study indicated that *F. ceranoides* could be a good source of phenolic compounds, comparing to other data obtained by Tierney et al. (2013) for *F. spiralis*. Note, that the sampling date from Tierney et al. was February 2009 and the collection date for this study was from September 2014 so, seasonality and other abiotic factors could influence the analysis results. Further studies are required for a complete analysis to make the comparisons more detailed.

### 3.2 Thin layer chromatography (TLC) for pigments

Thin-layer chromatography was used to determine the composition of the methanolic extracts (Fig.1).

The three main pigments were immediately resolved: chlorophyll *a*; fucoxanthin and chlorophyll *b*. The spot with the highest Rf corresponded to chlorophyll *a* (Rf=0.38) and it was only visible in the lyophilized samples. This suggested that sun drying could have degraded this pigment (Jeffrey, 1972; Jeffrey and Humphrey, 1975; Mikami and Hosokawa, 2013). The carotenoid pigment fucoxanthin (Rf=0.25) was visible in greatest quantities in the freeze-dried samples (LB>LA), not being detected in sample B. Finally, the spot with the smallest Rf corresponded to chlorophyll c (Rf=0.05). This pigment was present in all samples but most prominent in sample A, and not so much in the lyophilized samples. Once again, the drying process influenced the chemical composition of the resultant extracts. In all samples, it was possible to visualize *β*-carotene spots which eluted in front of the solvent, but whose concentration was too low to be detected at the end of the elution. Another point to note was the existence of another unidentified yellow-orange pigment, just below chlorophyll *a* in the freeze-dried samples (Rf=0.37). For further elucidation, in this case, it would be necessary to resort to more specific testing in order to obtain the specific identification of the pigment/xanthophyll.

### 3.3 Spectroscopic analysis

#### 3.3.1 UV/visible spectroscopy

The wavelengths of maxima and inflections in the UV/vis spectra of fractions allowed for the detection

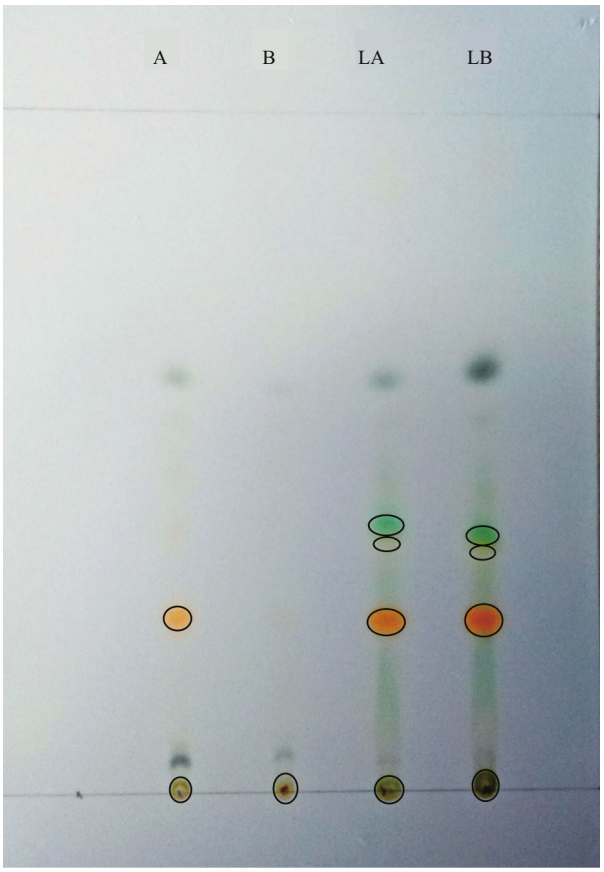


**Fig.1 Chromatographic profile of pigment plate**

of their major chemical constituents (Table 4), such as pigments and phlorotannins, as well as evaluate the effect of the drying processes applied.

Significant differences in the composition of the methanolic extracts were observed, such as the non-existence of the 430 nm maxima in the acetone fraction of the extract from B and shorthand (sh) maxima from sample A in the acetone fraction. The influence of the drying process was also evident, especially in the composition of the 70% acetone fraction of the methanolic extract.

The visible spectra were characteristic for the absorption maxima of fucoxanthin (Wright and Jeffrey, 1987), and more clearly in the lyophilized (LA: 434 nm; LB: 435 nm) rather than sun-dried samples. Sample A had an inflection in the zone of absorbance between the wavelengths of 430–435 nm which is a characteristic peak for fucoxanthin. Chlorophyll *a* and *c* were responsible for two other maxima observed in the visible zone (664/665 and 407/410/415/417 nm). They were found to vary in intensity according to the concentration of each of the chlorophylls (Jeffrey and Humphrey, 1975).

In the methanol fraction, in the visible zone, only

**Table 4 Absorbance maxima obtained during the spectrophotometric analysis of the samples**

| Extract | Fraction | Sample | UV (nm) | V (nm) |
|---|---|---|---|---|
| Methanolic | Acetone 70% | Met A | 214 | 665; 410; 444sh |
| | | Met B | 216 | 665; 408 |
| | | Met LA | 225 | 664; 434; 417 |
| | | Met LB | 232 | 664; 435; 415 |
| | | Phloroglucinol | 210; 268 | - |
| | Methanol | Met A | 209; 254 | 665; 406 |
| | | Met B | 208.4; 265 | 665; 407 |
| | | Met LA | 210; 269 | 665; 412 |
| | | Met LB | 211; 271 | 665; 409 |

- means on that sample, no peaks were detected in that assay.

maxima of absorbance from the chlorophylls where detected, since there is only a maximum around 400 nm, and this maximum varies amongst the samples analyzed. The analysis of the UV spectra allowed for the detection of the presence of characteristic bands of phenolic compounds, namely phloroglucinol, whose characteristic maximum at 210 nm was present in all samples. This was further validated by running a standard sample of phloroglucinol the result of which further supported by the works of Koivikko et al. (Koivikko et al., 2005; Koivikko, 2008). However, differences between the sun-dried and lyophilized samples were observed in the area between 254–271 nm, this suggested the occurrence of changes of phenolic compounds during the drying process in the sun (likely due to degradation or oxidation). This was most notable in sample A.

### 3.3.2 FTIR-ATR spectroscopy

FTIR-ATR analysis allowed the identification of chemical bonds types present in the samples. Since the extracts were not merely containing one compound, we can only speculate its complexity, more specifically the methanolic extract due to the lack of other published analytical studies and availability of standards to calibrate the analyses. It was decided to only run an FTIR-ATR analysis of the lyophilized samples.

In the methanolic extracts, we identified some fucoxanthin bonds as also indicated by Yip et al. (2014). The characteristic peaks were at the same values referenced by these authors. It was not possible to analyze the most characteristic peak of the allenic bridge since even a pure sample of fucoxanthin has a peak of low intensity. The peaks present in the samples

**Table 5 FTIR analysis of the methanolic extracts from lyophilized samples**

| Bonds | Wave number ($cm^{-1}$) | Sample | Compound |
|---|---|---|---|
| | 384 | LB | Phloroglucinol |
| | 386 | LA | Phloroglucinol |
| | 532 | LA, LB | Phloroglucinol |
| | 623 | LA, LB | Phloroglucinol |
| | 1 159 | LA | Phloroglucinol |
| | 1 163 | LB | Phloroglucinol |
| S=O | 1 244 | LB | - |
| S=O | 1 248 | LA | - |
| -C-H | 1 377 | LA, LB | Fucoxanthin |
| -C-H | 1 458 | LA, LB | Fucoxanthin |
| -C=O | 1 736 | LB | Fucoxanthin |
| -C=O | 1 738 | LA | Fucoxanthin |
| O-CH3 | 2 852 | LB | Fucoxanthin |
| O-CH3 | 2 854 | LA | Fucoxanthin |
| -CH | 2924 | LA, LB | Fucoxanthin |

- means no data available.

**Table 6 FTIR analysis of the aqueous extracts from lyophilized samples**

| Wave number ($cm^{-1}$) | Sample | Compound |
|---|---|---|
| 818 | LA, LB | Sulphate group |
| 1 028 | LA | Alginic acid |
| 1 030 | LB | Alginic acid |
| 1 232 | LA | Fucoidan |
| 1 236 | LB | Fucoidan |

(Table 5) which may belong to fucoxanthin were 1 377 $cm^{-1}$, 1 458 $cm^{-1}$, 1 736 $cm^{-1}$ (LB), 1 738 $cm^{-1}$ (LA), 2 852 $cm^{-1}$ (LB), 2 854 $cm^{-1}$ (LA) and 2 924 $cm^{-1}$. In the methanolic extract, there were also S=O type bonds in sample LA (1 248 $cm^{-1}$) and sample LB (1 244 $cm^{-1}$). We had identified some typical phloroglucinol bonds by analyzing a standard sample of phloroglucinol against the samples peaks, this was made without identifying the bonds, due to the lack of bibliographic references in this area.

Spectral analysis of the aqueous extracts (Table 6) and polysaccharides (Table 7) were based on information from Pereira et al. (2013) and Pereira and Ribeiro-Claro (2015).

In this case, both spectra presented alginic acid peaks (LA: 1 029 $cm^{-1}$; LB: 1 028 $cm^{-1}$), with more units of guluronic than mannuronic acid. Fucoidan was also present in the 1 227 $cm^{-1}$ (LB) and 1 232 $cm^{-1}$

**Table 7 FTIR analysis of the polysaccharides extracts from lyophilized samples**

| Wave number ($cm^{-1}$) | Sample | Compound |
|---|---|---|
| 818 | LA, LB | Sulphate group |
| 1 028 | LA | Alginic acid |
| 1 029 | LB | Alginic acid |
| 1 227 | LB | Fucoidan |
| 1 232 | LA | Fucoidan |

**Table 8 Antioxidant activity in the various extracts by the DPPH method (mean±SD; *n*=3)**

| Samples | Methanolic extract ($IC_{50}$ μg/mL) | Aqueous extract ($IC_{50}$ μg/mL) |
|---|---|---|
| A | 135.13±0.99 | 198.73±1.42 |
| B | 158.29±0.96 | 497.59±2.87 |
| LA | 121.89±1.52 | 303.60±0.99 |
| LB | 50.39±0.47 | 42.58±0.15 |

(LA) peaks which were characteristic of the sulphate ester groups, and there were also sulphate groups present as indicated by the 818 $cm^{-1}$ peak (Rupérez et al., 2002). The FTIR-ATR results showed that there were no great changes in the compounds between the lyophilized samples.

### 3.4 Antioxidant assays

#### 3.4.1 DPPH radical assay

The DPPH antioxidant assay is based on neutralizing the free radical DPPH by antioxidant compounds within the samples. The DPPH assays are used as one of the main antioxidant assays in pharmacognosy and used as a preliminary antioxidant assay before deepening any studies of bioactivity. Although DPPH is a synthetic radical, this assay is considered as a role model for the reaction of free radicals in the human body.

The sample with the highest antioxidant activity was LB, with similar values in both the methanolic and aqueous extracts (e.g. 50.4±0.47 μg/mL, 42.58±0.15 μg/mL) (Table 8). These results together with the phenolic content assay proved that the LB sample had more phenolic content in the methanolic extract, so it was possible to determine that the phenolic compounds were responsible for the highest antioxidant activity in this extract. Regarding the total phenolic content, the aqueous extract from the LB sample provided the greatest values.

Except for the aqueous extract from sample A,

**Table 9 Antioxidant activity in various extracts by the ABTS method in TEAC (mean±SD; *n*=3)**

| Sample | Methanolic extract (TEAC) | Aqueous extract (TEAC) | Polysaccharides (TEAC) |
|---|---|---|---|
| A | 14.43±0.002 | 2.68±0.006 | 10.21±0.035 |
| B | 16.82±0.005 | 3.86±0.007 | 9.74±0.007 |
| LA | 4.18±0.004 | 4.14±0.005 | 6.93±0.007 |
| LB | 2.42±0.004 | 1.00±0.004 | 10.36±0.005 |

extracts from sun-dried samples exhibited a lower antioxidant activity when compared to the lyophilized samples. For the lyophilized samples, it was clear that samples from zone B exhibited the highest antioxidant activity (Table 8).

Comparing these results with those obtained by de Carvalho (2013) in *F. spiralis* (methanolic extract: 113 μg/mL, aqueous extract: 53 μg/mL), it was verified that the LB sample of *F. ceranoides* had a higher antioxidant power than *F. spiralis* and, when the results were compared with Zubia et al. (2009), the lyophilized samples of zone B were identical to those obtained by these authors for a crude extract, from *F. ceranoides*. The antioxidant activity was linked to the phenolic content of the algae, although this relationship was more evident in some extracts than others.

3.4.2 ABTS (pH=7) radical assay

This assay is based on the reducing ability of the sample against ABTS radical cation in aqueous media allowing to analyze the antioxidant activity of polysaccharides (Rodriguez-Jasso et al., 2014).

In methanolic extracts, the drying method used seemed to be the most important factor in determining the antioxidant activity, since the lyophilized samples had higher activity than the sun-dried samples. As previously mentioned, this observation may suggest that compounds could be degraded during sun drying. In the aqueous extract, all the samples had different activities and no relationship could be established between these values and their origin, or the drying process. Again, the LB samples had the most reducing power amongst all of the different extracts tested (Table 9), especially the aqueous extract.

The polysaccharides extracted from the LA sample (TEAC=6.93) showed a greater activity than the other three samples, which also gave identical values. These results were very different from what was observed by the DPPH assay. This was most probably because ABTS method was in an aqueous medium so

**Table 10 Pearson's correlation test for phenolic content and antioxidant activity on methanolic extracts**

| | DPPH | ABTS | TPC |
|---|---|---|---|
| DPPH | - | 0.827 | -0.960* |
| ABTS | 0.827 | - | -0.642 |
| TPC | -0.960* | -0.642 | - |

*: the correlation is significant at the level of 0.05 (two extremities); - means no value.

**Table 11 Pearson's correlation test for phenolic content and antioxidant activity on aqueous extracts**

| | DPPH | ABTS | TPC |
|---|---|---|---|
| DPPH | - | 0.866 | -0.347 |
| ABTS | 0.866 | - | -0.006 |
| TPC | -0.347 | -0.006 | - |

- means no value.

was a higher efficacy to solubilize other algal compounds, such as fucoidans and alginic acid, that were precipitated in assays using an alcoholic medium such as used in the DPPH assay. Fucoidans may play a key role in this reaction because the sulphate in the O-2 group may react with ABTS (Barahona, 2011 in Rodriguez-Jasso et al., 2014).

To evaluate the correlation between the phenolic content and antioxidant activity of the methanolic extract, we executed a Pearson's correlation test (Table 10).

The results demonstrated a positive correlation between the phenolic content and antioxidant assay using DPPH, this strongly suggested that the phenolic compounds played an important role in such activity.

On the other hand, the lower correlation exhibited between phenolic content with the ABTS assay suggested that this activity is due to other compounds such as the pigments, such as fucoxanthin. This conclusion was further supported by Xia et al. (2013), who reported that purified fucoxanthin reacted better with ABTS ($EC_{50}$=0.03 mg/mL) than with DPPH ($EC_{50}$=0.14 mg/mL) and, in ABTS, the concentration level differed from DPPH (ABTS: 0.02 mg/mL to 0.08 mg/mL; DPPH: 0.1 mg/mL to 1 mg/mL). In the aqueous extracts, no relationship was found between the same parameters (Table 11), even between the antioxidant assays. In summarising a comparison between the results from the two methods used and published reports, it was concluded that some compounds (such as fucoidans and fucoxanthin) demonstrated more antioxidant activity in the ABTS radical assay and other compounds, such phenolics

had more affinity with the DPPH assay. In order to understand these relationships, these compounds need to be purified to clarify the differences observed in different assays.

## 4 CONCLUSION

The results of this study show that the drying methods tested affected the results of bioactivity and degradation of certain algal compounds. For example, solar drying (i.e. the traditional method) reduced antioxidant activity of the tested extracts. This observation was probably due to the degradation of the bioactive compounds. However, the sun-dried samples collected from the higher salinity zone could prove to be a useful biomass for the specific extraction of fucoxanthin, thereby avoiding the necessity for extra expense such as drier and freeze drier. The lyophilization method, which although is very expensive to use, maintained the bioactivity of compounds.

The extraction methods used in this study showed that was possible to do a general screening with a relatively small amount of biomass from one species to evaluate the potential of an alga species.

The results show that environmental conditions such as salinity and different drying methods could influence the chemical composition and consequently the bioactive status of the algal extracts.

It was also verified that the samples collected from a zone with lower salinity, in estuarine conditions, had the most pigments and phenolic compounds in the methanolic extract providing a greater antioxidant power. For the aqueous extract, there remains a need to clarify the composition of the extract in order to characterize the antioxidant molecules present such as fucoidans.

However, the samples collected from a zone of higher salinity produced a higher concentration of polysaccharides (probably because of the defensive response to the physical conditions of the site, such currents and waves, and salinity concentration). Further studies are required to clarify the relationships between the abiotic factors and the presence and composition of the polysaccharides content.

*Fucus ceranoides* is a well-adapted alga that can survive and live in extreme environments, and which is almost always abundant in the areas it occupies. The biggest problem for *F. ceranoides* to maintain its habitat seems to be the inter-species competition occurring with *F. vesiculosus*, where the latter fucoid species can take better advantage of higher salinities.

*Fucus ceranoides* could serve as an interesting model organism to test in aquaculture situations where lower salinity water is the input, for example, systems away from the coast or dependent on low salinity well water.

To further this study, it would be interesting to perform additional assays to provide a better understanding of those physiological mechanisms of *Fucus ceranoides*, which allow it to survive in low salinity where no other large brown algae are able to do so.

## 5 DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

## 6 ACKNOWLEDGMENT

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