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Potential uses of the brown seaweed *Cystoseira humilis* biomass: 2- Fatty acid composition, antioxidant and antibacterial activities

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Abstract
Seaweeds, or marine macroalgae, are rich in a large variety of natural compounds used in nutritional and pharmaceutical areas. In this study the brown seaweed *Cystoseira humilis* harvested from the Atlantic coast of Morocco has been investigated for fatty acid (FA) composition as well as for antioxidant and antibacterial potentials. The results revealed that polyunsaturated fatty acids (PUFAs) of *C. humilis* represented 47.67% of total FAs where arachidonic acid C20:4 (n-6) was the most abundant PUFA (18.1%) followed by eicosapentaenoic acid C20:5 (n-3) (11.79 %). *C. humilis* showed a low ω-6/ω-3 ratio, high unsaturation index (UI=191.42) and low atherogenicity and thrombogenic indices (AI=0.55 and TI= 0.04). Moreover, methanol extract of *C. humilis* exhibited high DPPH radical scavenging activity (82%) and a moderate Ferrous Ion-Chelating (FIC) ability (68%). The antibacterial activity was limited to *Staphylococcus aureus* and *Bacillus cereus* among all tested pathogenic bacterial strains. In conclusion, *C. humilis* exhibited promising a FAs profile and antioxidant activities which could be further enhanced by isolating these constituents in pure form for nutraceutical and pharmaceutical purposes.

Keywords: Fatty acids, DPPH scavenging and FIC ability, antibacterial activity, *Cystoseira humilis*, Morocco.

1. Introduction
Seaweeds are known to be a good source of healthy food due to a natural richness in minerals and vitamins as well as bioactive molecules content [1]. Many bioactive ingredients from seaweeds are documented for their benefits like some polysaccharides, polyphenols and lipids [2]. Seaweeds have low lipid content, ranging from 1 to 5% of dry matter [3]. Neutral lipids and glycolipids are the major lipid classes represented in seaweeds, and their proportion of essential fatty acids (FAs) is higher than of land plants [4]. They synthesize large amounts of long-chain polyunsaturated fatty acids (LC-PUFAs) [5, 6]. In most seaweeds, LC-PUFAs are mainly accumulated into complex polar lipids constituting membranes, while triacylglycerols (TAG) are predominantly constructed of saturated (SFAs) and monounsaturated (MUFAs) fatty acids [7, 8].
In recent years there has been an increase of the resistance of microorganisms to antibiotics that are commonly used in medical treatments. To overcome this problem, new therapeutic drugs from natural products have been explored [9]. Seaweeds are essential in nature and directly valuable to humans since they have antimicrobial, antiviral, antitumor, anticoagulant, fibrinolytic, and antioxidant properties [10-14]. Despite the fact that many algal compounds have medicinal properties, few of those compounds have shown real potential to be used as a nutraceutical or pharmaceutical. Phlorotannins are the most important group of bioactive substances that determine the pharmacological value of brown seaweeds [15].

In the present study the brown seaweed *Cystoseira humilis* was investigated for its antioxidant and antibacterial activities of methanolic extracts, as well as for their fatty acid composition and nutraceutical value.

2. Material and methods

2.1. Algal material and extract preparation

The seaweed *Cystoseira humilis* was collected from the Moroccan Atlantic coast at the south of El Jadida city (33°14'47.5"N 8°32'31.9"W) during spring 2015. Taxonomic characteristics and geographic distribution of this species in Morocco are described in Zrid et al [16]. Samples were washed with distilled water. Afterwards, algal biomass was freeze dried. The lyophilized samples were submitted to extraction with 80% methanol at 1:10 (m/v). The methanolic extracts of *C. humilis* were used to evaluate antimicrobial and antioxidant activities.

2.2. Fatty acid extraction, GC-MS composition and quality indices

The extraction of fatty acid methyl esters (FAME) was performed, according to a modified protocol of Lepage and Roy [17]. Fifty mg of lyophilized algal biomass was treated with 1.5 mL of derivatization solution (methanol/acetyl chloride, 20:1, v/v), after which 1 mL of hexane was added and the mixture heated for 1 hour at 90°C. Upon the samples being placed in an ice bath, 1 mL of distilled water was added to the mixture and the organic phase was removed and dried with anhydrous sodium sulfate. The extract was then filtered and evaporated, after that 500 µl of hexane was added to the extract before analysis by GC-MS. The extraction of FAME was performed in triplicate.

FAME were analyzed on a Bruker GC-MS (Bruker SCION 456/GC, SCION TQ MS) equipped with a ZB-5MS (30 m x 0.25 mm internal diameter, 0.25 µm film thickness, Phenomenex). A commercial standard (Supelco 37 Component FAME Mix‖ Sigma-Aldrich, Sintra, Portugal) was used for the identification and quantification of FAME. Values were expressed as % of total FAs.

The unsaturation index (U.I.) was calculated by multiplying the percentage of each fatty acid by the number of double bonds followed by summing up their contributions [18]. The atherogenicity (AI) and thrombogenicity (TI) indices were calculated according to the following equations [19].

$$AI= [(4*C14:0) + C16:0+C18:0] / [\sum MUFA+\sum PUFA\text{-}n6+\sum PUFA\text{-}n3]$$

$$TI= [C14:0 + C16:0+C18:0] / [0.5MUFA+0.5PUFA\text{-}n6+3PUFA\text{-}n3+PUFA\text{-}n3/ PUFA\text{-}n6]$$

2.3. DPPH radical scavenging activity

The antioxidant activity of *C. humilis* methanolic extract was established as diphenylpicrylhydrazyl (DPPH) free-radical scavenging according to the method of Blois [20] with slight modification. DPPH (0.06 mM) was dissolved in methanol and added to seaweed extract at different concentrations (5 to 100 mg/ml). The samples were incubated in the dark at room temperature for 30 min. After which the absorbance was measured at 517 nm using a spectrophotometer (UV-Visible Metashe 5200 HPC). The results were compared to a negative control (all reagents except the test extract) and positive controls (BHT and ascorbic acid). The percentage of DPPH radical scavenging was calculated with the following equation:

$$\text{DPPH scavenging activity (\%)} = [(Ac\text{-}As) /Ac] \times 100$$

where Ac is the absorbance of the negative control (methanol with DPPH solution) and As is the absorbance of the sample.

2.4. Ferrous Ion-Chelating ability

The iron ion-chelating activity was determined by the method of Dinis et al [21]. 2.75 m of distilled water was added to1.0 ml of methanolic extract (with different concentration) after wich the solution was mixed with 0.05 ml FeCl2 (2.0 mmol/l), 0.2 ml ferrozine (5.0 mmol/l) and. The mixture was shaken vigorously and incubated for 10 min. at room temperature in the dark. The absorbance of the iron ions-ferrozine complex was measured at 562 nm. The ability of each sample to chelate iron ions was calculated using the following equation:

$$\text{Chelating activity (\%)} = [1\text{-}(Asample – Ablank) / Acontrol] \times 100\%$$

EDTA was used as the positive control, FeCl2 solution substituted by distilled water was used as a blank, and the sample substituted by distilled water was used as a negative control.

2.5. Antibacterial activity

The antibacterial screening of *C. humilis* methanolic extract was tested against seven pathogenic bacteria including Gram positive species (*Staphylococcus aureus*, *Bacillus cereus*, *Enterococcus faecali*) and Gram-negative species (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* sp., *Klebsiella pneumoniae*) using the agar disc diffusion method [22]. A sterile saline solution was inoculated with 18–24 h growth culture of bacteria. The suspension was spread on Petri dishes containing Mueller-Hinton Agar (MHA). Then, sterile discs (6 mm in diameter), impregnated with 10µl of algal methanolic extract, were placed on the surface of Petri dishes separately inoculated with different tested strains. Gentamicin (15µg/disc) and ciprofloxacin (5µg/disc) were used as positive controls. Thereafter, the plates were incubated at 37°C for 24 h. The antibacterial activity was determined by measuring the diameter of the inhibition zone (mm) formed around the disc.

3. Results and discussion

3.1. Fatty acid methyl ester profile and nutraceutical perspectives

Seaweeds are in general a rich source of bioactive compounds such as polyunsaturated fatty acids (PUFAs) from ω-3 and ω-6 series [3,23], which are widely used in food and pharmaceutical industries [24]. FA composition of *C. humilis* is presented in Figure 1 and Table 1. The obtained results showed that saturated FAs (SFAs) content was about 33.44% with palmitic acid (C 16:0) as the most abundant SFA, which is in accordance with previous studies performed in other seaweeds species [25, 26]. Oleic acid (C18:1) was the predominant monounsaturated fatty acids (MUFAs) with a content of 16.03% of total FAs. This predominance of oleic acid was in accordance with results reported by Vizetto-Duart et al [27] for *C. humilis* collected from the Portugal coasts (10% of total FAs). It has been reported that the differences observed in the FAs composition of seaweeds may be related to the varying geographical origins of the samples, and/or to the environmental factors under which the samples were harvested [28]. In the studied *C. humilis*, polyunsaturated fatty acids (PUFAs) represented 47.67% of total FAs. Arachidonic acid C20:4 (ω-6) was the most abundant PUFAs (18.1%) followed by eicosapentaenoic acid C20:5 (ω-3) (11.79 %). Arachidonic acid has an enormous interest as a precursor for the biosynthesis of regulating/signalling molecules like prostaglandins, thromboxans and other bioregulators of many cellular processes [3]. Eicosapentaenoic acid acts as the forerunner of several substances such as prostaglandins, thromboxanes, and leukotrienes, which play an important role in regulating developmental and regulatory physiology [29]. The application of eicosapentaenoic acid in the food industry is related to different oxidative processes induced by light or free radical oxygen. During the autoxidation, EPAs are destabilized, and aldehydes might be generated leading to rancid tastes and smells. To avoid these kinds of problems microencapsulation of this FA by complex coacervation has been suggested [30]. Microencapsulation has the advantage of avoiding the addition of antioxidants to food and enabling the release of EPA and DHA only in the intestine [31].

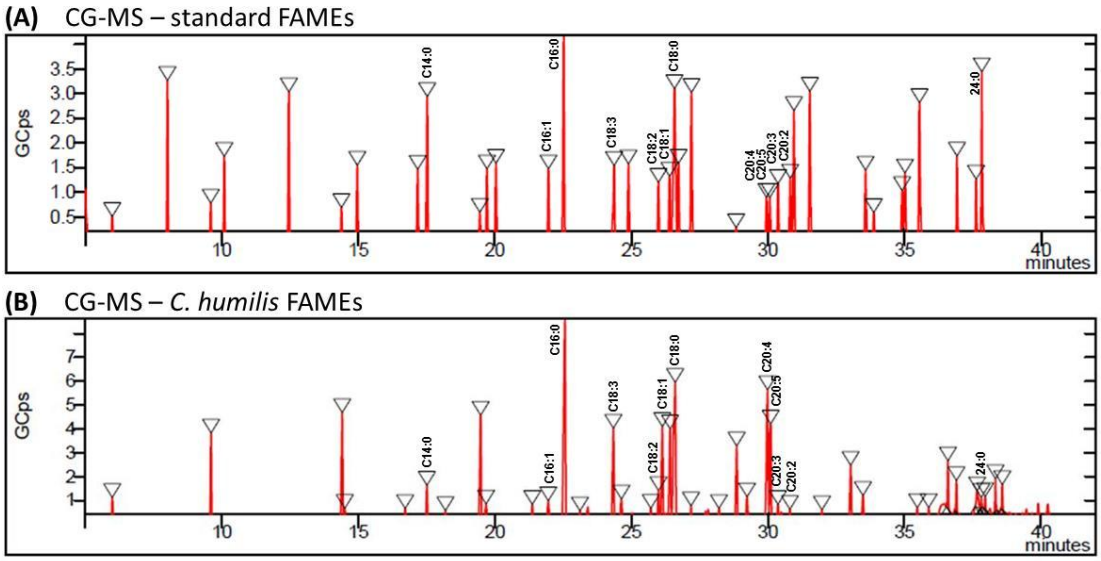

Figure 1: CG-MS spectra- Fatty acids methyl ester (FAMEs) of standard (A) and *Cystoseira humilis* (B).

Seaweeds are of potential value as sources of essential fatty acids, important in the nutrition of humans and animals [32]. A need for ω-3-fortified food has arisen because humans have mainly incorporated ω-6-rich cereals and vegetable oils into their diets together with other saturated fat foods, decreasing the intake of ω-3-PUFAs [33]. In the present study the ratio of ω-6/ω-3 was 3.04 (Table 1), which implies that *C. humilis* fatty acids could increase dietary supply of ω-3. In general, European and Western food products are rich in ω-6, while ω-6/ω-3 ratio recommended by the World Health Organization (WHO) for adult humans should be less than 10 as a whole in the diet [34, 35]. The ω-3 PUFAs are very important because they have been recognized to reduce the risk of cancer, arthritis, and mental health disorders such as dementia, depression, schizophrenia, Alzheimer's, and Parkinson's diseases [30,36-37]. FAs of seaweeds are also beneficial for the prevention of cardiovascular diseases and other chronic diseases, such as diabetes, hypertension, and autoimmune diseases in humans [38]. On other hand, *C. humilis* exhibits a high unsaturation index (UI=191.42), whereas atherogenicity (AI) and thrombogenic (TI) indices are low, about 0.55 and 0.04, respectively (Table 1). These AI and TI are lower than those reported by Vizetto-Duart et al [27] for the same species collected on the coast of Portugal. The obtained results suggest that *C. humilis* could be used in nutraceutical applications or in food products.

Table 1. Fatty acid methyl ester profile and nutritional indices for *Cystoseira humilis*.

Fatty acid			**% of total FAME**			
Myristic (C14:0)			1.57±0.24			
Pentadecanoic acid (C15:0)			nd			
Palmitic acid (C16:0)			29.22±1.68			
Stearic acid (C18:0)			1.26±0.09			
Arachidic acid (C20:0)			nd			
Behenic acid (C22:0)			nd			
Lignoceric acid (C24:0)			1.39±0.08			
Total SFA			**33.44**			
Pentadecenoic acid (C15:1)			nd			
Palmitoleic acid (C16:1)			2.86±0.05			
Oleic acid (C18:1)			16.03±1.20			
Eicosenoic acid (C20:1)			nd			
Docosenoic acid (C22:1)			nd			
Tetracosenoic acid (C24:)			nd			
Total MUFA			**18.71**			
Linoleic acid C18:2 (ω -6)			10.82±0.74			
Eicosadienoic acid C20:2 (ω -6)			1.34±0.13			
gamma-Linolenic acid C18:3 (ω -6)			3.48±0.28			
Eicosatrienoic acid C20:3 (ω -6)			2.14±0.18			
Arachidonic acid C20:4 (ω -6)			18.1±1.11			
Eicosapentaenoic acid C20:5 (ω -3)			11.79±0.91			
Nutritional indices						
PUFA/SFA	**∑ω-3**	**∑ω-6**	**∑ω-6/∑ω-3**	**UI**	**TI**	**AI**
1.42	11.79	35.88	3.04	191.42	0.04	0.55

FAME: fatty acids methyl esters; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; nd: not detected; UI: unsaturation index; AI: atherogenic index, TI: thrombogenic index.

3.2. Antioxydant activity

3.2.1. DPPH Radical scavenging activity

Radical scavenging is one of the mechanisms by which antioxidants inhibit oxidation, and a wide variety of *in vitro* methods have been used to explore the antioxidant potential of seaweeds [39]. Diphenylpicrylhydrazyl (DPPH) is a stable free radical commonly used for this purpose [40, 41]. *C. humilis* exhibited a significant antioxidant activity (82% at 1/ml) compared to ascorbic acid (83%) and BHT (79 %; Fig. 2). Moreover this species showed a low EC_{50} (0.58). This result is similar to those reported for other *Cystoseira* species (Table 2) except for *C. compressa*, which showed very low EC_{50} (0.21 mg/ml). It has been suggested that the antioxidant activity of brown seaweeds is related to their phenolic compounds or more specifically phlorotannins, terpenes

and alkaloids [42]. Several studies have mentioned antioxidant activity of compounds isolated from seaweeds like phloroglucinol, triphlorethol A [43], eckol, dieckol, 6,60-bieckol, 8,80-bieckol [43-46], phlorofucofuroeckol A, dioxinodehydroeckol [47] and diphlorethohydroxycarmalol [48, 46].

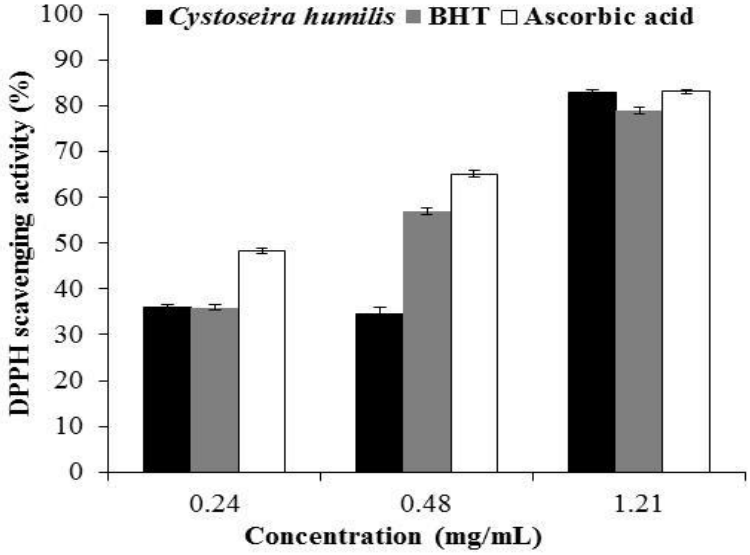

Figure 2: DPPH radical-scavenging activity of *Cystoseira humilis* extracts.

Table 2: DPPH radical scavenging activity (expressed as efficient concentration, EC_{50}) for *Cystoseira humilis* compared to other *Cystoseira* species.

Species	**EC_{50}** (mg/ml)	**Reference**
Cystoseira myrica	0.60	[49]
Cystoseira tamariscifolia	0.49	[50]
Cystoseira amentacea	0.40	[51]
Cystoseira barbata	0.56	[51]
Cystoseira compressa	0.21	[51]
Cystoseira humilis	**0.58**	**This study**

3.2.2. Ferrous Ion-Chelating activity

Transition metals ions such as Cu^{2+}, Fe^{2+} and Fe^{3+} are the primarily pro-oxidants that promote oxidation by decomposing lipid hydroperoxides into free radicals [39]. The most important type of secondary antioxidants are those that chelate transition metal ions by decreasing metal reactivity or by physically partitioning the metal away from lipids [52, 53]. In this study, methanol extract of *C. humilis* was tested for its Ferrous Ion-Chelating (FIC) activity. The obtained results showed moderate ferrous ion-chelating capacity compared to EDTA, an excellent chelator for ferrous ions (Fig. 3). The chelating capacity of EDTA was found to be 98.7% at a concentration of 0.05 mg/ml, while FIC ability of *C. humilis* extract varied from 47% at 0.05 mg/ml to 68% at 0.3 mg/ml. It has been reported that polyphenols derived from brown seaweeds are potent ferrous ion chelators and could form complexes with metal ions, as protection against toxic metal ions [44,54-56]. The metal chelating ability of polyphenols is related to the number, location of the hydroxyl groups and the presence of ortho-dihydroxy polyphenols [57-59]. Other algal components such as polysaccharides and proteins can be more effective chelators of ferrous ions than phenolic compounds [60, 61].

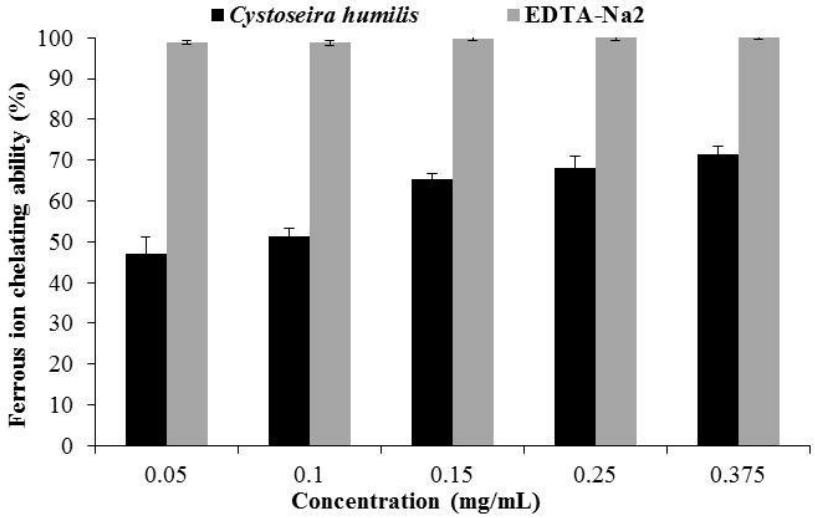

Figure 3: Ferrous ion-chelating activity of *Cystoseira humilis* methanolic extract.

Table 3: Antibacterial activity of *Cystoseira humilis* compared to other seaweeds and antibiotics.

	Inhibition zone diameter (mm) *				
	Seaweeds			Antibiotics	
Bacteria	*Cystoseira compressa*	*Cystoseira crinita*	*Cystoseira humilis*	Gentamicin (15 µg/disc)	Ciprofloxacin (5µg/disc)
Staphylococcus aureus	6.5	6	11	25	32
Bacillus cereus	NT	NT	9	20	31
Enterococcus faecalis	NT	NT	NI	NT	7
Escherichia coli	7	6	NI	20	10
Pseudomonas aeruginosa	NI	NI	NI	NT	NT
Klebsiella pneumoniae	NT	NT	NI	31	27
Salmonella sp.	NT	NT	NI	40	27
Reference	[59]		This study		

* Inhibition zone including disc diameter (6 mm); NI: no inhibition, NT: not tested.

3.3. Antibacterial activity

In recent years the resistance of microorganisms to general antibiotics increased. To overcome this problem, new therapeutic drugs from natural products have been explored [9]. In this study methanolic extract of *C. humilis* exhibited a moderate activity against *Bacillus cereus* and *Staphylococcus aureus* with inhibition zone diameters of 9 and 11 mm, respectively. No activity, contrariwise, was observed against *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Salmonella* sp. strains. These results are in accordance with those reported by Mhadhebi et al [62] showing no antibacterial activity of methanolic extracts of some *Cystoseira* species against tested bacteria (Table 3), whereas petroleum ether extracts of *C. sedoides* and *C. crinita* exhibited a moderate antibacterial activity against *Escherichia coli*. Consequently other works are underway to test antimicrobial activities of various solvent polarity extracts of *C. humilis* and expand the range of targeted active molecules. Earlier studies have suggested that antimicrobial activity depends on the type of extraction solvent used, but also on algal species [63, 64]. For example, the methanolic extract of *Dictyopteris polypodioides* (Dictyotale) harvested from the Atlantic coasts of Morocco showed high activity against most of the pathogens tested reached, with a maximum zone of inhibition of 36 mm observed against *Bacillus cereus* [65].

Conclusion

In the present study the antioxidant and antibacterial activities as well as fatty acid profile of *Cystoseira humilis* have been investigated. The methanolic extract of *C. humilis* exhibited interesting antioxidant activities with high DPPH radical scavenging activity and moderate ferrous ion-chelating ability. Extracts demonstrated antibacterial activity especially against *Staphylococcus aureus* and *Bacillus cereus*, while no activity was

detected against *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* sp. and *Klebsiella pneumonia*. *C. humilis* is rich in PUFAs (47.67% of total FAs) particularly arachidonic acid C20:4 (n-6), with a high degree of total unsaturation (UI=) and low atherogenicity and thrombogenic indices (AI=0.55 and TI= 0.04). Therefore, *C. humilis* could be considered a good potential source of bioactive and nutraceutical compounds for pharmaceutical, cosmetic and food industries.

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