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In vitro enzyme inhibitory properties, antioxidant activities, and phytochemical fingerprints of five Moroccan seaweeds

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ABSTRACT

The coastlines of Morocco have an abundant resource of diverse seaweeds but the bioactivity of many of these seaweeds is still unexplored. The aim of this study was to contribute to the evaluation of the antioxidant potential, using different in vitro systems and searching some fingerprints of phenolic constituents based on LC-MS/MS method of five seaweeds species obtained from Moroccan Atlantic Ocean: *Bifurcaria bifurcata, Cystoseira humilis, Cystoseira stricta, Fucus spiralis* and *Gelidium sesquipedale*. The total phenolic compounds, the antioxidant activity, anticholinesterase, tyrosinase inhibitory, urease inhibitory activities and the organic compounds were determined. The results showed that *Fucus spiralis* possessed the highest phenolic and flavonoid contents compared to the others. In addition, *Fucus spiralis* (IC_{50}: 13.25 ± 0.9 μg/mL) and *Cystoseira stricta* (IC_{50}: 13.58 ± 1.2 μg/mL) exhibited higher lipid peroxidation inhibitory activity. The highest1,1-diphenyl-2-picrylhydrazyl (DPPH) (IC_{50}: 47.23 ± 3.8 μg/mL), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) dia-mmonium salt (ABTS) (IC_{50}: 5.95 ± 0.6 μg/mL), and Cupric reducing antioxidant capacity (CUPRAC) (55.24 ± 0.04 μg trolox equivalents/mg) activity was measured in *Fucus spiralis*. Regarding metal chelating activity, *Bifurcaria bifurcata* was the best (IC_{50}: 45.22 ± 0.9 μg/mL) compared to the others. The highest acetylcholinesterase (IC_{50}: 28.92 ± 1.8 μg/mL), butyrylcholinesterase (IC_{50}: 11.72 ± 2.8 μg/mL), tyrosinase (IC_{50}: 6.19 ± 0.4 μg/mL) and urease (IC_{50}: 10.96 ± 1.3 μg/mL) inhibitory activities was found in *Fucus spiralis*. According to LC-MS/MS results, quinic acid, malic acid, fumaric acid, gallic acid, protocatechuic acid, 4-OH-benzoic acid, hesperidin, were detected in ethanol extracts of the five Moroccan seaweeds. This study demonstrated that seaweeds, particularly *Fucus spiralis* and *Cystoseira stricta* could be a promising source of antioxidants and phytochemicals which can be used in cosmetics, pharmaceutical and as nutritional food supplement in food industries to deleterious free radical induced stress, disorder and diseases.

1. Introduction

Morocco, whose Atlantic and Mediterranean coasts exceed 3500 km, constitutes an abundant richness of algal biomass to value and exploit in the pharmaceutical and agri-food sectors. The Moroccan algae are diverse and include more than 93 species of Chlorophyceae, 110 species of Phaeophyceae and 304 species of Rhodophyceae (Riadi and Kazzaz, 1998, 2000). However, the available scientific reports and knowledge on Moroccan algae species are very limited. The difference on chemical composition of the seaweeds nutrients depends on habitat, maturity state, species and environmental conditions (Cofrades et al., 2010). Seaweeds are therefore, a good source of different vitamins, essential amino acids, dietary fiber, essential fatty acids, hormones, minerals, and phenolic compounds (Senthilkumar et al., 2013; Stirk et al., 2014a, 2014b; Ambrozova et al., 2014). Furthermore, they are considered as a unique nutrient source especially in the low-calorie diets and healthier foods (Heiba et al., 1993; Cofrades et al., 2010; Mohamed et al., 2012). They are also used extensively in agriculture crops as plant biostimulants due to the presence of endogenous plant growth regulators (Stirk et al., 2014a, 2014b;

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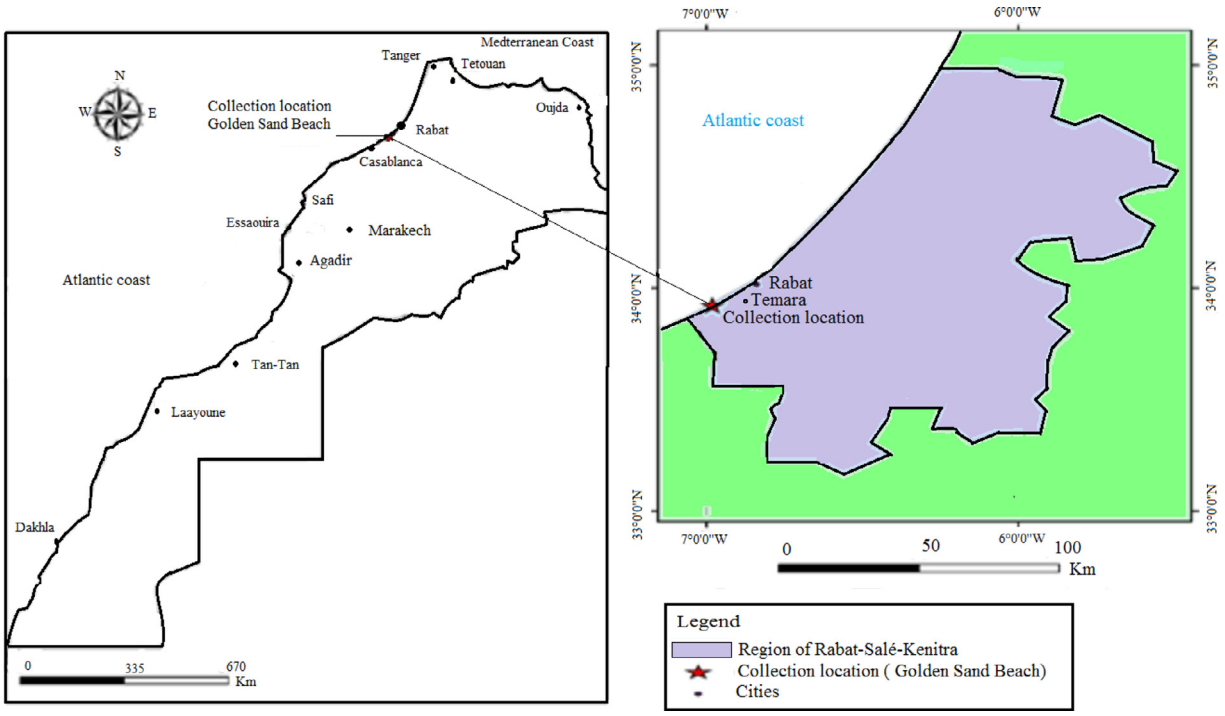

Fig. 1. Localization of collection point seaweeds at Golden Sand Beach.

Rengasamy et al., 2014). Recently, seaweeds have received special attention as a source of natural antioxidants (Senthilkumar et al., 2013).

Seaweeds have been used in pharmacological and food systems (Chan et al., 2011) due to their bioactive compounds such as phenolic compounds (Sabeena Farvin and Jacobsen, 2013; Machu et al., 2015; Agregán et al., 2017), phlorotannins (Wijesekara et al., 2011; Sathya et al., 2017; Shin et al., 2014), fucoxanthin (Yan et al., 1999; Kang et al., 2014) and polysaccharides (Zhang et al., 2010). Seaweeds present various therapeutic properties such as anti-inflammatory (Subash et al., 2016; Bitencourt et al., 2015), antimicrobial (Stirk et al., 2007; Rhimou et al., 2010; El Wahidi et al., 2015), antiviral (Bouhlal et al., 2010; Takebe et al., 2013), antitumoral (Shao et al., 2013; Zhai et al., 2014), radioprotective (Zhang et al., 2008; Shin et al., 2014) and neuroprotective (Rafiquzzaman et al., 2015; Custodio et al., 2016; Moodie et al., 2019).

Some of the synthetic antioxidants used in the food industry are suspected to cause cancer in rats (Grice, 1988). This suspicion led to a search for safe antioxidants from natural products. Similarly, some of the drugs being used against Alzheimer's Disease have side effects to the liver (Öztürk, 2012). Therefore, it is necessary to develop and utilize more effective and safe bioactive compounds from natural origins in the food and pharmaceutical industries.

To the best of our knowledge, this study is the first to characterize bioactivity in ethanol extracts from five Moroccan seaweeds. The seaweed studied are the most dominant species in the Moroccan Atlantic coast. Previous studies mainly focused on antibacterial and antifungal effects of these seaweeds. The objective of the present study was to undertake and investigate total phenolic and organic compounds by the establishment of the chemical fingerprint of five Moroccan seaweeds. In addition, the antioxidant potential and inhibitory activities on some enzymes, as therapeutic tools were evaluated.

2. Materials and methods

2.1. Collection and extraction of seaweeds

Seaweeds were collected in spring 2016 from Atlantic Coast 10 km south of Rabat-Morocco in front of Golden Sand Beach coast (33°92′N; 6°96′E) (Fig. 1) at low tide. The seaweeds were identified by Professor Moujahid A., Hassan First University, Department of Biology and Food Sciences according to the nomenclature adopted in the preliminary inventory of benthic algae of the Moroccan Coast (Boudouresque, 1984) and updated by Riadi and Kazzaz (2000).

Algal samples were identified as *Bifurcaria bifurcata* (Ross, 1958), *Cystoseira humilis* (Schousboe ex Kützing, 1860), *Cystoseira stricta* (Sauvageau, 1911), *Fucus spiralis* (Linnaeus, 1753) and *Gelidium sequipedale* (Clemente) (Thuret, 1876). Voucher specimens of all species were deposited in the National Herbarium (RAB) of the Moroccan Scientific Institute (Rabat, Morocco).

The seaweeds were transported to the laboratory in a cooler box, cleaned and washed with sea and fresh water to remove epiphytes, salts and sands. The samples were dried at 50 °C for 48 to 72 h. The dried samples were ground in an electrical grinder to obtain fine powder. Each powdered seaweed (25 g) was extracted using 70% ethanol (200 ml) at 60 °C for 2 h. The extraction procedure was repeated twice and the combined extracts were filtered through Buchner funnel under vacuum. The solvent was evaporated under reduced pressure and eventually lyophilized. The lyophilized extracts were stored at −20 °C until further used.

2.2. Determination of total phenolic content

The concentrations of phenolic content in all extracts were expressed as mg pyrocatechol equivalents (PEs), determined with Folin–Ciocalteu's reagent (FCR) according to the method of Slinkard and Singleton (1977). Briefly, 1 mL algal extract (1 mg extract/mL MeoH) was added to 46 mL distilled water and 1 mL FCR, and mixed thoroughly. After 3 min, 3 mL sodium carbonate (2%) was added to the mixture and shaken intermittently for 2 h at room temperature. The absorbance was read at 760 nm by a spectrophotometer (Shimadzu 1601, Japan). Pyrocatechol was used as standard and the results were expressed as mg pyrocatechol equivalents (PEs).

2.3. Determination of total flavonoid content

Measurement of flavonoid concentration of the extract was based on the method described by Tel et al. (2012), with a slight

modification (Öztürk et al., 2014). An aliquot of 1 mL algal extact (1 mg extract/mL MeOH) was added to test tubes containing 0.1 mL 10% aluminum nitrate, 0.1 mL 1 M potassium acetate and 3.8 mL methanol. After 40 min at room temperature, the absorbance was determined at 415 nm. The total flavonoid content was calculated from the standard quercetin calibration curve, and the result was expressed as μg quercetin equivalents (QEs).

2.4. Antioxidant activity

2.4.1. 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH free radical scavenging activity was determined by the DPPH method described by (Blois, 1958) with slight modifications (Öztürk et al., 2014). In its radical form, DPPH absorbs at 517 nm, but upon reduction by an antioxidant or a radical species, its absorption decreases. Ethanol was used as a control while butylated hydroxytoluene (BH) and α-tocopherol were used as antioxidant standards for comparison of the activity. The results were recorded as 50% inhibition concentration (IC_{50}). The sample concentration providing 50% DPPH scavenging effect (IC_{50}) was calculated from the graph of DPPH scavenging effect percentage against sample concentration.

2.4.2. 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging assay

The spectrophotometric analysis of $ABTS^{\cdot+}$ scavenging activity was determined according to the method of (Re et al., 1999) with slight modifications (Tel et al., 2013). The $ABTS^{\cdot+}$ was produced by the reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12 h. Before usage, the $ABTS^{\cdot+}$ solution was diluted to get an absorbance of 0.703 ± 0.025 at 734 nm with ethanol. Ethanol was used as a control, while BHT and α-tocopherol were used as antioxidant standards for comparison of the activity. The results were given as 50% inhibition concentration (IC_{50}). The sample concentration providing 50% $ABTS^{\cdot+}$ scavenging effect (IC_{50}) was calculated from the graph of $ABTS^{\cdot+}$ scavenging effect percentage against sample concentration.

2.4.3. β-Carotene/linoleic acid bleaching assay

The total antioxidant activity was evaluated using a β-carotene/linoleic acid test system (Sabudak et al., 2013), with slight modifications. β-Carotene (0.5 mg) in 1 mL chloroform was added to 25 μL linoleic acid and 200 mg Tween 40 emulsifier mixture. After evaporation of chloroform under vacuum, 100 mL distilled water saturated with oxygen was added by vigorous shaking. One hundred sixty microliters of this mixture were transferred into 40 mL samples at different concentrations. Ethanol was used as a control. BHA and α-tocopherol were used as antioxidant standards for comparison of the activity. As soon as the emulsion was added into each tube, the zero-time absorbance was measured at 470 nm using a 96-well microplate reader (Spectra Max 340PC^{384}, Molecular Devices, USA) and continued to be read every 30 min until the color of control disappeared. The last absorbance of the emulsion was read after incubation of the plate for 2 h at 50 °C. The bleaching rate (R) of β-carotene was calculated according to the following equation.

$$R = \frac{ln\frac{a}{b}}{t}$$

Where, ln = natural log, a = absorbance at time zero, b = absorbance at time t (120 min). The antioxidant activity (AA) was calculated in terms of percent inhibition relative to the control, using the following equation:

$$AA = \frac{R_{Control} - R_{Sample}}{R_{Control}} \times 100$$

2.4.4. Cupric-reducing antioxidant capacity

The cupric-reducing antioxidant capacity was determined according to the method of (Apak et al., 2004) with slight modifications. To each well, in a 96-well plate, 50 μL 10 Mm Cu(II), 50 μL 7.5 Mm neocuproine and 60 μL NH4Ac buffer (1 M, pH 7.0) solutions were added. Forty microliters of the extract at different concentrations was added to the initial mixture to make the final volume 200 μL. After 1 h, the absorbance at 450 nm was recorded against a reagent blank using a 96-well microplate reader (Spectra Max 340PC^{384}, Molecular Devices, USA). Results were given as absorbance and compared with those of BHA and α-tocopherol used as antioxidant standards.

2.4.5. Ferrous ion chelating activity

The chelating activity of the extracts on Fe^{2+} was measured as reported by Decker and Welch (1990), with slight modifications. The extract solution (80 μL dissolved in ethanol in different concentrations) was added to 40 μL 0.2 mM $FeCl_2$. The reaction was initiated by the addition of 80 μL 0.5 Mm ferene. The mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture reached equilibrium, the absorbance was measured at 593 nm. EDTA was used as a positive control for comparison of the activity.

2.6. Enzyme inhibitory activity

2.6.1. Anticholineterase activity

Acetylcholinesterase (AChE, Type-VI-S, EC 3.1.1.7, 425.84 U/mg) and butyrylcholinesterase (BChE, EC 3.1.1.8, 11.4 U/mg) inhibitory activities of ethanol extracts were determined according to previously described Ellman's colorimetric method (Ellman et al., 1961). For this purpose, 130 μL sodium phosphate buffer (100 mM, pH 8.0), 10 μL ethanolic extract at different concentrations, and 20 μL AChE or BChE enzymes in buffer were mixed and incubated for 15 min at 25 °C. Subsequently, 20 μL 0.5 mM DTNB (5,5′-dithiobis(2-nitrobenzoic acid) and 20 μL acetylthiocholine iodide (0.71 mM) or butyryl-thiocholine chloride (0.2 mM) was added for AChE, or BChE assay, respectively. Then the absorbance was measured at 412 nm using a 96-well microplate reader (SpectraMax 340PC^{384}, Molecular Devices, USA). Galantamine was used as standard compound (positive control) on two enzymes inhibitory effects evaluation (Sabudak et al., 2017).

2.6.2. Tyrosinase inhibition activity

Tyrosinase inhibitory activity of the extracts was measured using the method described by (Hearing, 1987) with slight modification. L-DOPA was used as a substrate. For this purpose, to the 96 well plate 150 μL potassium phosphate buffer (50 mM, pH 6.5), 10 μL tested extracts dissolved in absolute ethanol at different concentrations and 20 μL tyrosinase (13.3 U/well) were mixed. After 10 min incubation at room temperature, 8.4 mM L-DOPA was added then the absorbance was measured at 475 nm for 10 min using a 96-well microplate reader (Spectra Max 340PC^{384}, Molecular Devices, USA) (Sabudak et al., 2017). The well-known tyrosinase inhibitors kojic acid and L-mimosine were used as positive controls.

2.6.3. Urease inhibitory activity

Canavalia ensiformis (Jack bean) urease was used to determine inhibitory activity where urea was used as substrate (Zahid et al., 2015). The enzyme solution and substrate (0.1 M) were prepared in phosphate buffer (0.01 M, pH 8.2). To the well, 10 μL sample in ethanol, and 25 μL (8.3×10^{-3} U) urease, and 50 μL urea were added. After 15 min at 30 °C, the 45 μL phenol reagent containing 0.005% w/v sodium nitroprusside and 1% w/v phenol, and 70 μL alkali reagent containing 0.1% NaOCl and 0.5% w/v NaOH were added. The absorbance was measured kinetically at 630 nm using a 96-well microplate reader (Spectra Max 340PC^{384}, Molecular Devices, USA). The absorbance was measured kinetically at 630 nm. The thiourea was used as standard to compare the activity.

2.7. Identification and quantitation of phenolic compounds

The phenolic compounds and the organic acids of seaweeds were determined using LC-MS/MS instrument. The ethanolic extract was dissolved with methanol and diluted to 250 mg/L and then all samples were filtrated using 0.22-μm microfiber filter. LC−MS/MS (Nexera, Shimadzu, Kyoto, Japan) was equipped with DGU-20A3R degasser, LC-30AD binary pumps, SIL-30AC autosampler and CTO-10ASvp column oven. The chromatographic separation was performed on a C18 reversed-phase Inertsil ODS-4 (150 mm × 4.6 mm, 3 μm) analytical column ODS C18 column. The elution gradient consisted of mobile phase A (Water, 5 mM ammonium formate and 0.1% formic acid) and mobile phase B (methanol, 5 mM ammonium format and 0.1% formic acid). The elution was started with mobile phase A, and the gradients of mobile phase B. The optimizations of LC-MS/MS and the method validation parameters were the same as given previously (Ertas et al., 2015). The flow rate was 0.5 mL/min while the injection volume was 4 μL. Twenty-seven phenolic compounds including flavonoids, flavonoid glycosides, phenolic acids, phenolic aldehyde, coumarin and three non-phenolic organic acids were screened in the extracts.

2.8. Statistical analyses

All data on activity assays were the average of triplicate analyses, which were recorded as mean ± standard error of the mean (SEM). The significance between means were analyzed by ANOVA procedures by using SPSS software program ($p < 0.05$).

3. Results

3.1. Total phenolic and flavonoid content

The total phenolic content of the seaweed species ranged from 4.68 to 22.91 μg pyrocatechol equivalents per mg extract (PEs/mg extract). Among the five seaweeds studied, the phenolic content of *Fucus spiralis* was 3-fold higher than those of *Cystoseira humilis* and *Cystoseira stricta* and 2- and 5-fold higher *Gelidium sesquipedale, and Bifurcaria bifurcata* (Table 1). The flavonoid content of seaweeds ranged from 4.26 to 26.86 μg quercetin equivalents per mg extract (QEs/mg extract). The most flavonoid rich extract was the ethanol extract of *Fucus spiralis* which was about 3-and 4-fold higher than those of *Cystoseira stricta* and *Cystoseira humilis,* while *Gelidium sesquipedale* was nearly 5-fold lower than *Fucus spiralis* (Table 1). However, the lowest amount was observed in *Bifurcaria bifurcata,* which was 6.3-fold lower than that of *Fucus spiralis*.

3.2. Antioxidant activity

DPPH scavenging activity was consistently higher in ethanolic extracts of *Fucus spiralis*. These algae present a better activity being nearly 2-fold higher than those of *Gelidium sesquipedale* and *Cystoseira stricta*. In contrast, *Cystoseira stricta* and *Bifurcaria bifurcata* present a lower activity being 2.5 and 4.5-fold lower than that of *Fucus spiralis* (Table 2).

Fucus spiralis showed the highest ABTS scavenging activity, which was very close to those of α-tocopherol and BHT. The other seaweeds; namely, *Gelidium sesquipedale* and *Cystoseira humilis* also exhibited good results but lower than those of α-tocopherol, BHT and quercetin. However, the activity in *Bifurcaria bifurcata* was lower than that of ethanol extracts of all seaweeds, with 22-fold lower than that of *Fucus spiralis* (Table 2).

In β-carotene-linoleic acid assay, all seaweeds ethanol extracts exhibited the antioxidant activity with values lower than 75 μg/mL and none of them exhibited better activity than that of the antioxidant standards. The activity of *Fucus spiralis* and *Cystoseira stricta* were similar and exhibited the higher lipid peroxidation inhibition activity among all the tested extracts. *Gelidium sesquipedale* demonstrated the least activity which was about 6-fold lower than that of *Fucus spiralis* (Table 2).

All the seaweeds species had cupric reducing antioxidant capacity. In accordance with DPPH and ABTS results, *Fucus spiralis* had also the best antioxidant capacity. Decreasing the seaweeds antioxidants activity was nearly 2-fold in *Cystoseira stricta* and 4-fold in *Cystoseira humilis* and *Bifurcaria bifurcata*. However, the red algae showed an antioxidant activity with 6.7-fold which is lower than that of the brown algae *Fucus spiralis* (Table 2).

The ferrous ion chelating ability of seaweeds extracts exhibited variable activity. The ethanol extract of all seaweeds exhibited better metal chelating ability of ferrous ions than that of quercetin standards. The ethanol extract of the brown algae *Bifurcaria bifurcata* indicated the best metal chelating activity compared to that of the other species. *Fucus spiralis* and *Gelidium sesquipedale* exhibited relatively higher activities and showed nearly 1-fold lower than that of *Bifurcaria bifurcata*. The brown seaweeds *Cystoseira humilis* and *Cystoseira stricta* showed the lowest inhibitory activity, 3-and 4-fold, respectively, when compared to that of *Bifurcaria bifurcata* (Table 2).

Table 1
Total phenolic and total flavonoid contents of the extracts of the selected seaweeds.[a]

Extracts	Phenolic content μg PEs/mg extract[b]	Flavonoid content μg QEs/mg extract[c]
Cystoseira stricta	7.24 ± 0.03	8.53 ± 0.02
Cystoseira humilis	7.68 ± 0.05	6.72 ± 0.02
Fucus spiralis	22.9 ± 0.06	26.9 ± 0.03
Bifurcaria bifurcata	4.68 ± 0.04	4.26 ± 0.02
Gelidium sesquipedale	11.1 ± 0.03	5.84 ± 0.02

[a] Values expressed are means ± standard error of three parallel measurements ($p < 0.05$).
[b] PEs. pyrocatechol equivalents.
[c] QEs. quercetin equivalents.

3.3. Enzyme inhibitory activity

3.3.1. Anticholinesterase activity

Galantamine was used as a positive control to compare the activity as it is used to treat mild Alzheimer's patients. As expected, the antioxidant brown seaweed *Fucus spiralis* also demonstrated the highest AChE and BChE inhibitory activities. The brown seaweed *Bifurcaria bifurcata* which had the best chelating ability, also had good anticholinesterase activity. In contrast, *Cystoseira stricta* possessed a better inhibitory activity with 5-fold in BChE and about 3-fold in AChE lower when compared to than that of *Fucus spiralis*. The other seaweeds were inactive against both enzymes (Table 3).

3.3.2. Tyrosinase inhibitory activity

Except for *Bifurcaria bifurcata* and *Gelidium sesquipedale*, seaweed extracts possessed tyrosonase inhibitory activity. *Fucus spiralis* exhibited the highest inhibitory activity, 4- and 13-fold higher than *Cystoseira stricta* and *Cystoseira humilis* respectively. *Cystoseira stricta* and *Cystoseira humilis* also indicated good tyrosinase inhibitory activities compared to those of *Gelidium sesquipedale* and *Bifurcaria bifurcata* (Table 3).

3.3.3. Urease inhibitory activity

The brown algae *Fucus spiralis* was the most active extract among the other seaweeds. *Fucus spiralis* present a better urease inhibitory activity, being two times than the Thiourea standards. Thus, *Cystoseira stricta* was also exhibited good inhibitory activity. However, moderate activity was found in *Cystoseira humilis* and *Bifurcaria bifurcata*, 13-fold and 17-fold lower when compared to that of *Fucus spiralis* (Table 3).

Table 2
Antioxidant activity of extracts of seaweeds by the DPPH·, β-carotene-linoleic acid, $ABTS^{+}$, and metal chelating assays.[a]

Samples	DPPH assay IC_{50} (μg/mL)	β-carotene-linoleic acid assay IC_{50} (μg/mL)	ABTS assay IC_{50} (μg/mL)	Fe^{2+}- Ferrin assay IC_{50} (μg/mL)	CUPRAC (μg Trolox equivalents / mg extract)
Cystoseira stricta	78.73 ± 1.1	13.58 ± 1.2	113.4 ± 2.1	198.9 ± 5.9	24.06 ± 0.02
Cystoseira humilis	121.1 ± 1.3	27.58 ± 0.8	57.81 ± 1.3	136.1 ± 4.4	12.98 ± 0.03
Fucus spiralis	47.23 ± 3.8	13.25 ± 0.9	5.95 ± 0.6	55.01 ± 1.9	55.24 ± 0.04
Bifurcariabifurcata	208.5 ± 1.4	48.60 ± 3.2	130.6 ± 1.9	45.22 ± 0.9	12.92 ± 0.03
Gelidium sesquipedale	84.61 ± 3.9	75.36 ± 3.6	44.46 ± 2.4	83.73 ± 2.9	8.25 ± 0.01
α-Tocopherol[b]	12.26 ± 0.07	2.10 ± 0.08	4.87 ± 0.56	NT	–
BHT[b]	54.96 ± 0.99	1.34 ± 0.09	4.10 ± 0.06	NT	–
Quercetin[b]	2.07 ± 0.11	1.80 ± 0.10	1.18 ± 0.03	250.1 ± 0.07	–
EDTA[b]	NT	NT	NT	6.50 ± 0.07	–

NT= not tested.
[a] IC_{50} values represent the means ± standard error meaning of three parallel measurements ($p < 0.05$).
[b] Reference compounds.

3.4. Identification and quantification of phenolic compounds

Twenty-seven organic compounds were screened for the ethanol extracts of five Moroccan seaweeds by LC-MS/MS. Quinic acid was the most abundant compound of seaweed extracts which was detected in all studied seaweeds. The quinic acid ranged between 8535 and 415094.45 μg/g. Hesperidin and malic acid were also detected in all samples within the range of 67.42–73.48 μg/g and 366.4–616.9 μg/g, respectively. Fumaric acid, gallic acid, protocatechuic acid and 4-hydroxy-benzoic acid were also detected in some samples. According to LC-MS/MS results, quinic acid was the main component of *Fucus spiralis* and *Gelidium sesquipedale* and yielded nearly to 40% (w) of the extract. Since *Fucus spiralis* was the most active and *Gelidium sesquipedale* had relatively low activity, the quinic acid is probably not responsible for the activity. Similarly, malic acid is probably not responsible for the activities. The third common compound for all seaweeds was hesperidin, which is known as a strong antioxidant, and gives various activities. However, the yield was very low (>0.01%) (Table 4).

4. Discussion

Phenolic compounds (e.g. flavonoids, phenolic acids, and tannins) are considered as effective chain breaking antioxidants which may directly participate in antioxidative action (Shahidi et al., 1992). Hence, the total flavonoid and phenolic contents of the *Bifurcaria bifurcata, Cystoseira humilis, Cystoseira stricta, Gelidium sesquipedale* and *Fucus spiralis* extracts were determined as quercetin and pyrocatechol equivalents, respectively. Antioxidant compounds have an ability to inhibit the acetylcholinesterase and butyryl-cholinesterase enzymes (Atta-ur-Rahman and Choudhary, 2001; Moodie et al., 2019). Therefore, the acetylcholinesterase and butyrylcholinesterase inhibitory activity was screened beside the antioxidant activity assays. In addition, the urease inhibitory activity and tyrosinase inhibitory activity were also evaluated for the seaweeds extracts.

In a previous study, the phenolic content of *Fucus spiralis* was reported to be 1.15 ± 0.06 mmol equivalents of gallic acid/g dry weight, corresponding to 195.6 μg gallic acid equivalents per mg extract (Peinado et al., 2014). Furthermore, the methanolic extract of *Turbinaria ornata* and *Turbinaria conoides* exhibited a higher phenolic compound content with values of 16.64 ± 0.10 and 3.42 ± 0.35 mg of GAE/g, respectively (Chakraborty et al., 2013). The present results are in agreement with the previous study of Sabeena Farvin and Jacobsen, 2013, which showed that all the ethanolic extracts of the *Fucus* species had high total phenolic content with values ranging from 32 to 1920 mg/100 g dried seaweed. Flavonoids are a group of natural phenolic compounds exhibiting anti-inflammatory, anticancer, antiproliferative, antihypertensive, and antioxidant activity (Maheswari et al., 2016). Flavonoids also protect humans from coronary heart disease effects (Xiao et al., 2011). The content of flavonoids in *Cystoseira Barbara* varied from 5 to 8 mg QEs/g dried extract (Haddar et al., 2012).

DPPH is a stable free radical. In this assay, DPPH tests the scavenging ability of the antioxidants which are soluble in organic media. Herein, the higher radical scavenging activity was observed in the brown seaweed *Fucus spiralis* with inhibitory activity better than those of the BHT standards. In previous studies, brown seaweeds possessed the most potent radical scavenging activity against DPPH compared to other seaweeds (Andrade et al., 2013). The DPPH free radical scavenging of *Fucus spiralis* was 54.5 ± 0.4 mmol trolox/g DW (Peinado et al., 2014). Brown seaweed in general have better DPPH radical scavenging than red seaweed and *Fucus* species are good DPPH scavengers (Jimenez-Escrig et al., 2001; Wang et al., 2009).

ABTS tests the electron donating capacity of an antioxidant, which are soluble, both in lipophilic and hydrophilic media. Another advantage over the DPPH assay is that ABTS also tests compounds having steric hindrance (Demirkiran et al., 2013). In the present study, the best inhibitory activity was observed in *Fucus spiralis* with a value close to those of the α-tocopherol and BHT standards. In previous finding, ethanol extracts of brown

Table 3
Acetylcholinesterase (AChE), butyrylcholinesterase (BChE), tyrosinase and urease inhibitory activities of extracts of seaweeds.[a]

Samples	AChE assay IC_{50} (μg/mL)	BChE assay IC_{50} (μg/mL)	Tyrosinase assay IC_{50} (μg/mL)	Urease assay IC_{50} (μg/mL)
Cystoseira stricta	83.1 ± 2.1	69.4 ± 1.9	28.1 ± 2.8	25.9 ± 1.9
Cystoseira humilis	>200	>200	84.1 ± 0.9	148 ± 4.8
Fucus spiralis	28.9 ± 1.8	11.7 ± 2.8	6.19 ± 0.4	10.9 ± 1.3
Bifurcaria bifurcata	141 ± 1.3	177 ± 0.9	>200	191 ± 5.1
Gelidium sesquipedale	>200	>200	>200	>200
Galantamine[b]	0.005 ± 0.00	0.051 ± 0.00	NT	NT
Kojic acid[b]	NT	NT	0.67 ± 0.06	NT
L-Mimosine[b]	NT	NT	0.64 ± 0.04	NT
Thiourea	NT	NT	NT	23.8 ± 0.2

NT = not tested.
[a] IC_{50} values represent the means ± standard error meaning of three parallel measurements ($p < 0.05$).
[b] Positive standards.

Table 4
Identification and quantification of compounds of ethanol extract of seaweeds by LC–MS/MS.

Sample name	Retention time (min)	Parent ion (*m/z*)[a]	MS2 (Collision energy)[b]	μg analyte /g extract				
				Cystoseira stricta	*Cystoseira humilis*	*Fucus spiralis*	*Bifurcaria bifurcata*	*Gelidium sesquipedale*
Quinic acid	1.13	191.0	85 (22), 93 (22)	8535	12762.04	415094.45	59340.49	412594.82
Malic acid	1.23	133.1	115 (14), 71 (17)	468.3	590.1	366.4	450.4	616.9
Fumaric acid	1.48	115.0	71.4	–	16.33	–	–	–
Gallic acid	3.00	169.1	125 (14), 79 (25)	–	20.73	–	–	10.98
Protocatechuic acid	4.93	153.0	109 (16), 108 (26)	6.333	–	–	–	–
4-Hydroxy-benzoic acid	7.39	137.0	93, 65	25.11	146.3	56.58	–	–
Hesperidin	12.67	611.1	303, 465	73.48	67.02	66.41	70.35	62.47

[a] Parent ion (*m/z*): molecular ions of the standard compounds (mass to charge ratio).
[b] MS2 (CE): MRM fragments for the related molecular ions (CE refers to related collision energies of the fragment ions).

seaweed *Sargassum wightii* showed the highest activity in ABTS radical scavenging assay compared to green and red seaweed (Seenivasan and Indu, 2013).

Linoleic acid contains two double bonds at −9 and −12 positions which are easily oxidized by the singlet oxygen in the media and the antioxidant gives H· to the media to inhibit the radicalic reaction (Oztürk et al., 2009). Therefore, β-carotene is used to monitor the reaction. The total carotenoid contents of *Fucus serratus* was approximately 0.08% of the dried extracted cells, and fucoxanthin comprises about 70% of the total carotenoid (Liaaen-Jensen and Haugan, 1989). In this present work, *Fucus spiralis* and *Cystoseira stricta* containing higher amount of fucoxanthin had higher lipid peroxidation inhibition. This result is in accordance with the result of Holdt and Kraan (2011) where the fucoxanthin content ranges from 172 to 720 mg kg^{-1} DW in the brown seaweeds species, with a maximal concentration in *Fucus serratus*.

The CUPRAC assay tests the reducing capacity of the antioxidants, which have the ability to give electrons to the media. The method relies on the reduction of cupric to the cuprous form by an antioxidant (Özyürek et al., 2011). Based on this method, all studied algae exhibited the ability of reducing copper ions from Cu(II) to Cu(I). The Moroccan brown seaweed *Fucus spiralis* showed the highest reducing activity when compared to the other seaweeds. The assessment of the antioxidant activity of fucoidan extract from the brown seaweed *Padina distromatica* exhibited the ability of reducing copper in a concentration dependent manner (Paul, 2014). The highest antioxidant capacities in the CUPRAC assay were observed for some phenolic acids (Apak et al., 2008).

The complexation of excess amount of ferrous ions in the biological fluids is necessary; thus, the antioxidant capacity of seaweeds was evaluated according to the ferrous ions chelating assay. In this work, the ethanol extract of *Fucus spiralis* exhibited the best activity in all antioxidant activity assays except in the metal chelating activity assay. This suggests that *Fucus spiralis* mostly contains lipophilic and low molecular weight antioxidant compounds in good amounts. In the present study, the brown seaweed *Bifurcaria bifurcata* demonstrated the best metal chelating activity, although exhibiting relatively lower activity in other assays. The chemical complexity of the seaweed extracts can significantly interfer in the biological activities of natural products (Nickavar and Esbati, 2012). A similar result was reported for the Icelandic seaweed (*Palmaria palmata and Chondrus crispus*) extracts which showed the highest ferrous ion-chelating activity while the lower polyphenol content resulted in weaker DPPH free radical scavenging activity (Wang et al., 2009).

Previous studies highlighted high antioxidant activities and high phenolic contents in *Fucus* species such as *Fucus spiralis* and *Fucus vesiculosus,* and attributed these high activities to the presence of phlorotannins (Tierney et al., 2013; Wang et al., 2012). Since, the correlation was reported between antioxidant and anticholinesterase compounds, the anticholinesterase activity of seaweeds extracts was carried out against acetylcholinesterase (AChE) and butyryl cholinesterase (BChE) which are the chief enzymes of Alzheimer disease. The inhibition of acetylcholinesterase (AChE) enzyme, which catalyzes the breakdown of ACh, is considered to be a useful therapeutic approach for the symptomatic treatment of Alzheimer's disease (Pangestuti and Kim, 2011). In the present study, the findings suggest that among the tested Moroccan seaweeds, *Fucus spiralis, Bifurcaria bifurcata* and *Cystoseira stricta* had potential anticholinesterase compounds, which could be used in future as therapeutic agents for Alzheimer's disease. In another study on South African seaweeds, all seaweeds extracts tested had acetylcholinesterase inhibitory activity, among them *Dictyota humifusa* extracts were the most effective at inhibiting AChE (Stirk et al., 2007). In a recent study, two *Cystoseira* species; namely, *Cystoseira tamariscifolia* and *Cystoseira nodicaulis* were reported as potential anticholinesterase compounds (Custodio et al., 2016). Screening of AChE and BChE inhibitory activity on ethanol extracts of different seaweeds showed that extracts from *Cystoseira usneoides* and *Fucus spiralis* were potent inhibitors (Andrade et al., 2013). In another study, brown algae, such as *Ecklonia maxima, Ecklonia stolonifera* and *Ishige okamurae* were also able to inhibit AChE (Yoon et al., 2008; Yoon et al., 2009; Kannan et al., 2013).

The tyrosinase enzyme contains copper and is a polyphenol oxidase. It is linked to melanin hyperpigmentation of skin and the inhibition of tyrosinase plays an important role for vitiligo disease as well as other skin related diseases. Tyrosinase inhibitors are used as a whitening agent in cosmetics in clinical skin treatments (Shiino et al., 2001). Therefore, the compounds scavenging DPPH free radical such as some flavonoids and hydroxy benzenes inhibit tyrosinase enzyme (Momtaz et al., 2008). In the same way, marine products have been used in cosmetics and/or pharmaceuticals (Kim et al., 2017). In this study, *Fucus spiralis* was the most active algae against tyrosinase enzyme. In another study, methanol extracts of other brown algal species, such as *Cystoseira tamariscifolia* and *Cystoseira nodicaulis* strongly inhibit tyrosinase (Custodio et al., 2016). 7-Phloroeckol and dieckol isolated from the brown algae *Ecklonia cava* had higher inhibitory activity than those of commercial inhibitors such as arbutin and kojic acid against tyrosinase (Yoon et al., 2009; Heo et al., 2009). In vivo studies in mice suggested that fucoxanthin isolated from *Laminaria japonica* suppressed tyrosinase activity in ultraviolet B (UV-B)-irradiated guinea pig and melanogenesis in UV-B-irradiated mice (Thomas and Kim, 2013).

Urease enzyme has an important role in the development of gastric and peptic ulcer causing cancer (Mobley and Hausinger, 1989). Therefore, obtaining new urease inhibitors has become more attractive to treat or prevent urease mediated diseases. Brown seaweed contain sulfated polysaccharides (fucoidan compounds) which are natural substances inhibiting urease during *Helicobacter pylori* infection (Ayala et al., 2014). Herein, the urease enzyme inhibitory activity of five algae was studied. Among them, the brown algae *Fucus spiralis* was the most potent.

To further explore the phenolic profile of the Moroccan seaweeds, the extracts were analyzed by LC-MS/MS. The main components in ethanolic extracts were quinic acid, hesperidin and malic acid. The phenolic compounds tentatively identified from the ethanolic extract in the present study are in accordance with previous studies. Phenolic acids of the brown seaweeds *Ascophyllum nodosum, Bifurcaria bifurcata* and *Fucus vesiculosus* from Spain were predominantly composed of quinic acid derivatives (Agregán et al., 2017). Ethanolic extracts of *Fucus vesiculosus* and *Fucus serratus* contained trace levels of chlorogenic acids. Water extracts of all the *Fucus* species contained no *p*-hydroxybenzoic acid and syringic acid (Sabeena Farvin and Jacobsen, 2013).

Marine red and brown seaweed are an important source of bioactive secondary metabolite including phenols and polyphenols (Li et al., 2009). The brown seaweeds contain phlorotannins, flavonoids and condensed tannins such fucoxanthin, phloroglucinol, fucoidan (Thomas and Kim, 2013; Pádua et al., 2015). Red seaweed contains sulfated polysaccharides as major constituents (Fleita et al., 2015; Rodrigues et al., 2012). In a previous study regarding red and green seaweeds, a series of polyphenolic compounds such as *m*-hydroxybenzoic, protocatechuic and *p*-hyroxybenzoic acids were quantified in South African seaweeds (Rengasamy et al., 2015). Previous reports indicated the presence of several phenolic compounds, especially, ferulic, salicylic, coumaric, syringic, vanillic, gallic, caffeic, chlorogenic and *p*-hydroxybenzoic acids in 16 seaweeds (Fernando et al., 2016). However, in this study, the red algae *Gelidium sesquipedale* contained high amount of malic acid may be considered as a potential new source for malic acid. In the present study, the compounds elucidated using the LC-MS/MS instrument are insufficient to explain the activities tested herein. Therefore, further studies, particularly bioactivity guided fractionation is necessary to understand the origin of the activity.

5. Conclusion

The results presented herein are the first data on the antioxidant activity of *Cystoseira stricta*, and *Gelidium sesquipedale*, and the first information on the anticholinesterase activity of *Bifurcaria bifurcata, Cystoseira stricta* and *Gelidium sesquipedale*. The tyrosinase and urease inhibitory activities of all seaweeds were studied for the first time. In antioxidant activity methods, the best activity was observed in lipid peroxidation inhibition by the β-carotene–linoleic acid assay. Among the seaweeds, *Cystoseira stricta* and *Fucus spiralis* exhibited the best activity in all antioxidant tests, except for ferrous chelating activity. In four enzymes inhibitory activity. *Fucus spiralis* was the most active. *Cystoseira stricta* also exhibited similar inhibitory activity to that of *Fucus spiralis* against acetylcholinesterase, butyryl-cholinesterase, tyrosinase, and urease enzymes.

Development of tyrosinase inhibitors is becoming increasingly necessary in the cosmetic and pharmaceutical industry due to their usability in the treatment of hyperpigmentation related dermatological disorders, skin-whitening property and depigmentation after sunbum. In this study, it can be concluded that *Fucus spiralis*, and *Cystoseira stricta* are potential candidates as a natural source of tyrosinase inhibitors. The results indicated that the seaweeds commonly used as foods and as therapeutics since ancient times in maritime countries might be used in food and pharmaceutical industries as preservatives and/or therapeutic agents. However, further bioactivity studies and isolated constituents are needed.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Supplementary materials

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