

Seasonal variation in phenolic composition, antibacterial and antioxidant activities of *Ulva rigida* (Chlorophyta) and assessment of antiacetylcholinesterase potential

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Abstract The phenolic composition and antibacterial and antioxidant activities of the green alga *Ulva rigida* collected monthly for 12 months were investigated. Significant differences in antibacterial activity were observed during the year with the highest inhibitory effect in samples collected during spring and summer. The highest free radical scavenging activity and phenolic content were detected in *U. rigida* extracts collected in late winter (February) and early spring (March). The investigation of the biological properties of *U. rigida* fractions collected in spring (April) revealed strong antimicrobial and antioxidant activities. Ethyl acetate and *n*-hexane fractions exhibited substantial acetylcholinesterase inhibitory capacity with EC₅₀ of 6.08 and 7.6 µg mL⁻¹, respectively. The total lipid, protein, ash, and individual fatty acid contents of *U. rigida* were investigated. The four most abundant fatty acids were palmitic, oleic, linolenic, and eicosenoic acids.

Keywords *Ulvarigida* · Seasonal variation · Antimicrobial · Antioxidant · Acetylcholinesterase inhibition · Fatty acid

Introduction

Over the last decades, marine algae have received a lot of attention as functional food ingredients because of their richness in protein, vitamins, and minerals. One hundred grams of seaweeds provides more than the daily requirement of vitamins A, B₂, B₁₂, and C (Dhargalkar and Pereira 2005). In addition, seaweeds have traditionally been used in several countries in folk medicine for curing infectious diseases, gout, and eczema.

Hence, much attention has been paid to the development of innovative projects for the pharmaceutical applications from seaweeds. Marine algal compounds exhibit various biological activities such as anticoagulant (Wijesinghe et al. 2011), antiviral (Artan et al. 2008), antioxidant (Cox et al. 2009), anticancer (Namvar et al. 2012), and antiinflammatory (Kazlowska et al 2010) activities.

Antioxidants are able to protect the human body from several diseases such as cancer, inflammation, diabetes, rheumatoid arthritis, and cardiovascular diseases generated by the reactions of the reactive oxygen species (ROS) with biological molecules. ROS are highly genotoxic/mutagenic and damage cellular macromolecules such as DNA, proteins, and lipids (Mantena et al. 2008). The use of synthetic antioxidants to prevent free radical damage involves toxic side effects (Botterweck et al. 2000). The stability of seaweed to oxidation during storage and the absence of the oxidative damage in the structural components suggest the presence of highly developed protective antioxidant defense systems (Zubia et al. 2007). In addition, the emergence of multiple drug-resistant bacterial infections is an escalating problem worldwide with resistance of pathogenic bacteria to widely used antimicrobial agents (Heuer et al. 2009). Food-borne diseases and food spoilage have been also recognized as two of the most important concerns for the food industry. Therefore, the need to develop additional natural antimicrobial agents is considered a public health priority in both pharmaceutical and food industries. Accordingly, algae extracts and their derived bioactive secondary metabolites like various polyunsaturated and monounsaturated fatty acids, phenolic components, terpenoids, and polysaccharides offer the opportunity in this regard (Suganthi et al. 2009). A strategic extraction of secondary metabolites would increase the potential for new discoveries targeting high value-added products.

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Ulva rigida C. Agardh (Ulvaceae) is a substantial part of the total biomass of the benthos along the Tunisian coast. This alga is used in aquaculture since it contains protein, pigments, vitamins, minerals, and unknown growth factors (Nakagawa et al. 1987). For this reason, it is of great interest to investigate the *U. rigida* secondary metabolites and biological activities in order to promote new industrial applications. However, the production of algae secondary metabolites is often controlled by physical and biological factors such as climate, reproductive state, locality, and seasonality. Sampling throughout the year is essential to characterize biological activity due to seasonal variation. To our knowledge, insufficient attention has been dedicated to evaluating the possible temporal variation in antibacterial and antioxidant activities of *U. rigida*. The main objectives of the present work are (1) evaluation of the seasonal variation of the antibacterial and antioxidant activities of the hydroalcoholic extracts of *U. rigida* collected monthly for 12 months from the Tunisian coast; (2) assessment of antioxidant, antimicrobial, and antiacetylcholinesterase activities of the different *U. rigida* fractions; and (3) investigation of the chemical composition of this alga.

Materials and methods

The alga *U. rigida* was collected monthly for 12 months, between winter (September) 2008 and summer (August) 2009, from Sidi Mansour Sfax (Tunisia, 35°14'58.36" N, +11°7'17.75" E). A voucher specimen (LBPes URA01) was deposited in the Laboratory of Biopesticides of the Centre of Biotechnology of Sfax. Extracts of air-dried *U. rigida* were prepared by two methods. Firstly, 300 g of air-dried *U. rigida* thallus collected monthly (12 samples) was extracted with 80 % ethanol–water for 48 h at room temperature. The ethanol was evaporated at 40 °C under vacuum with a rotary evaporator and the remaining aqueous phase was lyophilized to produce a hydroalcoholic extract (EtOH–H₂O extract) (40 g). These extracts were used to study the seasonal variation of the antibacterial and antioxidant activities. Secondly, 200 g of air-dried thallus of *U. rigida* collected in spring (April) was extracted (for 24 h) in a Soxhlet apparatus with 1.5 L methanol at 70 °C. The mixture was filtered and then concentrated to produce methanol extract (total extract 26.6 g). Twenty-five grams of the dried methanolic extract was suspended in 100 mL distilled water and was sequentially partitioned with *n*-hexane (3×250 mL), chloroform (3×250 mL), ethyl acetate (3×250 mL), and *n*-butanol (3×250 mL) to obtain a *n*-hexane fraction (HexF), chloroform fraction (CHCl₃F), ethyl acetate fraction (EtOAcF), butanolic fraction (ButOHF), and water fraction (WF), respectively. Samples were filtered through a filter paper in a Buchner funnel, evaporated at reduced pressure at 40 °C, and then dissolved in the appropriate solvent. The

remaining aqueous layer was lyophilized to give a water fraction. The stock solutions were kept at 4 °C in the dark until further analysis.

Antimicrobial, antioxidant, and antiacetylcholinesterase activities

Seasonal variation of the antibacterial activity of the *U. rigida* hydroalcoholic extracts, prepared monthly for 12 months, was evaluated against two Gram-positive bacteria, *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 25923, and two Gram-negative bacteria, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. The antibacterial activity of spring *U. rigida* crude extract and fractions was investigated against 11 bacterial strains and six fungal strains obtained from international culture collections (ATCC) and the local culture collection of the Centre of Biotechnology of Sfax, Tunisia. They included seven Gram-positive bacteria, *B. subtilis* ATCC 6633, *Bacillus cereus* ATCC 14579, *S. aureus* ATCC 25923, *S. aureus* ATCC 6536, *S. epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, and *Listeria monocytogenes* (food isolate 2132), and four Gram-negative bacteria, *E. coli* ATCC 25922, *E. coli* ATCC 8739, *P. aeruginosa* ATCC 27853, and *Klebsiella pneumoniae* CIP 32147. The following fungal strains were also tested: *Aspergillus niger* CTM 10099, *Fusarium graminearum* (ISPAVE 271), *Fusarium oxysporum* (CTM 10402), *Fusarium culmorum* (ISPAVE 21 W), *Alternaria alternata* (CTM 10230), and *Candida albicans* ATCC 10231. The bacterial strains were cultivated in Muller–Hinton agar (MH) (Oxoid Ltd, UK) at 37 °C except for *Bacillus* species which were incubated at 30 °C. The fungi were cultured on potato dextrose agar medium and incubated at 28 °C. Working cultures were prepared by inoculating a loopful of each tested bacteria in 3 mL MH broth and were incubated at 37 °C for 12 h. For the test, the turbidity of the overnight broth was adjusted to 0.5 McFarland standards (1–1.5 10⁸ CFU mL⁻¹) by the Densimat spectrophotometer (BioMérieux, Italy) and then diluted in Mueller–Hinton broth to a final inoculum concentration of 10⁷ CFU mL⁻¹. Fungal spore suspensions were collected from the surface of each fungal colony by gently scraping with a loop and suspended in 10 mL potato dextrose broth. Each suspension was mixed vigorously by vortexing for 15–20 min. The spore suspension stock was diluted to obtain a concentration of 10⁶ spores mL⁻¹ (measured by Thoma blade).

Antibacterial and antifungal tests were performed by the agar well diffusion method as described by Andrews (2005) using sterile Mueller–Hinton media (Bio-Rad, France) for bacterial strains and potato dextrose agar (Bio-Rad, France) for antifungal tests. One hundred µL of freshly prepared cell suspension adjusted to 10⁷ CFU mL⁻¹ for bacteria and 10⁶ spores mL⁻¹ for fungi were inoculated onto the surface of agar plates. Thereafter, wells with 6 mm in diameter were punched

in the inoculated agar medium with sterile Pasteur pipettes and 50 μL of extracts (50 mg mL^{-1}) was added to each well. Sterile DMSO (20 %) and ethanol (50 %) were negative controls of the solvent used to resuspend the extract also tested in the bioassays. Gentamicin (10 $\mu\text{g/well}$) was used as positive control for bacteria while amphotericin B (20 $\mu\text{g/well}$) was used as positive control for fungal strains. The plate was allowed to stand for 2 h at 4 °C to permit the diffusion of the extracts followed by incubation at 37 °C for 24 h for bacterial strains and 72 h for fungi at 28 °C. The antimicrobial activity was evaluated by measuring the zones of inhibition (clear zone around the well) against the tested microorganisms. All tests were repeated three times.

The antioxidant activity of spring (April) *U. rigida* extracts and fractions was assayed by radical scavenging activity, β -carotene bleaching test, and DNA nicking assay. Radical scavenging activity was determined by the spectrophotometric method based on the reduction of a methanol DPPH solution (Blois 1958). Briefly, 0.5 mL of the extract, in the concentration range of 0.1–2 mg mL^{-1} , diluted in methanol was added to 1 mL DPPH methanol solution (0.1 mM). The mixture was shaken vigorously and left to stand at room temperature for 30 min in the dark. Then, the absorbance was measured at 517 nm against a blank by a spectrophotometer (Bio-Rad SmartSpec™ plus). DPPH radical scavenging activity (%) was calculated according to the formula: DPPH radical scavenging activity (%) = $[(\text{OD}_{\text{blank}} - \text{OD}_{\text{sample}}) / \text{OD}_{\text{blank}}] \times 100$, where OD_{blank} is the absorbance of the control reaction containing all reagents except the tested compound. $\text{OD}_{\text{sample}}$ is the absorbance of the test compound. Extract concentration providing 50 % inhibition (EC_{50}) was calculated from the graph plotting inhibition percentage against extract concentration. Ascorbic acid was used as positive control. Tests were carried out in triplicate.

The antioxidant activity of the spring (April) *U. rigida* extracts was evaluated by the spectrophotometric β -carotene bleaching test (Juntachote and Berghofer 2005). A stock solution of β -carotene–linoleic acid mixture was prepared as follows: 0.5 mg β -carotene was dissolved in 1 mL chloroform (HPLC grade) for which 25 μL linoleic acid and 200 mg Tween 20 were added. Chloroform was completely evaporated using a vacuum evaporator. Then, 100 mL distilled water with oxygen (30 min at a flow rate of 100 mL min^{-1}) was added with vigorous shaking. Aliquots of 4 mL of this mixture were mixed with 200 μL of the extracts in the concentration range of 0.1–4 mg mL^{-1} . The emulsion system was incubated for 3 h at room temperature. The same procedure was repeated with the synthetic antioxidant, butylated hydroxytoluene (BHT), as positive control in the concentration range of 5–150 $\mu\text{g mL}^{-1}$ and a blank containing 200 μL of ethanol. After this incubation period, absorbance of the mixtures was measured at 470 nm. Antioxidative capacity of the extracts was compared with those

of BHT and a blank. Antioxidant activity is expressed as percent inhibition relative to control (0.2 mL ethanol) using the equation:

$$\text{Antioxidant activity(\%)} = [1 - (\text{OD}_0 - \text{OD}_t / \text{OD}_0 - \text{OD}_t)] \times 100$$

where OD_0 and OD'_0 are the absorbance values measured at zero time for the test sample and control, respectively, while OD_t and OD'_t are the corresponding absorbance values measured after incubation for 3 h.

A DNA nicking assay was performed as described by Lee et al. (2002) with minor changes. DNA damage protective activity of *U. rigida* fractions was checked on pBlue-script II vector. Plasmid DNA was isolated by SpinKlean™ Plasmid MiniPrep Kit (BIOMATIC). The experiments were performed in a volume of 20 μL in a microcentrifuge tube containing 3 μL of intact plasmid DNA (50 ng), 5 μL of each extract (250 μg), and 7 μL 1 \times TE buffer (10 mM Tris–HCl and 1 mM EDTA pH 7.2). The reaction mixture was incubated firstly for 10 min before adding 5 μL of Fenton's reagent (30 mM H_2O_2 , 50 μM ascorbic acid, and 80 μL FeCl_3), followed by incubation for 45 min at 30 °C. Untreated plasmid DNA, treated plasmid DNA with Fenton's reagent only, and treated plasmid DNA with Fenton's reagent and quercetin at 100 mM were used as controls. After incubation, 10 μL of each reaction mixture was mixed with 3 μL of gel loading dye (0.15 % bromophenol blue and 40 % sucrose) and immediately loaded into an agarose gel (1 %) and electrophoresed in a horizontal slab gel apparatus in Tris/acetate/EDTA gel buffer for 1 h (50 V). The gel was photographed on a UV transilluminator 312 nm (Vilber Lourmat, France).

The acetylcholinesterase (AChE) inhibitory activity was determined according to the method described by Mata et al. (2007) using electric eel acetylcholinesterase (EC 3.1.1.7, type VS), acetylthiocholine iodide (AChI), and 5'-dithio-bis-2-nitrobenzoic acid (DTNB). The galanthamine-HBr was used as positive control. The reaction containing 300 μL of Tris–HCl buffer (pH 8), 100 μL of organic fractions of *U. rigida* collected in spring (April) at different concentrations (1–250 $\mu\text{g mL}^{-1}$), and 50 μL of the enzyme solution (0.28 UI mL^{-1}) was incubated for 15 min at 37 °C. Subsequently, 75 μL of a solution of AChI (23 $\mu\text{g mL}^{-1}$) and 475 μL of 3 mM DTNB were added, and the final mixture was incubated for 30 min at 37 °C. Absorbance of the mixture was measured at 405 nm. A control mixture was prepared using 100 μL of a solution, similar to the sample mixture but with the solvent instead of the extract. Percent of inhibition was calculated as: $I\% = 100 - [A_{\text{sample}} / A_{\text{control}}] \times 100$, where A_{sample} is the absorbance of the extracts and essential oil containing reaction and A_{control} is the absorbance of the reaction control. Tests were carried out in triplicate.

Analytical methods

Total phenolic content was determined using the Folin–Ciocalteu method (Waterman and Mole 1994) adapted to a microscale. Phloroglucinol was used as a standard for the calibration curve. The phenolic content was expressed as mg phloroglucinol equivalent/gram of dry extract (mg PHG g^{-1}) using the linear equation based on the calibration curve.

The following three parameters are measured: moisture, protein, and fat content. The moisture was prepared from the dried sample of spring *U. rigida* (April). The samples were ground into a fine powder with a mechanic grinder (Moulinex, France) and then ashed by heating at 525 °C for 5 h (AOAC 2000). Protein content was calculated from nitrogen content as follows: $\text{N (\%)} \times 6.25$ using the Kjeldahl method (AOAC 2000). The fat matter was measured from 5 g of spring *U. rigida* (April) using a Soxhlet extraction apparatus with 150 mL petroleum ether as a solvent at the boiling point for 8 h. Fatty acid composition was determined by the analytical methods described in the European Parliament and of the European Council in EEC regulation 2568/91 (1991). Fatty acids were converted to fatty acid methyl esters (FAMES) by vigorous shaking of 0.1 g total fat in 3 mL *n*-hexane with 0.4 mL methanolic potassium hydroxide (2 N). The FAMES were then analyzed in an Agilent 6890 N Network Gas Chromatograph system (Agilent Technologies, France) equipped with an HP-5MS Phenyl Methyl Siloxane capillary column (30 m \times 250 $\mu\text{m} \times$ 0.25 μm) and a 5975 B Insert MSD mass selective detector. Helium (constant flow, 1 mL min^{-1}) was used as a carrier gas. The temperatures of the injector and detector were 250 and 240 °C, respectively. The following temperature program was applied: 120 °C for 5 min, increase of 3 °C min^{-1} to 180 °C, increase of 10 °C min^{-1} to 220 °C, and 220 °C for 31 min. The identification of compounds was achieved by comparing the retention times with those of authentic compounds and the spectral data obtained from the Wiley and NIST libraries.

Statistical analysis

Experimental results concerning this study were expressed as means \pm standard deviation of three parallel measurements. The significance of difference was calculated by Student's *t* test, and values $p < 0.05$ were considered to be significant. Correlation and regression analysis was carried out using Microsoft Excel.

Results and discussion

Seasonal variation of the antibacterial and antioxidant activities

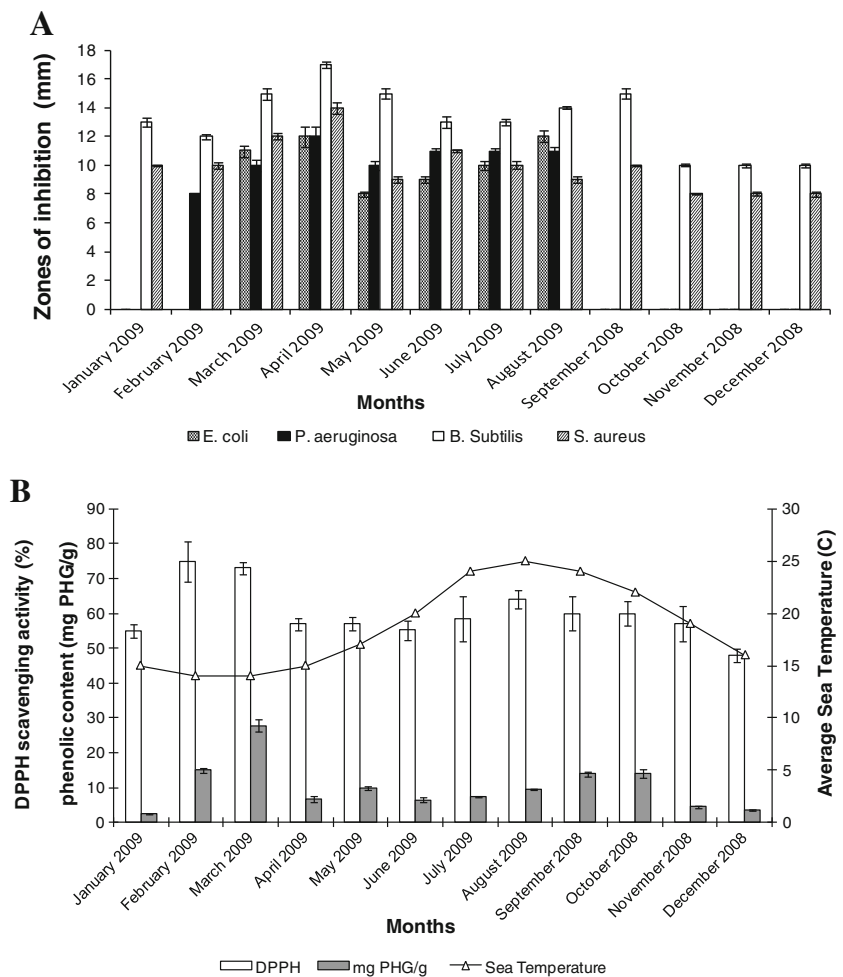
Antibacterial activity against the two tested Gram-positive bacteria was observed throughout the year with differences

of the inhibition zones from 1 month to another (Fig. 1a). However, this activity varied monthly against *E. coli* and *P. aeruginosa*, with a full attenuation in some periods. The highest inhibitory activity was detected with the material collected during spring and summer, with a decline in activity when the collection was made during autumn and winter (Fig. 1a). Results obtained from the agar well diffusion method indicated that *B. subtilis* and *S. aureus* were the most sensitive microorganisms showing the largest inhibition zones in April (spring). Analysis of the variance revealed that both the seasonal and bacterial strain variations exhibited a significant ($p < 0.05$) effect on the antibacterial activity of *U. rigida*. *Ulva fasciata*, collected from the east coast of South Africa, exhibited a strong antibacterial activity in autumn (May), low activity in late winter (July) and spring (September–November), and no activity in summer (Stirk et al. 2007). The discordance in the results could be attributed to the geographic location. There are seasonal and geographical variations in the antimicrobial activity of seaweeds collected from the Mangalore coast of India with the highest activity using algae collected during the post-monsoon season (from October to November) (Vidyavathi and Sridhar 1991).

The changes in antibacterial activity of the investigated *U. rigida* hydroalcoholic extracts, with regard to seasonal variations, might be attributed to a possible variation in the quantities and/or qualities of active secondary products. Numerous studies have noticed a wide variation in the production of chemical defense molecules of terrestrial plants associated with physical (temperature and light) and biological factors, like life cycle and herbivore pressure. Season and water pollution influence the sterol composition of *U. rigida* and *Enteromorpha linza* (Popov et al. 1985).

Antioxidant activity measured using the DPPH test revealed significant differences ($p < 0.05$) during the year (Fig. 1). The highest activity was detected in late winter (February) and early spring (March) compared to those of the late summer (August) and the early autumn (September–October). In spring and summer (April–July), there was a decline in the antioxidant activity (Fig. 1b). The scavenging of these ROS is part of *U. rigida* acclimatization. The antioxidant activity was significantly greater in samples collected during cold months with water temperatures less than 15 °C. In opposition, temperate months, with temperatures greater than 20 °C such as in summer (June–August) and in autumn (September–October), showed a decline in the antioxidant activity. These results indicate that antioxidant activity increases with the decrease of temperature. Acclimatization of algae to low temperatures generally involves several fold increases in the antioxidant enzyme activities (superoxide dismutase, glutathione reductase) and photosynthetic capacity (Dudgeon et al. 1995).

Fig. 1 Seasonal variation of *U. rigida* hydroalcoholic extracts. **a** Antibacterial activity against Gram-positive and Gram-negative bacteria and **b** antioxidant activity. Results are given as mean ± SD (*n*=3)



Seasonal variation of the phenolic compounds

The biological activities of *U. rigida* could be due to the presence of diverse phytochemicals including phenolics, and the Folin–Ciocalteu method was used for the quantification of the total phenolic content. The late winter and early spring extracts (February and March) showed higher phenolic content than those collected in the other months (Table 1). The variation in the activity observed for samples collected monthly for 12 months resembled to the variation observed in total phenolic content. This result might be due to the phenolic compounds that could act as antiradical or antioxidant molecules able to protect cells from biochemical damage caused by ROS. This finding is also in agreement with numerous literature data that reported the effect of phenolic compounds in the protection of the algal thallus against photodestruction by UV radiation (Connan et al. 2006). Analogous up-regulation in antioxidant capacity was observed in algae and many terrestrial plant species (Siatka and Kašparová 2010).

Since the hydroalcoholic extracts of spring *U. rigida* (April) showed a strong antibacterial activity, we have investigated in more detail the antimicrobial activity of the methanolic extract

and derived fractions (HexF, CHCl₃F, EtOAcF, ButOHF, WF) against a panel of microorganisms. The antioxidant and anti-acetylcholinesterase activities were also investigated.

Total phenolic contents and biological activities of spring *U. rigida* fractions

The phenolic compounds are commonly found in terrestrial and marine plants and have been reported to have several biological activities including antioxidant and antimicrobial properties. The total phenolic contents of spring *U. rigida* fractions (April) were determined and are presented in Table 1. The highest content of total phenolic was found in ethyl acetate fraction followed by chloroform fraction, whereas the contents obtained with butanol and water extracts were much smaller. This result showed that the total phenolic content depends on the type and polarity of the extracting solvents.

The in vitro activity of spring *U. rigida* (April) was qualitatively assessed by the determination of the inhibition zone diameters. Total extract showed an antibacterial activity against 90 % of the tested bacteria and failed to show a prominent antifungal activity (Table 2). The chloroform fraction showed

Table 1 Total phenolic content and EC₅₀ values of DPPH free radical scavenging, β -carotene–linoleate model, and acetylcholinesterase inhibition of *U. rigida* total extract and derived fractions

Extracts	TPC ^a (mg PHG g ⁻¹)	EC ₅₀ (mg mL ⁻¹) ^b		EC ₅₀ (μ g mL ⁻¹)
		DPPH assay	β -Carotene assay	
Total extract	9 \pm 0.71	>2	2.7 \pm 0.07	32.93 \pm 4.92
<i>n</i> -Hexane	–	0.65 \pm 0.08	1.5 \pm 0.09	96.67 \pm 6.48
CHCl ₃ F	13 \pm 1.10	0.95 \pm 0.14	2.1 \pm 0.07	0
EtOAcF	19 \pm 0.80	1 \pm 0.07	1.75 \pm 0.16	100 \pm 9.54
ButOHF	6 \pm 0.31	>2	>4	14.97 \pm 1.1
Water F	5 \pm 0.62	>2	2.9 \pm 0.2	3.2 \pm 0.85
Ascorbic acid	–	0.0036 \pm 0	–	–
BHT	–	–	0.048 \pm 0	–
Gаланthамин	–	–	–	19.9 \pm 0.48

Ascorbic acid, BHT, and galanthamin were used as standards. Each value represents the mean \pm SD ($n=3$)

AChE acetylcholinesterase (AChE) inhibition, – not tested, BHT butylated hydroxytoluene

^a TPC (mg PHG g⁻¹): total phenolic content expressed as mg of phloroglucinol acid equivalent per g of dry plant extract

^b EC₅₀ values corresponding to the amount of extract required to scavenge 50 % of radicals present in the reaction mixture

the widest range of antibacterial activity against both Gram-positive and Gram-negative bacteria. Ten out of the 11 tested bacterial strains were inhibited by chloroform fraction followed by the ethyl acetate and *n*-hexane fractions. Moderate effects were observed using butanol and water fractions against all tested bacteria. Although Perez et al. (1990) found that the extract of *Ulva lactuca* had no antimicrobial activity, our results showed that the organic extract of *U. rigida* inhibited most of the tested microorganisms. This discordance could be due to not only to subspecies differences, but also to the production of bioactive compounds related to the seasons and organic solvents used in the extraction. Moreover, results of antibacterial activity indicated that Gram-positive bacteria are more sensitive to the extracts than Gram-negative bacteria. The outer membrane of the Gram-negative bacteria acts as a barrier to many environmental substances including antibiotics (Tuney et al. 2006). Although diffusion agar is a common method of testing the bacterial sensitivity to plant extracts and antibiotics, the use of this method to determine the susceptibility of fastidious or slow-growing organisms present some drawbacks.

The scavenging activity of the spring *U. rigida* extracts was dose dependent. Ascorbic acid, used as positive control, exhibited 100 % inhibition of the DPPH radical at 0.01 mg mL⁻¹. The antioxidant efficiency can be also evaluated by the determination of the EC₅₀ value corresponding to the amount of the extract or fraction required to scavenge 50 % of DPPH radicals present in the reaction mixture. High EC₅₀ values indicated low antioxidant activity. The most potent radical scavenger fraction was the *n*-hexane fraction followed by those of chloroform and ethyl acetate fractions (Table 1). Butanol and water fractions showed higher EC₅₀ values (> 2 mg mL⁻¹) and consequently a lower

antioxidant capacity. Therefore, compounds with the strongest radical scavenging activity in *U. rigida* are of low to medium polarity. Furthermore, the highest content of total phenolic was found in ethyl acetate and chloroform fractions. These results were in favor of the implication of phenolic compounds in the antioxidant activity of *U. rigida* extracts by hydrogen transferring reaction.

In order to provide additional data on the antioxidant potential of spring *U. rigida* fractions, β -carotene bleaching assay was carried out. This test simulates the polyunsaturated fatty acid oxidation of the membrane lipid components. The linoleic acid free radical attacks the highly unsaturated β -carotene models. The ability of spring *U. rigida* extracts and the positive control, BHT, to prevent lipid peroxidation is presented in Table 1. The order of the antioxidant activity comparing the amount of extract required to the inhibition of 50 % linoleic acid peroxidation present in the mixture (EC₅₀) was found to be *n*-hexane > ethyl acetate > chloroform > water (Table 1). Butanol fraction showed a weak antioxidant activity compared to the BHT used as positive control. There was a significant positive association ($p<0.05$) between the DPPH radical scavenging ability and β -carotene bleaching capacity. Furthermore, a positive correlation ($R^2=0.98$) between the antioxidant activity and the phenolic contents was also observed. From these results, it is clear that *U. rigida* exhibited a capacity to reduce lipid oxidation and may have a therapeutic effect. Algae components present benefits in controlling inhibition of lipid oxidation in the rat and liver and inhibition of apoptosis and other protective mechanisms in cancer patients (Shon et al. 2003).

In order to study the protective effects of spring *U. rigida* fractions on hydroxyl radical mediated DNA strand breaks,

Table 2 Antimicrobial activities of *U. rigida* extracts and derived fractions against fungi and foodborne and spoiling bacteria

Extracts/fractions	Inhibition zone diameter (mm) ^a						
	Total extract	HexF	CHCl ₃ F	EtOAcF	ButOHF	WF	Genta ^b
Strains							
Bacterial strains							
Gram positive							
<i>Bacillus subtilis</i> ATCC 6633	10.3±0.3	08.5±0	08.3±0.1	09.3±0.2	10±0.0	19.3±0.6	20.5±0.2
<i>Bacillus cereus</i> ATCC 14579	14.6±0.5	14.3±0.8	16.5±0.5	11.6±0.6	–	–	20.3±0.4
<i>Staphylococcus aureus</i> ATCC 25923	08.5±0.3	–	–	12.5±0.5	9.5±0.5	09±0.2	25.5±1.1
<i>Staphylococcus aureus</i> ATCC 6536	10.5±0.5	15.6±1.3	18±0.5	–	–	11.6±0.8	16.5±0.8
<i>Staphylococcus epidermis</i> ATCC 12228	16.3±0.7	20.5±0.6	14.5±0.3	12.3±0.6	–	–	20±0.5
<i>Enterococcus faecalis</i> ATCC 29212	10.3±0.5	–	15.3±0.4	09±0	10.3±0.4	–	12±0.2
<i>Listeria monocytogenes</i> (food isolate)	10.5±0.5	21.5±1.0	13±0.5	–	–	–	20±0.7
Gram negative							
<i>Escherichia coli</i> ATCC 25922	10.3±0.4	– ^d	14.5±0.5	10.5±0.3	–	–	21±1.2
<i>Escherichia coli</i> ATCC 8739	– ^d	–	10.3±0.4	–	–	–	20±1
<i>Pseudomonas aeruginosa</i> ATCC 27853	10.6±0.5	–	12.66±0.6	08±0	–	–	18±0.6
<i>Klebsiella pneumoniae</i> CIP 32147	08.5±0.4	09.3±0.5	09.5±0.3	–	13.6±0.8	–	12±0.5
Fungal strains							
<i>Aspergillus niger</i> CTM 10099	07±0.10	–	–	–	–	–	15.3±0.9
<i>Fusarium graminearum</i> ISPAVE 271	–	–	–	–	–	–	14.5±0.5
<i>Fusarium culmorum</i> ISPAVE 21 W	–	12.6±0.2	–	–	–	16.5±0.5	14±0.3
<i>Fusarium oxysporum</i> CTM 10402	– ^d	11.6±0.8	–	10.5±0.5	08.5±0.2	–	12.5±0.8
<i>Alternaria alternata</i> CTM 10230	–	–	–	–	–	–	14.6±0.7
Yeast strain							
<i>Candida albicans</i> ATCC 10231	–	–	–	11.5±0.5	12.5±0.5	10.3±0.2	16±2.1

Each value represents the mean ± SD (n=3)

HexF *n*-hexane fraction, CHCl₃F chloroform fraction, EtOAcF ethyl acetate fraction, ButOHF butanol fraction, WF water fraction

^a Diameter of inhibition zone of various *U. rigida* extracts including diameter of disc 6 mm

^b Genta: gentamicin was used as a standard antibiotic at 15 µg mL⁻¹

^c Amph: amphotericin B was used antifungal standard at 20 µg mL⁻¹

^d Activity not detected

a free pBluescript II vector DNA breaks system in vitro was used. The supercoiled plasmid DNA was broken by hydroxyl radical generated from the Fenton reaction into three forms (supercoiled, open circular, and linear form). The effects of *U. rigida* ethyl acetate and chloroform fractions on pBluescript II DNA cleavage were demonstrated by the gel electrophoretogram (Fig. 2). The plasmid DNA was mainly of the supercoiled form in the absence of Fenton's reagent and converted into the relaxed circular and linear form after Fenton's reaction. The effects of *U. rigida* fractions and quercetin, known as free radical scavenger metal chelators, on the free radical-induced DNA damage were investigated in lanes 3, 4, 5, and 6 (Fig. 2). Quercetin and *U. rigida* fractions have a protective effect on the cleavage of DNA at least by 50 % except for butanol fraction which was inactive at the tested concentration. Thus, *U. rigida*-derived fractions are able to protect DNA from damage caused by

the hydroxyl radical. This effect might be due to their chelating activity on iron or hydroxyl radical scavenging activity or both.

Cholinesterase inhibitors represent the most promising therapeutic agents for Alzheimer's disease. Inhibition of AChE is considered as a promising approach for the treatment of

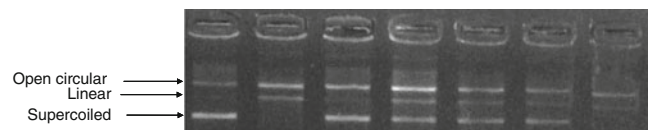


Fig. 2 Effect of *U. rigida* extracts on Fe²⁺/H₂O₂-mediated DNA strand breaks: lane 1, control of pBluescript II DNA; lane 2, DNA damage control (in the presence of Fenton reagents); lane 3, DNA in the presence of the quercetin used as positive control (100 µM); lanes 4–7, DNA in the presence of *n*-hexane, chloroform, ethyl acetate, and *n*-butanol fractions, respectively

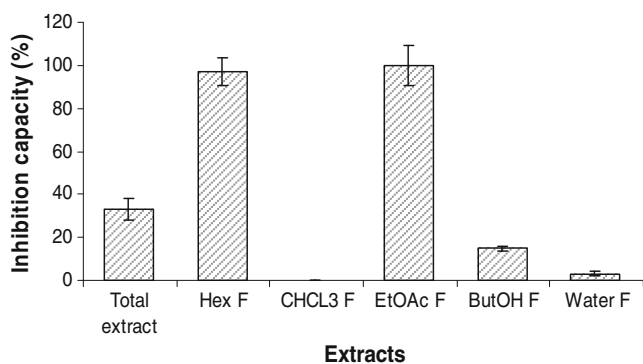


Fig. 3 Effect on acetylcholinesterase activity of *U. rigida* total extract and derived fractions. Values are means \pm SD ($n=3$)

Alzheimer's disease and for possible therapeutic applications in the treatment of Parkinson's disease, aging, and myasthenia gravis. Cholinesterase plays a key role in the hydrolytic degradation of acetylcholine, which is the primary neurotransmitter in the sensory and neuromuscular system regulation of cholinergic nervous transmission (Howes and Houghton 2003). Therefore, spring *U. rigida* total extract and derived fractions were evaluated for their ability to inhibit acetylcholinesterase. The *n*-hexane and ethyl acetate fractions showed a strong AChE enzyme inhibition (Fig. 3). At this concentration, total extract and butanol and water fractions exhibited low activity. Due to the high activity of the ethyl acetate and *n*-hexane fractions in the AChE assay, the inhibition effect of these two fractions at different concentrations ($1\text{--}100\ \mu\text{g mL}^{-1}$) was evaluated, and the EC_{50} values (the inhibitory dose that reduced 50 % of AChE activity) determined. The AChE inhibition showed an increase in inhibition with increasing concentrations of the *U. rigida* fractions. The best inhibitory activity was exhibited by the EtOAc fraction with an EC_{50} of $6.08\ \mu\text{g mL}^{-1}$ followed by the *n*-hexane fraction (EC_{50} of $7.6\ \mu\text{g mL}^{-1}$). The activity of both EtOAc and *n*-hexane fractions indicated that the inhibitory principles have low to moderate polarity. A number of studies have recently shown AChE inhibitory activity of several marine algae species. Among the most potent algae, the red *Ecklonia stolonifera* showed an inhibition of 45.97 % toward AChE at $100\ \mu\text{g mL}^{-1}$ (Yoon et al. 2008). *Hypnea valentiae* and *Ulva reticulata*, two species from Tamil Nadu, India, were able to inhibit AChE activity with EC_{50} values of 2.6 and $10\ \text{mg mL}^{-1}$, respectively (Suganthi et al. 2009). Thus, we noted the strong antiacetylcholinesterase of the Tunisian *U. rigida* compared to the *U. reticulata* collected from India. Algae can be used to enhance cognitive function and to alleviate other symptoms associated nowadays with Alzheimer's disease.

Chemical composition of *U. rigida*

Moisture content, protein, and lipid of the spring *U. rigida* were investigated. The fatty acid composition of *U. rigida*

Table 3 Chemical characteristics and fatty acid composition of *U. rigida*

Component	Content
Ash content (%)	19.10 \pm 0.62
Crude protein (%)	1.83 \pm 0.02
Lipids (%)	0.37 \pm 0.01
Fatty acids (%)	
Myristic (C14:0)	tr.
Palmitic (C16:0)	54.41 \pm 1.10
Palmitoleic (C16:1)	5.82 \pm 0.64
Stearic (C18:0)	1.39 \pm 0.15
Oleic (C18:1)	18.35 \pm 0.28
Linoleic (C18:2)	3.12 \pm 0.02
Linolenic (C18:3)	7.22 \pm 0.10
Arachidic (C20:0)	4.01 \pm 0.28
Eicosenoic (C20:1)	5.67 \pm 0.46
Σ SAFA	59.81 \pm 1.53
Σ MUFA	29.84 \pm 1.38
Σ PUFA	10.34 \pm 0.24

Values are means \pm SD of three determinations

SAFA saturated fatty

acids, MUFA monounsaturated fatty

acids, PUFA polyunsaturated fatty acids, tr: trace amounts (less than 0.2 %)

showed nine fatty acids, and five were unsaturated (Table 3). The most abundant fatty acids were palmitic acid, oleic acid, and linolenic acid. Among the detected fatty acids, unsaturated acids constitute 40.18 % of the total composition. These values are close to those obtained with *U. lactuca* and *Durvillaea antarctica* (Ortiz et al. 2006). These fatty acids make *U. rigida* specifically prone to oxidation and may have beneficial effects in health and in the control of chronic diseases. The chemical composition and properties of *Ulva* species had not been well investigated compared to the red and brown algae. The main constituent of Ulvaceae species is the typical polysaccharide, known as ulvan. Moreover, a survey of the chemical composition showed a wide range of compounds, particularly fatty acids, terpenes, sterols, and polyphenolic compounds. Seven labdane diterpenoids with potential antibacterial activity were isolated from *U. fasciata* and an antiinflammatory agent identified as 3-O- β -glucopyranosylstigmasta-5,25-diene from *U. lactuca* (Chakraborty et al. 2010). Polyunsaturated fatty acids isolated from *U. fasciata* and *U. pertusa* showed a strong algicidal activity on phytoplankton (Alamsjah et al. 2008).

Conclusion

In conclusion, significant variation in antimicrobial and antioxidant activities was demonstrated by the hydroalcoholic

extract of the green alga *U. rigida* collected monthly for 12 months. The highest antimicrobial activity was detected with the material collected during spring and summer, with a decline in activity in autumn and winter. A strong antioxidant activity was detected in late winter (February) and early spring (March). The variation in the activity corresponds to the variation observed in total phenolic content. These differences can be attributed to the seasonal changes in abiotic factors such as climate, salinity, temperature, pollution, and epiphytic organisms and also to different stages of plant metabolism. Methanol extract and derived fractions of spring *U. rigida* were tested for antifungal, antibacterial, and antioxidant activities and AChE inhibitory capacity. *U. rigida* showed a strong antimicrobial activity against a panel of microorganisms and potent antioxidant and anti-AChE activities. Further investigations to the isolation of specific bioactive compounds through bioassay-guided fractionation and their characterization as well as studies evaluating their safety are needed. *U. rigida* is an ideal candidate for cost-effective, readily exploitable natural phytochemical compounds and new therapeutic drugs. This seaweed is also rich in proteins and has low total lipid content and polyunsaturated fatty acids. These properties make the green alga *U. rigida* a healthy food for human and animal nutrition.

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