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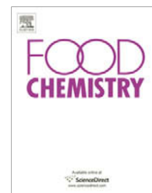

Codium tomentosum and *Plocamium cartilagineum*: Chemistry and antioxidant potential

Patrícia Valentão [a], Pedro Trindade [a], Daniela Gomes [a], Paula Guedes de Pinho [a], Teresa Mouga [b], Paula B. Andrade [a,*]

[a] REQUIMTE/Department of Pharmacognosy, Faculty of Pharmacy, Porto University, R. Aníbal Cunha, 164, 4050-047 Porto, Portugal
[b] Escola Superior de Turismo e Tecnologia do Mar, Instituto Politécnico de Leiria, Santuário N.ª Sra. Dos Remédios, Apartado 126, 2524-909 Peniche, Portugal

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ABSTRACT

The chemical composition of green *Codium tomentosum* Stackhouse and red *Plocamium cartilagineum* (Linnaeus) P.S. Dixon seaweeds from the Atlantic Ocean surrounding Portugal was studied. For the first time, the profile of organic acids was analysed in these matrices: seven and four organic acids were detected in green and red species, respectively. In *P. cartilagineum* these compounds were present in vestigial amounts, whilst *C. tomentosum* exhibited a higher content, with oxalic acid being the main compound. Phenolics, UV-absorbing compounds, were absent in both species. Volatiles profile was also determined for the first time and a total of 41 compounds were identified, which included alcohols, aldehydes, esters, halogenated compounds, ketones, monoterpenes (namely terrestrial ones), norisoprenoid derivatives, amongst others. Norisoprenoid derivatives and aldehydes were predominant. The main volatiles in green and red seaweeds were limonene and benzophenone, respectively. Additionally, both species revealed considerable antioxidant activity against both reactive oxygen (superoxide radical) and reactive nitrogen (nitric oxide) species, in a concentration-dependent manner.

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1. Introduction

The interest in marine organisms as a potential and promising source of pharmaceutical agents has increased during the last years (Celikler, Vatan, Yildiz, & Bilaloglu, 2009). Although fresh and dried seaweeds are extensively consumed, particularly by coastal peoples in several countries, they are considered as under-exploited resources (Fayaz et al., 2005).

Intertidal marine seaweeds live in harsh environments where they are subjected to repeated immersion and emersion due to tidal fluctuations. As result, they are periodically exposed to air, experiencing a variety of potentially stressful environmental conditions, including nutrient limitation, intense light, rapid temperature fluctuation, osmotic stress and desiccation (Burritt, Larkindale, & Hurd, 2002), which leads to the formation of free radicals and other oxidising agents (Zubia, Robledo, & Freile-Pelegrin, 2007). However, the absence of oxidative damage in the structural components of macroalgae (i.e., polyunsaturated fatty acids) and their stability to oxidation during storage suggest that their cells have protective antioxidant defence systems (Zubia et al., 2007). The use of seaweed species as alternative sources of natural antioxidant compounds has attracted the attention of biomedical scientists (Celikler et al., 2009). In fact, seaweeds and their extracts have demonstrated strong antioxidant activity (Yuan & Walsh, 2006). Proteins with antioxidative properties, phenolic compounds, such as flavonoids and coumarins, tocopherols, nitrogen-containing compounds including alkaloids, chlorophyll derivatives, amino acids and amines, as well as other compounds like carotenoids, ascorbic acid, glutathione and uric acid, are powerful antioxidant molecules found in macroalgae (Celikler et al., 2009).

Phytochemicals like phenolic compounds, organic acids and volatiles are known to influence the organoleptic properties of plant foods (Le Pape, Grua-Priol, Prost, & Demaimay, 2004; Ribeiro, Valentão, Baptista, Seabra, & Andrade, 2007). Additionally, these compounds may be involved in the protection against various diseases, due to their antioxidant potential (Celikler et al., 2009). A constant supply of distinct phytochemicals provides a most complete antioxidant support for diseases prevention, through overlapping or complementary effects (Ribeiro et al., 2007). Moreover, there is consumer demand for natural sources of antioxidants due to concerns over the potential toxic effects of synthetic antioxidants (Zubia et al., 2007). So, to obtain optimal health benefits, it has been suggested to adopt a diet composed of a variety of natural phytochemical sources, like seaweeds (Celikler et al., 2009).

Codium tomentosum (Chlorophyta) and *Plocamium cartilagineum* (Rhodophyta) are marine species found in the intertidal zone. As far as we know, there are no studies concerning samples of these

* Corresponding author. Tel.: +351 222078934; fax: +351 222003977.
E-mail address: pandrade@ff.up.pt (P.B. Andrade).

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species collected in the Atlantic Ocean. The limited number of studies on these species, from other sites, have concentrated on the fatty acid composition (Khotimchenko, 2003), the antioxidant, and antigenotoxic potential of a crude ethanolic extract (Celikler et al., 2009), anti-tumourigenic (El-Masry, Mostafa, Ibrahim, & El-Naggar, 1995) and hypoglycemic activities (Lamela, Anca, Villar, Otero, & Calleja, 1989) for *C. tomentosum*, and the presence of volatile halogenated metabolites in *P. cartilagineum* (Kladi, Vagias, & Roussis, 2004).

The present study aims to determine the organic acids, phenolic compounds and volatiles in the aqueous extract of *C. tomentosum* and *P. cartilagineum*. This was achieved by HPLC/UV, HPLC/DAD and GC/MS, respectively. In order to assess the antioxidant potential of these species, antiradical activities were also characterised by different biochemical methods, namely by evaluating their 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\cdot}$), superoxide radical ($O_2^{\cdot -}$) and nitric oxide ($^{\cdot}$NO) scavenging abilities. To the best of our knowledge, this is the first report on organic acids, phenolic compounds, volatile compounds and antioxidant activity of aqueous extracts of *C. tomentosum* and *P. cartilagineum*.

2. Materials and methods

2.1. Standards and reagents

Oxalic, ketoglutaric, malic, malonic, fumaric, quinic, lactic, shikimic, succinic and citric acids, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\cdot}$), nicotinamide adenine dinucleotide (NADH), phenazine methosulphate (PMS), nitroblue tetrazolium chloride (NBT), sulphanilamide, (*E*)-2-hexenal, (*E*)-2-octenal, (*E*)-2-nonenal, (*E*,*E*)-2,4-decadienal, benzophenone, limonene, safranal, β-cyclocitral, β-homocyclocitral, geranyl acetone and eugenol were obtained from Sigma Chemical Co. (St. Louis, MO, USA). (*E*,*Z*)-2,6-Nonadienal, (*E*)-2-decenal, methyl dihydrojasmonate, linalool, terpineol and β-ionone were from SAFC (Steinheim, Germany). Aconitic, ascorbic, tartaric and pyruvic acids and *o*-cymene were purchased from Extrasynthése (Genay, France) and acetic acid from Fisher Scientific (Loughborough, UK). *N*-(1-Naphthyl)ethylenediamine dihydrochloride, methanol, formic and phosphoric acids were from Merck (Darmstadt, Germany) and sulphuric acid from Pronalab (Lisbon, Portugal). Sodium nitroprussiate dehydrate (SNP) was from Riedel-de Haën (St. Louis, MO). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Sampling

Samples were collected along the coast of west Portugal. *C. tomentosum* Stackhouse (Chlorophyta) was picked at S. Felix da Marinha (Espinho) in June 2008 and *P. cartilagineum* (Linnaeus) P.S. Dixon (Rhodophyta) was collected at Praia do Porto de Areia Norte (Peniche) in September 2008. Samples were placed on ice and immediately transported to the laboratory in ice-boxes. Seaweeds were then cleaned, washed with sea water to remove epiphytes and encrusting material and kept at −20 °C, prior to their lyophilisation in a Labconco 4.5 Freezone apparatus (Kansas City, MO, USA). The dried material was powdered. Each sample corresponds to a mixture of three individuals.

2.3. Preparation of extracts

Aqueous extracts were prepared by putting ca. 3.0 g of dried material in 500 ml of water. The mixture was boiled for 20 min and then filtered through a Büchner funnel. The extracts obtained were then frozen and lyophilised. The lyophilised extracts were kept in a desiccator, in the dark.

2.4. HPLC/UV analysis of organic acids

The lyophilised extracts were redissolved in sulphuric acid 0.01 N and 20 μl were subjected to HPLC analysis. The separation of organic acids was achieved with an analytical HPLC unit (Gilson), using an ion exclusion column (Nucleogel Ion 300 OA; 300 × 7.7 mm) in conjunction with a column heating device at 30 °C. Elution was carried out isocratically with sulphuric acid 0.01 N, at a flow rate of 0.2 ml/min. Detection was performed with an UV detector set at 214 nm. Organic acids quantification was achieved by the absorbance recorded in the chromatograms relative to external standards.

2.5. HPLC/DAD analysis of phenolics

The lyophilised extracts were redissolved in water and 20 μl were subjected to HPLC analysis. This was performed in an analytical HPLC unit (Gilson), using a C18 Spherisorb ODS2 (25.0 × 0.46 cm; 5 μm particle size) column from Waters (Ireland). The solvent system used was a gradient of water–formic acid (19:1) (A) and methanol (B), starting with 5% methanol and installing a gradient to obtain 15% B at 3 min, 25% B at 13 min, 30% B at 25 min, 35% B at 35 min, 45% B at 39 min, 45% B at 42 min, 50% B at 44 min, 55% B at 47 min, 70% B at 50 min, 75% B at 56 min, and 80% B at 60 min, at a solvent flow rate of 0.9 ml/min. Detection was achieved with a Gilson diode array detector (DAD). Spectral data from all peaks were accumulated in the range 200–400 nm. The data were processed using Unipoint System software (Gilson Medical Electronics, Villiers le Bel, France).

2.6. Headspace–solid phase microextraction (HS–SPME)

Several commercial fibres can be used to extract volatiles. According to recommendations of the supplier (Supelco, Bellefonte, PA, USA) and to our knowledge (Guedes de Pinho et al., 2008, 2009), Blue – divinylbenzene/PDMS (DVB/PDMS) coated (50/30 μm) fibre is the most suitable for the intended compounds. It was conditioned by inserting it into the GC injector; both temperature and time used were in accordance to Supelco's recommendation procedure: 250 °C for 30 min.

Approximately 0.250 g of lyophilised aqueous extract was placed in a 15 ml vial and 5 ml of 10% ethanol were added. The vial was sealed with a polypropylene hole cap and PTFE/silicone septa (Supelco, Bellefonte, PA, USA). The mixture was then magnetically stirred (360 rpm), at 50 °C, for 5 min. The DVB/PDMS fibre was then exposed to the headspace for 60 min, with agitation (360 rpm). Afterwards, the fibre was pulled into the needle sheath and the SPME device was removed from the vial and inserted into the injection port of the GC system for thermal desorption. After 2 min the fibre was removed and conditioned in another GC injection port for 20 min, at 250 °C. Samples were analysed in triplicate.

2.7. Gas chromatography/mass spectrometry analysis

GC/MS analysis was performed using a Varian CP-3800 gas chromatograph (USA) equipped with a Varian Saturn 4000 mass selective detector (USA) and a Saturn GC/MS workstation software version 6.8. A VF-5 ms (30 m × 0.25 mm × 0.25 μm) column from Varian was used in the analyses. To check the identity of some of the compounds found with this column, a Stabilwax-DA fused silica (60 m × 0.25 mm, 0.25 μm) column (Restek, USA) was also used. The injector port was heated to 220 °C. The injections were performed in split less mode. The carrier gas was Helium C-60

(Gasin, Portugal), at a constant flow of 1 ml/min. The oven temperature was set at 40 °C for 1 min, then increasing 2 °C/min to 220 °C and held for 30 min. All mass spectra were acquired in electron impact (EI) mode. Ionisation was maintained off during the first 2 min. The ion trap detector was set as follows: the transfer line, manifold and trap temperatures were 280, 50 and 180 °C, respectively. The mass ranged from 40 to 350 *m*/*z*, with a scan rate of 6 scan/s. The emission current was 50 μA, and the electron multiplier was set in relative mode to auto tune procedure. The maximum ionisation time was 25,000 μs, with an ionisation storage level of 35 *m*/*z*. The analysis was performed in Full Scan mode.

Compounds were identified by comparing the retention times of the chromatographic peaks with those of authentic compounds analysed under the same conditions, and by comparison of the retention indices (as Kovats indices) with literature data (Hidehito, Hidenobu, & Daizo, 2002; Kajiwara, Hatanaka, Kawai, Ishihara, & Tsuneya, 1987, 1988; Kambourova, Bankova, & Petkov, 2003; Kamenarska et al., 2002, 2006; Kladi et al., 2004; Le Pape et al., 2004). The comparison of MS fragmentation pattern with those of pure compounds and mass spectrum database search was performed using the National Institute of Standards and Technology (NIST) MS 05 spectral data base. Confirmation was also conducted using a laboratory built MS spectral database, collected from chromatographic runs of pure compounds performed with the same equipment and conditions. The relative areas (RAs) of individual components, in relation to total identified compounds, are expressed as percentage of lyophilised aqueous extract. Chromatographic peaks' areas were determined by re-constructed Full Scan chromatogram using for each compound some specific ions. By this way some peaks which are co-eluted in Full Scan mode (resolution value less than 1) can be integrated with a value of resolution higher than 1.

2.8. DPPH· scavenging activity

The disappearance of DPPH· was monitored spectrophotometrically at 515 nm on a Multiskan Ascent plate reader (Thermo-Electron Corporation), following a described procedure (Ferreres et al., 2008). For each extract, a dilution series (five different concentrations) was prepared in a 96-well plate. The reaction mixtures in the sample wells consisted of 25 μl of lyophilised extract dissolved in water and 200 μl of 150 μM DPPH· dissolved in methanol. The plate was incubated for 30 min at room temperature after addition of DPPH·. Three experiments were executed, each one of them consisting of three determinations. IC values were estimated by interpolation of the nonlinear regression curve built using the mean values of the three experiments.

2.9. Superoxide radical ($O_2^{\cdot-}$) scavenging activity

The effect of the lyophilised extracts on the superoxide radical-induced reduction of NBT was monitored spectrophotometrically in a Multiskan Ascent plate reader in kinetic mode, at 560 nm. Superoxide radicals were generated by the NADH/PMS system according to a described procedure (Ferreres et al., 2008). All components were dissolved in phosphate buffer (19 mM, pH 7.4). Three experiments were executed, each one of them consisting of three determinations. IC values were estimated by interpolation of the nonlinear regression curve built using the mean values of the three experiments.

2.10. Nitric oxide (·NO) scavenging activity

The ability of the aqueous lyophilised extract to scavenge nitric oxide radicals was evaluated spectrophotometrically in a Multiskan Ascent plate reader according to a previously described method (Ferreres et al., 2008). A dilution series (five different concentrations) was prepared in a 96-well plate. The reaction mixtures in the sample wells consisted of dissolved lyophilised extract and SNP. The plates were incubated at 25 °C for 60 min under light. Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2% H_3PO_4) was then added and the absorbance of the chromophore formed during the diazotisation of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine was determined at 560 nm. Three experiments were executed, each one of them consisting of three determinations. IC values were estimated by interpolation of the nonlinear regression curve built using the mean values of the three experiments.

3. Results and discussion

Considering the toxicity of organic solvents, and for further comparison to what happens in a nutritional context, from a nutritional/biological point of view it is preferable to use aqueous extracts when assessing the antioxidant potential of a given species. Furthermore, some organic solvents, for example methanol, interfere in several assays, which render them impossible to be used in some of the antioxidant activity assays performed herein. A lyophilised extract of each species was prepared in order to be used in all assays, thus avoiding possible variation occurring during the extractive process. In addition, the use of lyophilised extract allows a better preservation of the extract during storage (it is not possible to test them immediately after their preparation), reducing the possibility of its alteration by, for example, hydrolysis or even evaporation of the solvent. So, we proceeded to the characterisation of the extract that was effectively tested for bioactivity.

Whilst the exact reason for the production of organic acids in seaweeds is unknown, recent evidence has suggested that it is associated with plant tolerance to environmental stress (López-Bucio, Nieto-Jacobo, Ramiréz-Rodríguez, & Herrera-Estrella, 2000).

The two species showed different organic acids profile. *C. tomentosum* was characterised by the presence of oxalic, aconitic, ketoglutaric, pyruvic, malic, malonic and fumaric acids (Fig. 1A), whilst *P. cartilagineum* presented oxalic, ketoglutaric, pyruvic and acetic acids (Fig. 1B). All of these compounds are described for the first time in these species. None of the samples revealed the presence of citric, succinic, quinic, ascorbic, shikimic, lactic or tartaric acids.

C. tomentosum aqueous lyophilised extract exhibited a ca. 11 g kg^{-1} concentration of organic acids, in which oxalic acid was the main compound, representing ca. 60.9% of total identified organic acids, followed by malic acid (ca. 31.3% of total acids, Table 1). Aconitic, malonic and fumaric acids were the minor compounds (Table 1). The quantification of the identified compounds in the red algae was not possible, due to their low levels. The high oxalic acid content may be important for the protection of the species against pathogens and epiphytes by affecting taste, texture, and calcium availability (Libert & Franceschi, 1987). Furthermore, this compound may act as a pH regulator and osmoregulator (Libert & Franceschi, 1987).

Phenolic compounds are secondary metabolites that have diverse biological activities, such as in defence mechanisms of plants under different environmental stress conditions like wounding, infection, excessive light or UV irradiation (Muchuweti et al., 2007). Intertidal seaweeds, when uncovered during low tide, are vulnerable for incurring photoinhibition or damage by photosynthetically active radiation or UV radiation. To avoid photodamage, these marine organisms may change their metabolism and produce a large number of active compounds capable of absorbing UV radiation, including polyphenols (Takeshi, Yumiko, & Joko,

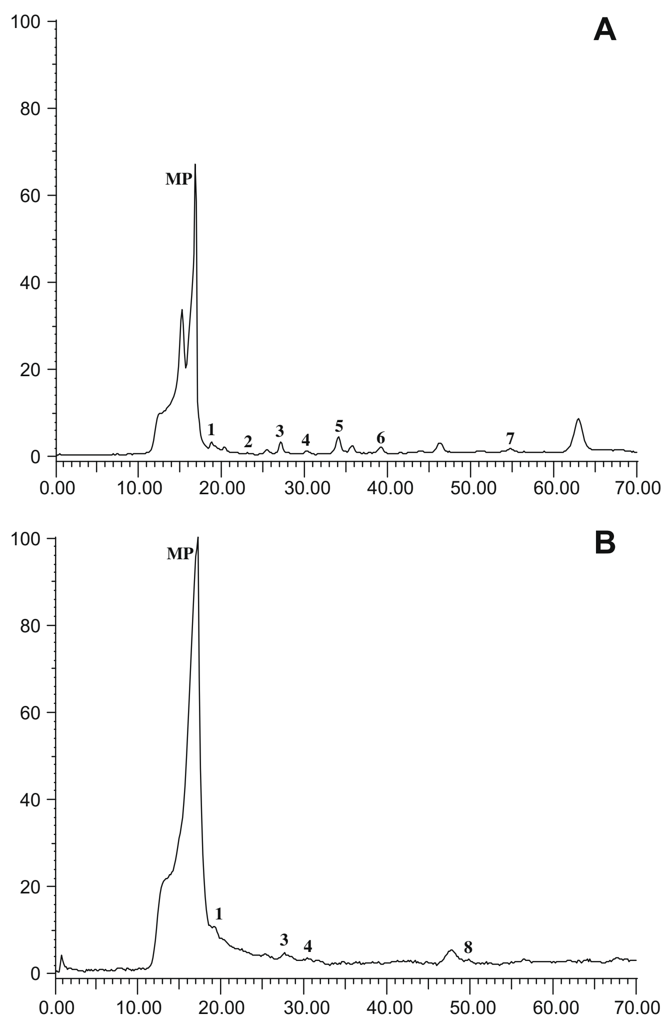

Fig. 1. HPLC–UV chromatograms of organic acids of (A) *C. tomentosum* and (B) *P. cartilagineum*. Detection was at 214 nm. Peaks: (MP) mobile phase; (1) oxalic acid; (2) aconitic acid; (3) ketoglutaric acid; (4) pyruvic acid; (5) malic acid; (6) malonic acid; (7) fumaric acid; and (8) acetic acid.

Table 1
Organic acids in *C. tomentosum* hot-water extract (mg of organic acid kg^{-1} of lyophilised extract).[a]

	Organic acid	Mean	SD
1	Oxalic	6505.8	796.0
2	Aconitic	nq	nq
3	Ketoglutaric	284.2	20.0
4	Pyruvic	549.2	10.4
5	Malic	3341.4	914.1
6	Malonic	nq	nq
7	Fumaric	nq	nq
	$\sum$	10,680.6	

[a]Results are expressed as means of three determinations. SD, standard deviation; $\sum$, sum of the determined organic acids; and nq, not quantified.

2005). Therefore, in this study the phenolic profile of *C. tomentosum* and *P. cartilagineum* was tentatively characterised by HPLC/DAD. However, no phenolics were detected, which was further confirmed by a negative result after addition of 20% sodium hydroxide solution to the aqueous extracts. The absence of this kind of compounds due to their degradation cannot be invoked, once the preparation of aqueous extract by boiling has already been used by our group before (Ferreres et al., 2008; Ribeiro et al., 2007), and several phenolic compounds were determined. The production of other UV-absorbing compounds, such as mycosporin-like amino acids, already described for species of the same phylum (Holzinger & Lütz, 2006), instead of phenolic compounds, may explain the absence of these compounds.

Volatiles investigations in plants often report biologically active compounds, which have defensive functions or are attractants, repellents, grazing inhibitors, insecticides, etc. (Kamenarska et al., 2002). There is very limited data about these compounds on marine algae and no previous study has considered the volatile composition of *C. tomentosum*. In studies concerning to *P. cartilagineum*, only volatile halogenated metabolites were reported (Kladi et al., 2004).

Forty-one compounds were identified (Table 2, Figs. 2 and 3) and, as far as we know, all of them are described for the first time in both *C. tomentosum* and *P. cartilagineum*. These compounds belong to different classes: one alcohol (1), nine aldehydes (2–10), six esters (11–16), two halogenated compounds (17–18), two ketones (19–20), five terpenes (21–25), ten norisoprenoid derivatives (26–35) and six other volatiles (36–41). From these, 37 were found in *C. tomentosum* and 17 in *P. cartilagineum*.

Carotenoids represent a diverse group of pigments widely distributed in nature. They serve as accessory pigments to harvest light for photosynthesis (Lu & Li, 2008). Moreover, these isoprenoid pigments play essential roles in nutrition and protection against photooxidative damage in algae (Lu & Li, 2008). The presence of carotenoids can give rise to volatile compounds, norisoprenoids, which constituted the main group of volatiles in the analysed species (Table 2). Norisoprenoids resulting from the oxidative cleavage of carotenoids are signals in algae development, serve as antifungal agents, and contribute to their flavour and aroma (Lu & Li, 2008). All of the ten norisoprenoid derivatives detected were present in the analysed Chlorophyta, whilst only some of them were noticed in the Rhodophyta, namely α-ionone (29), geranyl acetone (30), β-ionone (32), 2,3-epoxy-β-ionone (33) and dihydroactinidiolide (34). 2,3-Epoxy-β-ionone was the second major compound identified in the red algae (Table 2). β-Ionone (32), 2,3-epoxy-β-ionone (33) and dihydroactinidiolide (34), both metabolites of β-carotene, have been reported as the most common marine seaweeds norisoprenoids (Kamenarska et al., 2002, 2006). However, α-ionone (29) and hexahydrofarnesyl acetone (35) were also previously mentioned in many other algae species (Kajiwara et al., 1987; Kamenarska et al., 2002, 2006; Rzama, Benharref, Arreguy, & Dufourc, 1995). As the identified norisoprenoids are mainly metabolites deriving from the cleavage of β-carotene, it is possible to assume that this carotenoid was predominant in the analysed species.

Terpenoids are the most important volatile compounds of many terrestrial plants (Yassaa et al., 2008). It is worth considering that terpenes are common metabolites in algae and their formation by the condensation of isopentenyl pyrophosphate units is, in this regard, comparable to terrestrial metabolism (Yassaa et al., 2008). Unlike terrestrial mechanisms, marine terpene biosynthesis is thought to involve halogens through cyclisation reactions. Halogen-induced cyclisation of a slightly modified monoterpene precursor yields a halogenoterpene that can then be further halogenated (Yassaa et al., 2008). This process is common in marine organisms, but there is no literature evidence of terrestrial emissions of haloterpenes, and few works evidence that marine macroalgae produce monoterpenes with the same structures as those found in terrestrial plants (Yassaa et al., 2008). However, in this study, five terpenes, all of them found in terrestrial plants (Grayson, 1997), were identified and no halogenated terpene was detected. Although it is known that halogenated terpenes are synthesised by marine algae, as mentioned above, Abreu and Galindro (1996) indicated that the production of those compounds by a

Table 2
Average content (in percentage) of volatile compounds identified in lyophilised aqueous extracts of the two intertidal macroalgae.

Number	Compound	RT[a] (min)	QI[b] (*m*/*z*)	RA[c] (%) C. tomentosum	RA[c] (%) P. cartilagineum
Alcohols					
1	(*Z*)-4-Hexenol[d]	4.669	55/67/82	nd	0.718
Aldehydes					
2	(*E*)-2-Hexenal[d,e]	5.928	55/69/83	1.329	0.184
3	(*E*)-2-Heptenal[d]	8.763	57/70/83	1.245	0.229
4	(*E*)-2-Octenal[d,e]	11.769	70/93	0.762	0.208
5	(*E*,*Z*)-2,6-Nonadienal[d,e]	14.566	41/67/70	1.520	nd
6	(*E*)-2-Nonenal[d,e]	14.767	55/70/93	0.789	nd
7	(*E*)-2-Decenal[d,e]	17.679	83/70	0.634	nd
8	(*E*,*E*)-2,4-Decadienal[d,e]	19.239	81/152	3.476	nd
9	Dodecanal[d]	21.569	82/96/155	0.941	nd
10	Pentadecanal[d]	26.489	57/82/96	1.329	nd
Esters					
11	Hexyl hexanoate[d]	20.920	84/99/117	0.415	nd
12	Isopropyl laurate[d]	26.738	102/183/200	1.131	0.203
13	Methyl dihydrojasmonate[d,e]	27.362	83/93/151	3.714	7.299
14	Isopropyl myristate[d]	31.007	129/228	0.705	nd
15	Methyl palmitate[d]	33.047	87/143/270	0.257	nd
16	Isopropyl palmitate[d]	34.903	61/256/257	0.106	nd
Halogenated compounds					
17	Benzyl chloride[d]	8.565	91/126	nd	0.692
18	4-Bromo-1-cyclohexene[d]	10.397	81/93	nd	4.923
Ketones					
19	2-Dodecanone[d]	21.160	71/85/126/184	0.381	nd
20	2-Octadecanone[d]	27.780	71/127/169	0.339	nd
Monoterpenes					
21	Limonene[d,e]	10.859	68/93	28.060	nd
22	o-Cymene[d,e]	12.505	91/119	0.167	nd
23	Linalool[d,e]	12.963	93/121	3.087	nd
24	Menthone[d]	14.710	112/139/154	nd	4.299
25	α-Terpineol[d,e]	15.830	93/121/136	1.545	nd
Norisoprenoid derivatives					
26	Safranal[d,e]	15.957	91/107	0.983	nd
27	β-Cyclocitral[d,e]	16.530	109/137/152	0.481	nd
28	Citral[d]	17.846	69/84/137	1.568	nd
29	α-Ionone[d]	21.965	93/121	5.307	7.157
30	Geranyl acetone[d,e]	22.554	107	0.517	7.588
31	β-Homocyclocitral[d,e]	22.703	81/107/151	0.222	nd
32	β-Ionone[d,e]	23.385	177	2.059	7.044
33	2,3-Epoxy-β-ionone[d]	23.464	123/177	8.597	15.867
34	Dihydroactinidiolide[d]	24.698	111/137/180	8.837	9.240
35	Hexahydrofarnesyl acetone[d]	31.371	95/109	3.938	nd
Others					
36	Benzothiazole[d]	16.787	69/108/135	5.499	4.152
37	Methylethylmaleimide[d]	16.900	124/139	0.977	nd
38	Nonionic acid[d]	17.763	60/73	1.063	nd
39	Eugenol[d,e]	20.165	164	1.746	12.933
40	Decanolactone[d]	23.059	85/128/152	5.173	nd
41	Benzophenone[d,e]	27.019	77/105/182	1.099	17.258
Identified compounds				37	17
Alcohols				0	1 (0.718)
Aldehydes				9 (12.025)	3 (0.621)
Esters				6 (6.328)	2 (7.502)
Halogenated compounds				0	2 (5.615)
Ketones				2 (0.720)	0
Monoterpenes				5 (32.859)	1 (4.299)
Norisoprenoid derivatives				10 (32.509)	5 (46.896)
Others				6 (15.557)	3 (34.343)

[a]RT, retention time.
[b]QI, quantification ions.
[c]RA (%) = relative area in percentage.
[d]MS, tentatively identified by NIST05.
[e]Identified by comparison with reference compound.
nd, not detected.

given species depends on collection location and season. May be in our case those features were not the most appropriate ones for the production of those compounds by our samples. On the other hand, those compounds are usually extracted by organic solvents (Abreu & Galindro, 1996; Kladi et al., 2004) and, in our case, an aqueous extract was used. So, the use of a much more polar solvent may

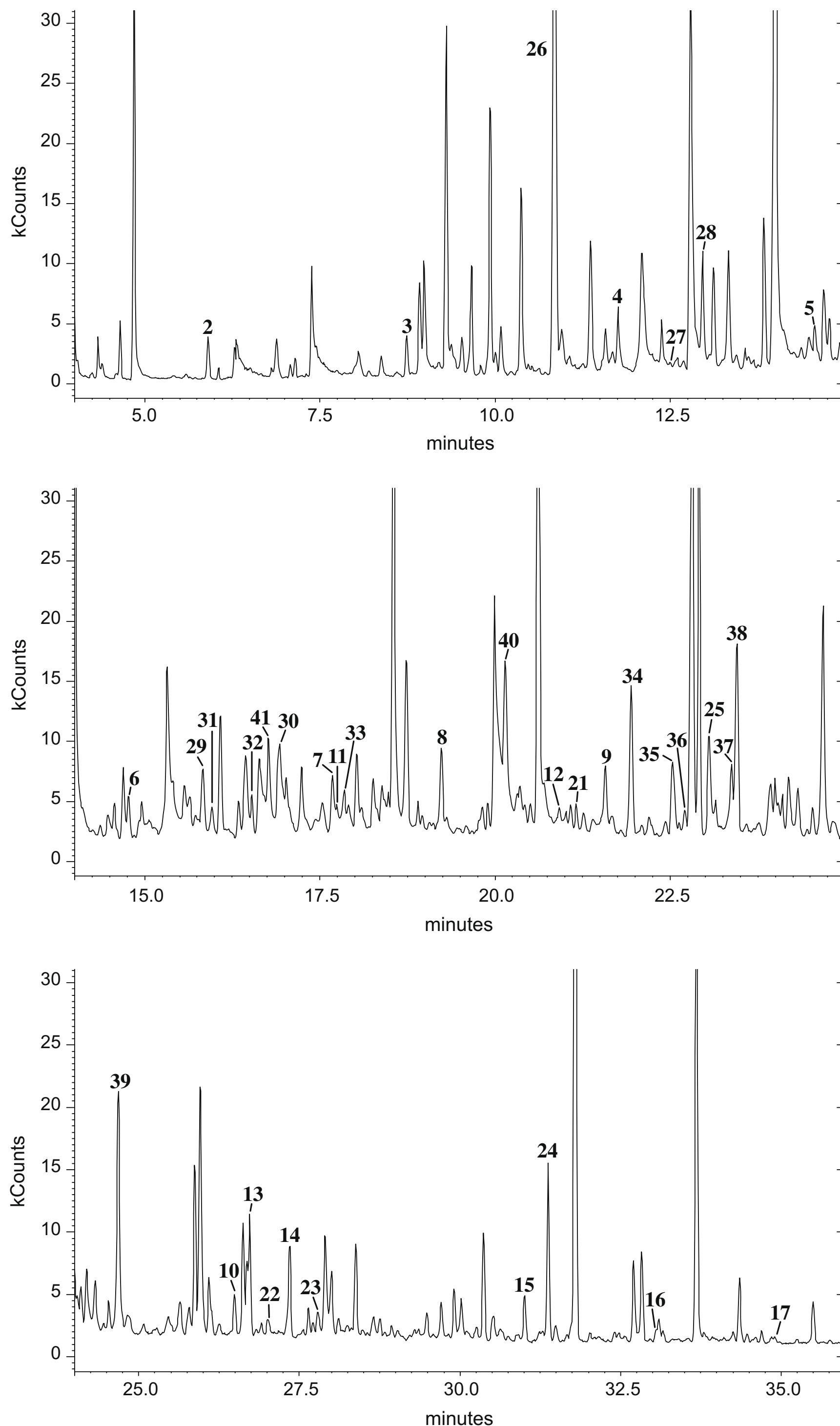

Fig. 2. Chromatographic profile of HS–SPME combined with GC/MS using divinylbenzene/PDMS fibre of the green algae *C. tomentosum*. Identity of compounds as in Table 1.

also justify the absence of this kind of compounds in our extract. In *P. cartilagineum* menthone (24) was the only identified terpene. The terpenoid composition of *C. tomentosum* was more complex, and the major identified compound was limonene (21) (Table 2). This compound has been found to be chemopreventive and chemotherapeutic against many rodent solid tumour types (Crowell & Gould, 1994).

Carbonyl compounds often possess allelopathic functions in plants, serving as attractants, repellents or pheromones (Kamenarska et al., 2006). Their role in algae is not known, but their significant concentrations and diversity are an indication that they could also exert some important allelopathic role (Kamenarska et al., 2006). Aldehydes constituted the second most abundant group of volatile compounds. All of the identified aldehydes could arise from the polyunsaturated fatty acids (Schwab, Davidovich-Rikanati, & Lewinsohn, 2008) and all of them are present in *C. tomentosum*, whilst the red algae only exhibited (*E*)-2-hexenal (2), (*E*)-2-heptenal (3) and (*E*)-2-octenal (4). (*E*,*E*)-2,4-Decadienal (8), the major aldehyde in *C. tomentosum* (Table 2), suppresses the development of copepod eggs (Kamenarska et al., 2006). The

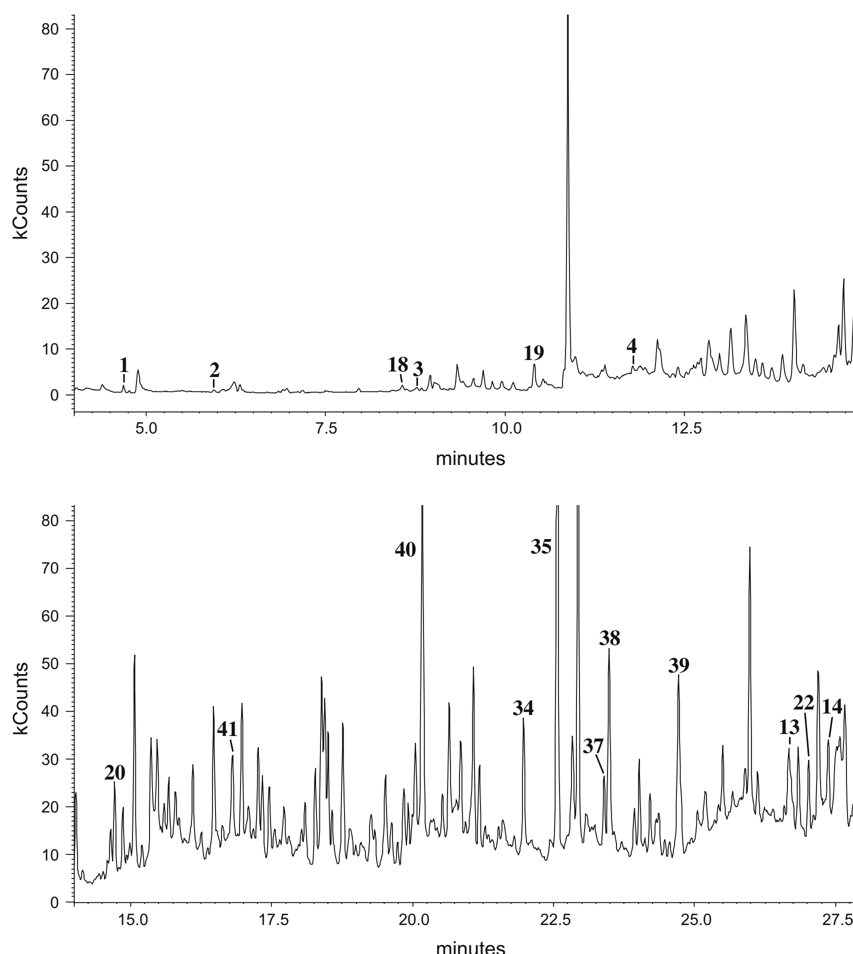

Fig. 3. Chromatographic profile of HS–SPME combined with GC/MS using divinylbenzene/PDMS fibre of the red algae *P. cartilagineum*. Identity of compounds as in Table 1.

diversity of aldehydes in the analysed green algae can be explained by the higher concentration of fatty acids previously described in species of *Codium* genus (Khotimchenko, 2003).

(*E*)-2-Hexenal (2), formed by enzymatic degradation of unsaturated fatty acids, (*Z*)-4-hexenol (1), the only identified alcohol, resulting from the reduction of the corresponding aldehyde, and hexyl hexanoate (11), formed by esterification of most abundant alcohols with common metabolic carboxylic acids, contribute to the so-called "green odour" (Ruther, 2000). The presence of these compounds indicates that the lipoxygenase pathway can be activated in the analysed algae (Ruther, 2000).

Other carbonyl compounds, namely esters, were also detected (Table 2, Figs. 2 and 3). All of the identified carbonyl compounds are fatty acid derivatives (Schwab et al., 2008) and were found in *C. tomentosum*, whilst only isopropyl laurate (12) and methyl dihydrojasmonate (13) were present in *P. cartilagineum*. These compounds appear to be common components of many marine algae (Kamenarska et al., 2006). Esters produced by terrestrial plants take an active part in plant-insect relationships and these compounds might have similar functions in algae (Kamenarska et al., 2006). Methyl dihydrojasmonate (13), the main ester in both species (Table 2), has been related with metabolites responsible for the protection mechanisms of plants, as phenolic compounds, terpenoids and alkaloids (Kim, Chen, Wang, & Choi, 2006). The presence of methyl palmitate (15) and isopropyl palmitate (16), palmitic acid derivatives, in *C. tomentosum* is not surprising, once palmitic acid appears to be the most abundant fatty acid in this species (Khotimchenko, 2003).

Two aliphatic methyl ketones, 2-dodecanone (19) and 2-octadecanone (20), were found in the analysed green seaweed but not in *P. cartilagineum* (Table 2). These compounds may be derived from the biosynthesis and/or degradation of fatty acids, such as palmitic, oleic and linoleic, previously mentioned as the main fatty acids in *C. tomentosum* (Khotimchenko, 2003), perhaps by decarboxylation of the β-ketoacid, an intermediate in both pathways (Schwab et al., 2008). In *P. cartilagineum*, only polyhalogenated homosesquiterpenic fatty acids were identified (Kladi et al., 2004), which may explain the absence of ketones in this species.

Two simple halogenated compounds, benzyl chloride (17) and 4-bromo-1-cyclohexene (18), were identified, for the first time, in the analysed Rhodophyta (Table 2). All of the halogenated metabolites already described in this species have very complex structures (Kladi et al., 2004). No halogenated compound was detected in the Chlorophyta. This can be explained by the fact that, of all marine seaweeds, Rhodophyta possess the highest abundance of unique biosynthetic pathways for organohalogen production (Kladi et al., 2004). The detection of halogen-containing compounds in red algae is an indication of the presence of haloperoxidase in the species: the emission of this kind of compound is only possible when the biological halogenation occurs by algal haloperoxidases (Kamenarska et al., 2006). These compounds are produced by algae as allelochemicals, which possess antibacterial and antifungal effects, suppress the development of other algae and induce larval settlement (Kladi et al., 2004). The common presence of these metabolites in marine organisms is due to the abundance of chloride and bromide ions in seawater (Kladi et al., 2004).

Amongst other miscellaneous volatiles, one sulphur containing compound, benzothiazole (36), was also identified in the two analysed species (Table 2). This compound was previously identified in other algae, namely in *Padina pavonia* (Kamenarska et al., 2002), but its functions in the organism are not clear. Methylethylmaleimide (37) is a simple alkaloid identified in the analysed green algae (Table 2), which originates from photo-oxidation of chlorophyll (Xian, Chen, Liu, Zou, & Yin, 2006).

One free fatty acid, nonanoic acid (38), was also detected (Table 2). Free fatty acids have different functions in organisms, where they serve as energetic substrates and allelophatic agents (Kamenarska et al., 2006). It is also known that the antibiotic activity of some algae species might be attributed to the presence of a mixture of free fatty acids (Kamenarska et al., 2006).

Eugenol (39), the simplest phenylpropanoid, is a phenolic aroma component identified in both analysed species, being the third main compound in *P. cartilagineum* (ca. 12.9%) (Table 2). It is known that phenylalanine is its initial precursor, and one of the steps of its synthesis implies the formation of ferulic acid (Koeduka et al., 2006). Many reports suggest this compound, in general, acting as antimicrobial and antianimal toxin with analgesic properties for humans (Koeduka et al., 2006).

Decanolactone (40) was observed only in *C. tomentosum* (Table 2). Many bioactivities have been attributed to lactones, including insecticidal, herbicidal, antimicrobial and cytotoxic effects (Viana, Oliveira, Brum, Picada, & Pereira, 2007). This compound, in mice, has revealed a dose-dependent effect on the central nervous system, including hypnotic, anticonvulsant and hypothermic activities (Viana et al., 2007); however, as far as we know, no studies report these activities in the analysed Chlorophyta.

Benzophenone (41), the immediate precursor of xanthones in higher plants (Liu, Falkenstein-Paul, Schmidt, & Beerhues, 2003), was the main compound in *P. cartilagineum* (ca. 17.2%), being also found in *C. tomentosum* (Table 2, Figs. 2 and 3). The biosynthesis of benzophenones and xanthones involves benzoic acids (benzoyl-CoA and 3-hydroxybenzoyl-CoA) that arise biosynthetically from cinnamic acid, itself supplied by phenylalanine (Liu et al., 2003). This compound is, by far, one of the most widely used conventional low molecular weight photoinitiator for UV-curing (Decker, 1996). However, it has some disadvantages: it is known for its relatively strong odour, yellowing, and exceptional ability to migrate and be extracted from cured products (Decker, 1996).

Hydrocarbons have been described as abundant volatiles of seaweeds (Kambourova et al., 2003). However, in the analysed species, hydrocarbons were not found. Kamenarska et al. (2006) concluded that probably the hydrocarbons' concentrations can be connected with the evolutionary level of the algae: highest hydrocarbons concentrations were present in evolutionary advanced algae, whilst in the lowest ones only traces were found. The absence of hydrocarbons in the two analysed species seems to point that they correspond to a lower evolutionary degree.

The identified secondary metabolites are mainly simple metabolites. The absence or the little amounts of complex compounds can be explained by the salt water that markedly suppresses biosynthetic pathways of secondary metabolism (Kambourova et al., 2003).

As described above, in the studied lyophilised extracts a significant number of compounds possessing biological activity were identified. In order to confirm this assumption, we additionally investigated the antioxidant capacity of the lyophilised extracts from *C. tomentosum* and *P. cartilagineum*. Nevertheless, it is difficult to compare the antioxidant activity between the two studied species since they were collected at different periods and origins. In fact, the production of antioxidant compounds is influenced by several factors, both extrinsic (herbivory pressure, irradiance, depth, salinity, nutrients, etc.), and intrinsic (type, age and reproductive stage) (Connan, Delisle, Deslandes, & Ar Gall, 2006).

Basic knowledge on the efficacy of compounds in extracts to quench free radicals can be inferred from the DPPH$^{\cdot}$ assay. The screening of the lyophilised aqueous extracts of *C. tomentosum* and *P. cartilagineum* revealed a weak concentration-dependent antioxidant capacity only for the green algae (IC_{10} at 794 µg/ml) (Fig. 4A). These results are quite surprising because these species live in highly dynamic upper littoral areas, characterised by numerous stress factors (i.e. high irradiance, temperatures and desiccation). However, Choo, Snoeijs, and Pedersen (2004) described *Enteromorpha ahlneriana* and *Cladophora glomerata*, two intertidal algae, as more stress-susceptible and stress-tolerant species, respectively, and explained the relation between antioxidant

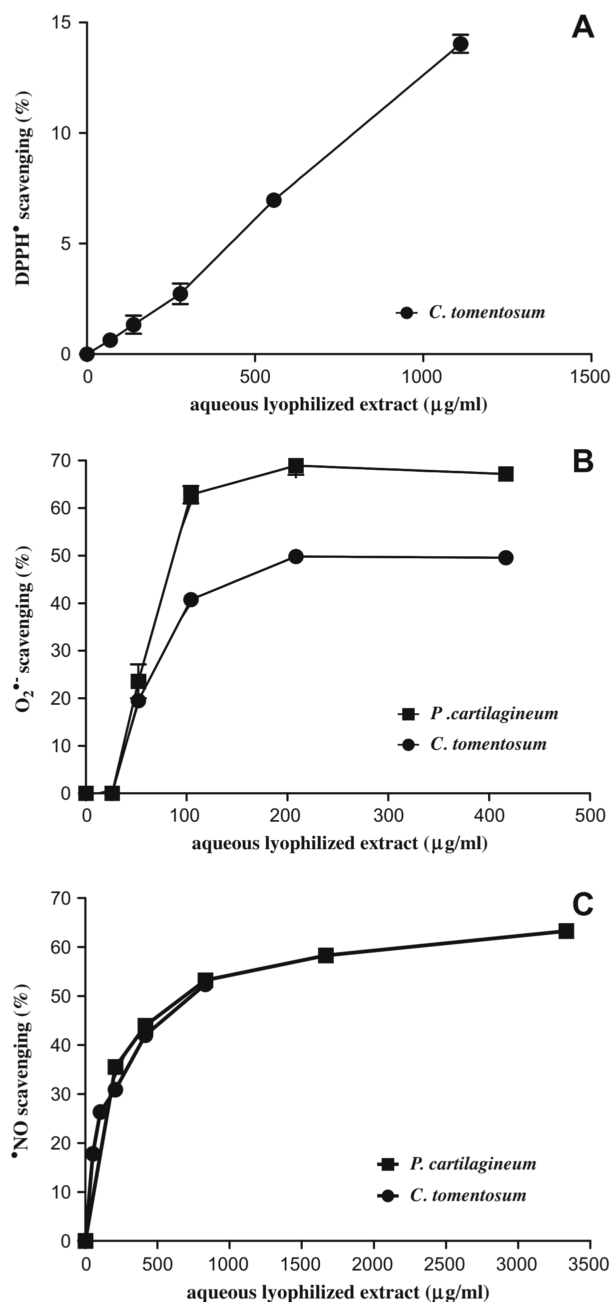

Fig. 4. Effect of green and red algae aqueous extracts on the scavenging of (A) DPPH$^{\cdot}$ (only green algae aqueous extract); (B) superoxide radical; and (C) nitric oxide. Values show mean ± SE from three experiments performed in triplicate.

defence and their life strategy. So, it may be that antioxidative mechanisms in *P. cartilagineum* are less expressed.

In the present work, extracts from *C. tomentosum* and *P. cartilagineum* were tested for their ability to scavenge the superoxide radical and nitric oxide, reactive oxygen and nitrogen species, respectively. Superoxide radical is one of the most effective free radicals, implicated in cell damage as precursor of main reactive oxygen species, like hydroxyl radical and peroxynitrite, contributing to the pathological process of many diseases (Zubia et al., 2007). The aqueous extracts of the two seaweeds exhibited a concentration-dependent superoxide radical scavenging capacity: the red algae revealed to be more effective (IC_{25} at 54 μg/ml) than the green species (IC_{25} at 66 μg/ml) (Fig. 4B).

Nitric oxide is involved in several physiological processes, like blood pressure control, neural signal transduction, platelet function and antimicrobial defence (Furchgott, 1999; Ignarro, 1999). Despite the beneficial effects, an overproduction of this reactive species is associated with several types of biological damage (Beckman, 1996). In addition, it reacts rapidly with superoxide radical to form peroxynitrite, a major damaging oxidant produced *in vivo* (Beckman, 1996). The analysed extracts displayed quite similar protective activity against nitric oxide, which was concentration dependent: the scavenging ability followed the order red algae (IC_{50} at 688 μg/ml) > green algae (IC_{50} at 737 μg/ml) as shown in Fig. 4C. Thus, besides the scavenging capacity observed for both superoxide radical and nitric oxide, the two seaweeds may also prevent the formation of other biologically important oxidative species resultant from the reaction of those two, like peroxynitrite and hydroxyl radical.

In conclusion, both *C. tomentosum* and *P. cartilagineum* showed the presence of organic acids and a great variety of volatile compounds. These biological active compounds may contribute to the observed antioxidant activities, suggesting that these intertidal macroalgae developed a defence system, which reflects an adaptation to harsh environment. So, these two marine species can be good sources of active components. Additionally, the observed antioxidant activity can potentially elevate their beneficial value as human food or additives, and expand their dietary market.

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