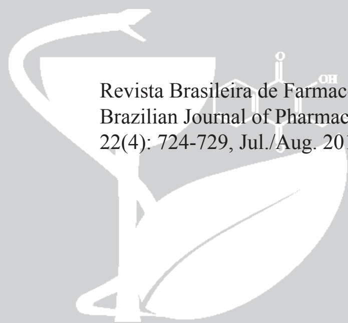

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Antioxidant activity and chemical composition of the non polar fraction of *Gracilaria domingensis* (Kützing) Sonder ex Dickie and *Gracilaria birdiae* (Plastino & Oliveira)

Thais Guaratini,[1] Norberto P. Lopes,[2] Eliane Marinho-Soriano,[3] Pio Colepicolo,[1] Ernani Pinto[4*]

[1]Departamento de Bioquímica, Instituto de Química de São Paulo, Universidade de São Paulo, Brazil,
[2]Departamento de Física e Química, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto-SP, Brazil,
[3]Departamento de Oceanografia e Limnologia, Universidade Federal do Rio Grande do Norte, Brazil,
[4]Departamento de Análises Clínicas e Toxicológicas, Faculdade de Ciências Farmacêuticas Universidade de São Paulo, Brazil.

Abstract: *Gracilaria domingensis* (Kützing) Sonder ex Dickie and *Gracilaria birdiae* (Plastino & Oliveira) (Gracilariales, Rhodophyta) are seaweeds that occur on the Brazilian coast. Based on their economic and pharmaceutical importance, we investigated the antioxidant activity of the methanolic, ethyl acetate and hexane extracts of both species. The hexane extracts display a high antioxidant activity and comparative analyses indicated *G. birdiae* as the most active species. Chemical investigation of these fractions showed several carotenoids and fatty acids, as well as cholesterol and sitosterol derivatives. HPLC-DAD analysis of *G. birdiae* showed violaxanthin (0.04 $\mu g.mg^{-1}$ of dry material), antheraxanthin (5.31 $\mu g.mg^{-1}$), aloxanthin (0.09 $\mu g.mg^{-1}$), zeaxanthin (0.45 $\mu g.mg^{-1}$) and β-carotene (0.37 $\mu g.mg^{-1}$) as the major carotenoids. *G. domingensis* showed a similar carotenoid profile, however, with much lower concentration than *G. birdiae*. Gas chromatography coupled to mass spectrometry was used to determine other nonpolar compounds of these seaweeds. The main compounds detected in both studied species were the fatty acids 16:0; 18:1 Δ^{9}; 20:3 $\Delta^{6,9,12}$, 20:4 $\Delta^{5,8,11,14}$. We found no specificity of compounds in either species. However, *G. birdiae*, presented higher contents of carotenoids and arachidonic acid than *G. domingensis*.

Introduction

Seaweeds have been used in traditional medicine for centuries and some genera, including *Gracilaria*, present species that are economically important since they are used in the pharmaceutical, nutraceutical, cosmetic and food industries (Blouin et al., 2011; Gressler et al., 2010, Holdt & Kraan, 2011). Fresh and dried seaweeds are extensively commercialized because they are rich in proteins, fatty acids, vitamins, and minerals (Cardozo et al., 2007; Fayaz et al., 2005; Gressler et al., 2011). As described for plants, nutrient and chemical composition of seaweeds may vary due to species, temperature oscillation and geographic area (Cardozo et al., 2007; Gobbo-Neto & Lopes, 2007, 2008).

Previous work applied simultaneous fluorescence and O_2 evolution experiments for calculation of the percentage of carotenoids in *G. domingensis* and *G. birdiae* (Andersson et al., 2006). The comparison suggested a lower content of carotenoids in *G. domingensis* compared to *G. birdiae*. *Gracilaria* is economically important for agar production in Brazil; however, there is little quantitative information on the individual carotenoids and the fatty acid composition of these two species (Carnicas et al., 1999; Guaratini et al., 2005; Guaratini et al., 2007; Pinto et al., 2011). Valuable products extracted from algae have been studied at different levels (Pinto et al., 2003; Cardozo et al., 2008).

Regarding natural products produced by macroalgae and the correlation with environmental conditions, sensitive analytical procedures for the separation, identification and quantification of the major metabolites in apolar and polar extracts are needed for

more detailed analyses and future studies. In the present study, we employ HPLC-DAD and GC-MS for the analyses of the carotenoid and fatty acid contents in two wild *Gracilaria* species (*G. domingensis* and *G. birdiae*) that occur in Brazil. A comparison of the antioxidant activities of hexane and ethyl acetate extracts of *G. domingensis* and *G. birdiae* was also carried out.

Materials and Methods

Chemicals

All solvents were HPLC or reagent grade, from Sigma Chemical Company (St. Louis, MO, USA), Tedia (São Paulo, Brazil) and Mallinckrodt (Paris, KY, USA). Deionized water was obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA). Stock solutions of antheraxanthin (0.625 mg L^{-1}), lutein (1.296 mg L^{-1}), violaxanthin (0.814 mg L^{-1}), and zeaxanthin (0.591 mg L^{-1}) in ethanol were purchased from DHI Water & Environment (Copenhagen, Denmark) and stored at -80°C. β-Carotene, astaxanthin and chlorophyll *a* and *b* were purchased from Sigma (St. Louis, USA).

Algae material

Gracilaria domingensis (Kützing) Sonder ex Dickie (Gracilariales, Rhodophyta) and *Gracilaria birdiae* Plastino & Oliveira (Gracilariales, Rhodophyta) were collected at Praia do Cotovelo in Natal, Brazil (5° 55' 33.6" N – 35° 9' 14.4" W), and stored at -80 °C until extraction. Voucher samples were collected by Prof. Dr. Eliane Marinho-Soriano, Federal University of Rio Grande do Norte, RN.

Antioxidant activity

DPPH (1,1-diphenyl-2-picrylhydrazyl) solution were prepared in methanol, as previously published (Yokozawa et al., 1998; Pellat et al., 2004). In a 96-well microplate, 10 µL of a 1 mM solution of DPPH and appropriate volumes of antioxidant (to obtain final concentrations of 0.1, 0.5, 1.0, 1.5 and 2.0 mg/mL) were added and the final volume of 100 µL completed with methanol. For each concentration of the antioxidant, corrections were made for the absorbance of the reference (samples without DPPH solution) and comparisons made to the rate in the absence of any added antioxidant (negative control). After preparation, samples were placed in an Elisa reader at 25 °C in the dark. The samples were incubated for different time intervals (10 to 130 min with 10-min intervals) and then standardized to the same reaction time for all extracts. Triplicates were performed for each concentration and the readings were made at 517 nm (Pellati et al., 2004; Yokozawa et al., 1998).

For the peroxidation assay, linolenic acid micelles were prepared with 2.6 mM of sodium dodecyl sulfate (SDS) in 0.01 M sodium phosphate buffer, pH 7.4. An aliquot of 10 µL of methanol solution of the antioxidant to be tested at different concentrations was added to 2 mL of lipid suspension in a quartz cuvette. After 10 min incubation at 50 °C, lipid peroxidation was initiated with 10 µL of 0.07 M 2,2'-azobis(2-amidinopropane) (ABAP), also prepared in phosphate buffer. The kinetics of formation of lipoperoxides was accompanied spectrophotometrically at 234 nm for 15 min (Chicaro et al., 2004).

Preparation of samples for HPLC analysis

The samples were prepared as previously published (Guaratini et al., 2009). In resume, the homogeneous, freeze-dried samples were weighed, dissolved in methanol:acetone (1:1, v:v) and sonicated for 15 min. The extracts were then centrifuged and filtered through a 0.45 µm membrane (Millex HN nylon, 13 mm, Millipore). Aliquots (50 µL) of the extract (3 mg mL^{-1}) were injected into the HPLC system. The pigment content was expressed as µg *per* mg of dry weight (µg mg^{-1} DW).

HPLC equipment

The HPLC instrument consists of a Shimadzu SCL-10A*VP* system equipped with two LC-10AD pumps, a SIL-10AD*VP* automatic sample injector, a DGU-14A degasser, and a SPD-M10A*VP* photodiode-array detector. The Coulochem III ED electrochemical detector (ESA Inc., Chelmsford, MA), coupled in series with the photodiode-array detector, consisted of a guard cell (Model 5021) and an analytical cell (Model 5010) with two porous graphite working electrodes, palladium reference electrodes and platinum counter electrodes.

The analyses were carried out as previous reported (Guaratini et al., 2009). Chromatographic separations were carried out on a C_{30} column (Ultracarb, 250 x 4.6 mm, 5 µm, Phenomenex) at 1.0 mL min^{-1} and room temperature, using as mobile phase: (A) methanol:water:1 M ammonium acetate buffer, pH 4.6 (90:8:2), and (B) methanol:methyl-tert-butyl ether (MTBE):1 M ammonium acetate buffer, pH 4.6 (30:68:2). The gradient elution was performed as follows: a linear increase from 5 to 10% of solvent B (0-15 min); maintaining 10% B for 10 min; a linear gradient (10 min) to 15% B followed by another linear gradient (5 min) to 40% B and then an increase to 45% of solvent B in 2 min, an isocratic elution for 20 min and an increase to 100% B in 1 min and maintaining 100% B for 5 min, for a total run time of 68 min. The chromatographic parameters, capacity factors (*k*) and separation factors (α) were calculated to

evaluate the separation efficiency. The injection volume of standards and samples was 50 µL and all ultraviolet-visible spectra were recorded from 200 to 800 nm. For quantitative analyses, chromatograms were integrated at 445nm. The ED was operated in the DC-mode and the settings were as follows: +850 mV at the guard channel (to improve the baseline signal by oxidizing interfering compounds possibly present in the mobile phase) and +600 mV at the first cell (channel 1). For confection of the hydrodynamic voltammograms, data were acquired in channel 1, which was set at different potentials in each run (+100 to +900 mV, in 50 mV increments).

Preparation of samples for CG-MS analysis

Ten milliliters (10 mL) of dichloromethane (J.T.Baker, Phillipsburg, NJ, USA) were added to 1.0 g (DW) of each evaporated hexane extract of algae and submitted to an ultrasonic bath for 30 min followed by filtration. Subsequently, 2.0 mL of 1.0 M sodium methoxide were added to the extract and shaken occasionally during 5 min at 65 °C. After cooling, 1.0 mL of water was added and the mixture was extracted with 1.0 mL of chloroform (J.T.Baker, Phillipsburg, NJ, USA) by shaking for one minute and centrifuging at 3,000 rpm to extract methyl esters of the fatty acids. The chloroform extraction was further repeated two times. The chloroform phases were combined (3.0 mL) and the solvent evaporated under a flow of nitrogen gas. Samples were suspended in 1.0 mL of ethyl acetate (J.T.Baker, Phillipsburg, NJ, USA), dried with anhydrous sodium sulfate (Sigma Inc., St. Louis, MO, USA).

GC-MS analysis

Samples were analyzed by GC-MS with a Shimadzu QP2010 with ionization source of 70 eV and fragmentation by electron impact (EI). Samples of 1.0 µL of each sample were injected at 220 °C on a DBWAX column (30 m x 0.25 mm x 0.25 µm). The analysis occurred in the splitless mode, with 1 min sample time, a column flow of 1.3 mL min^{-1}, a linear velocity of 41.4 cm s^{-1} and scan between *m/z* 40 and *m/z* 500. The oven temperature started at 50°C, was increased at 20°C • min^{-1} to 200 °C, kept at this temperature for 5 min, then increased at 5 °C min^{-1} to 230 °C and kept at this temperature for 30 min. For identification of the compounds, the peaks were compared with standards, when available, always consulting the WILEY7 library.

Results and Discussion

The DPPH system generate a stable free radical and is often used in preliminary studies, mainly for screening extracts to indicate antioxidant capacity. This procedure is fast and, at the same time, sensitive (Yokozawa et al., 1998). Hydrogen donor molecules present in the extract that react with the DPPH radical cause a decrease in the intensity of absorption at 517 nm and can help identify extracts with anti-radical compounds. This reaction is dependent on exposure and, after a time course analysis, we established a reaction time of 30 min to test all extracts and determine the IC30 and IC50 values (minimum inhibitory concentration of 30 or 50% of the radical, respectively) (results are presented in Table 1). *G. birdiae* extracts showed better results. The hexane fractions from *G. birdiae* required approximately 0.6 $mg.mL^{-1}$ and 0.8 $mg.mL^{-1}$ extract for 50% inhibition of DPPH radical. An additional lipid peroxidation assay was carried out to confirm the antioxidant activity. Lipid peroxidation is initiated by any species capable of abstracting a hydrogen atom from the lipid methylene groups (Gutteridge & Halliwell, 1990). The method used in this work used micelles of linolenic acid in buffer and SDS. The linolenic acid in the micelles reacts with the radical initiator ABAP and the procedure measures the kinetics of lipoperoxide formation. When antioxidants are included in this system, there is a consequent reduction in the formation of lipoperoxides and, therefore, the rate of reaction (Chicaro et al., 2004) decreases. The initial rates of formation of lipoperoxides were plotted versus the concentrations tested for each extract, to obtain a slope value that can be compared to the positive control (α-tocopherol solution). From these data, we determined the relative antioxidant activity of

Table 1. Antioxidant activity of the *Gracilaria birdiae* and *G. domingensis* extracts determined from the inhibition of DPPH formation. Data are presented as IC30 and IC50, from five independent experiments.

Extracts	*G. birdiae*		*G. domingensis*	
	IC30	IC50	IC30	IC50
hexane	0.387	0.610	>2.0	>2.0
hexane:ethyl acetate	0.486	0.833	>2.0	>2.0
ethyl acetate	0.665	1.193	>2.0	>2.0
butanol	0.372	1.466	1.432	>2.0
methanol/H2O	>2.0	>2.0	1.551	>2.0

each extract, compared to the activity of α-tocopherol, as presented in Table 2. In these trials, as in the DPPH assay, the highest activities were detected in the more apolar extracts of the alga *G. birdiae*. In previous work, Nahas et al. (2007) also observed a higher activity in the less polar extracts by testing different species of algae, reinforcing our observation.

Table 2. Antioxidant activity of the *Gracilaria birdiae* and *G. domingensis* extracts by the lipoperoxidation inhibition assay (ABAP).

Extracts	*G. birdiae*	*G. domingensis*
hexane	3.5	-
hexane:ethyl acetate	2.4	1.3
ethyl acetate	0.75	1.0
butanol	1.02	1.4
methanol/H2O	0.29	0.74

These biological results stimulated the investigation of the apolar extracts (hexane) of both algae. A previously published methodology was applied for the identification and quantification of apolar compounds using co-injection of authentic standards to confirm their presence (Table 3). Overall, the screening analysis and the preliminary chemical profile confirmed the possibility of grouping the hexane fraction with the hexane/acetate fraction of each individual species. The qualitative analysis of the pooled fractions revealed a similar profile with five major carotenoids in each species (see Table 4); however, the level of carotenoids is higher in *G. birdiae* than in *G. domingensis*. These results are in agreement with the data obtained of Andersson et al. (2006). Some differences can be explained in terms of biotic and abiotic parameters (Gobbo-Neto & Lopes, 2007), such as the strains studied, the age and time of collection, and the tissue analyzed. In this case, both species were collected at the same time and place and these data suggest that the variations are more related to the strains studied.

Table 3. Carotenoids identified in *Gracilaria birdiae* and *G. domingensis* by HPLC-DAD with authentic standard comparison. Peak number (P_n.), Retention time (R_t), Maximum wavelength (λ_{max}), Separation Factor (α).

Pigments	P_n	R_t (min)	λ_{max} (nm)	α
violaxanthin	5	17.1	438	7.1
antheraxanthin	8	26.5	445	11.6
aloxanthin	9	30.5	451	13.5
zeaxanthin	12	40.1	450	18.1
cantaxanthin	13	44.8	474	20.3
β-carotene	16	66.8	451	30.8

Finally, GC-MS analyses were carried out to confirm the presence of other antioxidant constituents in the pooled fractions (Table 5). Several previous studies described the composition of these primary metabolites in algae (Apt & Behrens, 1999; Arendt et al., 2005; Cardozo et al., 2002; Sanina et al., 2004). Although controversial in the literature, fatty acid profiles have been used as a chemotaxonomic tool to identify brown, red and green algae (Dawczynski et al., 2007). A comparative study of algae found the predominance of saturated fatty acids in red algae, while brown algae were richer in unsaturated fatty acids (Sánchez-Machado et al., 2004). In our analysis, we observed a predominance of unsaturated fatty acids with saturated to unsaturated ratios of 1:4 and 1:1 for *G. birdiae* and *G. domingensis*, respectively. Palmitic acid (C16) was found as one of the major constituents (10.75% *G. birdiae* and 29.98% *G. domingensis*), which confirms results in the literature for other red macroalgae (Dawczynski et al., 2007). High levels of arachidonic acid (C20: 4 5,8,11,14 Δ), an essential fatty acid that makes up the family of ω-6, have been described for some species of *Gracilaria* (46 to 62%) (Khotimchenko, 2005). Of the two species of *Gracilaria* studied in this work, *G. birdiae* presented this fatty acid in large quantities (52%), while *G. domingensis,* although collected under the same conditions, contained only 13%. Note also the presence of acids that comprise the family of ω -3 and 9, some of which are present in relevant quantities, such as oleic acid (C18: 1 Δ 9), which accounts for 12 to 14% of the total fatty acids analyzed.

Table 4. Carotenoid quantification (μg of carotenoids/mg of dry algae) in *Gracilaria birdiae* and *G. domingensis* by HPLC-DAD.

Pigments	P_n	*G. birdiae*	*G. domingensis*
violaxanthin	1	0.044	0.021
antheraxanthin	2	-	0.224
aloxanthin	3	0.09	0.01
zeaxanthin	4	0.445	0.249
cantaxanthin	5	0.022	-
β-carotene	6	0.365	0.166

The results of the study show that extracts of Brazilian *Gracilaria* species present antioxidant activity and contain essential fatty acids and carotenoids (β-carotene is a precursor of vitamin A); thus, these algae can be considered to be functional foods. This also has implications for the utilization of these species as a source of antioxidants in applications requiring these properties.

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Table 5. Percentage of apolar constituents from hexane extracts of *Gracilaria birdiae* and *G. domingensis* by GC-MS.

R_t	Fatty acids	%	
		G. birdiae	*G. domingensis*
7.36	lauric (C12)	0.13	nd
8.63	myristic (C14)	3.55	4.43
9.45	pentadecanoic (C15)	1.03	2.68
10.52	palmitic (C16)	10.75	29.98
10.77	palmitoleic (C16:1 Δ7)	0.18	2.88
10.84	palmitoleic (C16:1 Δ9)	0.67	4.70
11.04	palmitoleic (C16:1 Δ11)	0.06	2.80
11.83	margaric (C17)	0.80	nd
12.63	cyclopentane tridecanoic	nd	9.10
13.53	stearic (C18)	1.94	1.91
13.93	oleic (C18:1 Δ9)	12.88	14.00
14.04	octadecenoic (C18:1 Δ11)	2.77	2.10
14.27	octadecenoic (C18:1 Δ13)	nd	nd
14.73	linoleic (C18:2 Δ9,12)	1.43	nd
15.28	octadecatrienoic (C18:3 Δ6,9,12)	0.22	nd
15.87	linolenic (C18:3 Δ9,12,15)	0.24	1.40
17.03	eicosanoic (C20)	0.13	0.66
17.40	eicosenoic (C20:1 Δ11)	0.51	0.47
18.23	eicosadienoic (C20:2 Δ11,13)	2.16	0.82
18.72	eicosatrienoic (C20:3 Δ6,9,12)	5.90	2.76
19.17	arachidonic (C20:4 Δ5,8,11,14)	52.86	13.26
20.48	eicosapentenoic (C20:5 Δ5,8,11,14,17)	0.31	0.39
20.65	docosanoic (C22)	0.38	0.27
21.14	docosenoic (C22:1, Δ13)	0.11	0.58
23.03	tricosanoic (C23)	0.22	nd
25.99	lignoceric (C24)	0.31	0.54

nd: not detected. R_t is retention time

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***Correspondence**

Ernani Pinto
Departamento de Análises Clínicas e Toxicológicas, Faculdade de Ciências Farmacêuticas Universidade de São Paulo
Av. Professor Lineu Prestes, 580 05508-900 São Paulo-SP, Brazil
ernani@usp.br
Tel. +55 11 3091 1505
Fax: +55 11 3091 3661