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Antioxidant potential of methanolic and aqueous extracts of Chnoospora minima, Padina gymnospora and Sargassum cymosum (Ochrophyta, Phaeophyceae)

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Research Article

Keywords: antioxidant, brown seaweeds, crude extract, functional ingredients, in vitro assays, screening

Posted Date: November 20th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3621912/v1

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Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Journal of Applied Phycology on December 27th, 2023. See the published version at https://doi.org/10.1007/s10811-023-03160-3.

Abstract

Brown seaweeds are reported to have high antioxidant activity, due to the rich composition in phenolic compounds. In this way, they present potential as functional ingredients and additives for food, feed, cosmeceutical and pharmaceutical industries. The objective of this study was to evaluate the antioxidant potential of methanolic and aqueous extracts of three species of brown algae by five *in vitro* assays, in order to contribute to screening of functional foods ingredients, and to the search for natural antioxidants from marine alga biomass matrix. The ABTS, DPPH, FRAP and iron-chelating assays were used for analysis of antioxidant activity, and the Folin-Ciocalteu assay for the quantification of total phenolic compounds. The methanolic and aqueous extracts of *Padina gymnospora* and *Sargassum cymosum* showed up to 50% of antioxidant potential for the five assays, and *Chnoospora minima* presented antioxidant potential up to 50% only for ABTS assay. Comparing the extracts, aqueous extracts of *C. minima* and *S. cymosum* presented higher antioxidant activities and phenolic compounds than methanolic extracts, whereas for *P. gymnospora* the methanolic extracts presented greater activities. To integrate the results of antioxidant potential, a total antioxidant capacity index was calculated, classifying the extract potential in reactivity order. It was verified that the methanolic extract of *P. gymnospora* had the highest antioxidant activity and content of phenolic compounds, indicating the potential of this species in the search for natural antioxidant substances and suitable candidate for further studies as food and functional ingredients.

1. Introduction

Antioxidants are substances capable of inhibiting or retarding the degradation of organic molecules caused by reactive oxygen species (ROS), which are highly unstable and produced by redox reactions (Sies 1993). In organisms, ROS are normally produced by cellular metabolism, but in excess can damage cellular constituents, that have been related to cell aging, apoptosis and the emergence of many cellular and metabolic disorders. To combat ROS, organisms have different antioxidant mechanisms, including enzymatic and non-enzymatic defenses (Mallick and Mohn 2000). The enzymatic antioxidant mechanisms include mainly superoxide dismutase, peroxidase, catalase and glutathione reductase, while the non-enzymatic mechanisms include substances such as ascorbic acid, carotenoids, phenolic compounds, sulfated polysaccharides and tocopherols.

In normal physiological conditions, there is a balance between ROS and antioxidant mechanisms. However, exogenous factors can lead to oxidative stress and cause degradation of DNA and other biomolecules by overproduction of reactive species or deficiency of antioxidant mechanisms (Das and Roychoudhury 2014).

Seaweeds, in the aquatic environment, are naturally exposed to variation of nutrients, light, CO_2 and O_2 concentrations, temperature, desiccation and salinity, and are prone to oxidative stress (Mallick and Mohn 2000). To ensure their survival, several species have stress response mechanisms with high antioxidant capacity (Cofrades et al. 2010). In general, brown algae have high antioxidant activity due to the presence of phenolic substances, especially phlorotannins (Catarino et al. 2021). Studies by Zubia et al. (2007) and Cofrades et al. (2010) relate the antioxidant activity in extracts of brown algae to the high amount of phenolic compounds, presenting potential as functional foods and in the conservation of food products.

Functional foods are those that, besides their nutritional properties, have a proven effect on health promotion and/or disease prevention (LeBlanc et al. 2018). The specific ingredients present in these foods, responsible for their beneficial effect, are called nutraceuticals. In this sense, the consumption of foods rich in antioxidant substances, such as phenolic compounds, is related to the reduction of the incidence of different types of cancer, cardiovascular and chronic respiratory diseases (Van Duyn and Pivonka 2000).

In addition to the therapeutic effects, antioxidant compounds can be used as ingredients in the preservation of food products, as the oxidation of organic molecules by reactive species is one of the main mechanisms for reducing food shelf time (Petcu et al. 2023). Synthetic antioxidant, such as BHA and BHT, are widely used in the preservation of food products, but their safety concerns have led to legislative restrictions due to doubts over their toxic and carcinogenic effects (Gulcin 2020). For these reasons, research has been conducted to find sources of natural antioxidants with no toxic effects.

The *in vitro* study of the antioxidant potential of brown algae extracts may lead to the discovery of novel functional foods and antioxidant substances from natural sources. Antioxidant substances have different mechanisms of action, so it is recommended to use different in vitro assays for a more complete analysis of the antioxidant potential of extracts (Frankel and Meyer 2000).

Recent studies have highlighted the antioxidant potential of brown seaweeds extracts from the Brazilian coast (Polo and Chow 2022; Urrea-Victoria et al. 2022; Harb et al. 2023). In this study, we aim to evaluate the antioxidant potential of methanolic and aqueous extracts of three species of brown algae collected from the Brazilian coast through five different *in vitro* assays. Our goal is to contribute to the screening of functional food ingredients and to the search for natural antioxidants from marine algal biomass matrix.

2. Materials and methods

2.1 Collection of biological material and extraction procedure

The biological material was collected in the south coast of Espírito Santo (ES), Brazil, in March 2016. *Chnoospora minima* was collected at Praia da Cruz (21°02,116'S; 40°48,812'O), municipality of Marataízes, and at Parati Beach (20°48,456'S; 40°36,575'O), municipality of Anchieta. *Padina gymnospora* and *Sargassum cymosum* were collected at Marataízes Beach (21°02,620'S; 40°49,453'O), municipality of Marataízes.

The biological material was collected in the intertidal region, paying attention to healthy individuals. The seaweeds were screened and cleaned of macroepiphytes, washed with fresh water and partially air dried, protected from the sun. Later, they were placed in plastic bags and transported to the laboratory, where they were dried in oven with air circulation at 40°C for 48 h. The dried material was ground in a knife mill (30 mesh sieve; Fortinox STAR FT 80), each species was divided into five subsamples ($n = 5$) and each subsample was individually extracted (five technical replicates) by simple and serial maceration with five solvents of increasing polarity (hexane, dichloromethane, ethyl acetate, methanol and water).

The macerations were performed three times with each solvent. For maceration with organic solvents (1:2 *w*/*v*), two solvent changes occurred every 24 h at room temperature. The aqueous maceration (1:4 *w*/*v*) occurred in water bath at 80°C and the solvent was changed twice every 5 h. The extracts were filtered and pooled per solvent, always maintaining the individuality of each five subsample. The organic extracts were concentrated in water bath (40°C) and finally freeze-dried. The aqueous extracts were directly concentrated by freeze-dried. Only methanolic and aqueous crude extracts were used for antioxidant potential analysis, since they had the highest extract yield, which made it possible to perform the subsequent antioxidant assessment.

Some fresh specimens were used in the preparation of exsiccates (n = 5), which were identified by Dr. Fábio Nauer using morphological characteristics. After species identification, the exsiccates were deposited in the Herbarium of the Institute of Biosciences of the University of São Paulo (SPF) and received the following voucher numbers: SPF 58244 and SPF 58246 (*C. minima*), SPF 58243 (*P. gymnospora*) and SPF 58248 (*S. cymosum*).

2.2 Antioxidant potential

To analyze the antioxidant potential of methanolic and aqueous extracts of *C. minima*, *P. gymnospora* and *S. cymosum*, five colorimetric assays were performed (n = 5): ABTS radical scavenging; DPPH radical scavenging; ferric reducing antioxidant power (FRAP); metal chelating activity; and quantification of total phenolic compounds with Folin-Ciocalteu reagent. Assays were conducted in 96-well microplates with a final volume of 300 µL and absorbance's were measured on a UV-vis spectrophotometer (EpochTM 2-BioTek®, USA). The ABTS radical scavenging assay was performed according to Torres et al. (2017) and Santos et al. (2019) at a wavelength of 734 nm. The DPPH radical scavenging assay followed the method of Pires et al. (2017a) and Santos et al. (2019) at 517 nm. The FRAP assay was performed as Urrea-Victoria et al. (2016) and Santos et al. (2019) at 595 nm. The metal chelating activity was performed as Harb et al. (2016) and Santos et al. (2019) at 562 nm. The reducing power of the Folin-Ciocalteu reagent was performed as described by Pires et al. (2017b) and Santos et al. (2019) at 760 nm.

The concentrated extracts were dissolved in 10% DMSO to a stock concentration of 15 mg mL^{-1}. A preliminary test was performed for the five assays at a final concentration up to 400 µg mL^{-1}, to subsequently select other extract concentrations for evaluating the dose dependence and estimating the EC_{50} (effective concentration at which the extract 50% of the assessed effect after a specific reaction time). For extracts that presented antioxidant activity greater than 40% in the preliminary test, the assays were conducted in five concentrations up to a maximum concentration of 1000 µg mL^{-1}, varying according to species and extract.

For the antioxidant assays, sample blanks were prepared, replacing the reactive solution volume of each assay with 10% DMSO, since the sample coloration (photosynthetic pigments) may interfere with the absorbance of the reaction. The absorbance values of sample blanks were discounted from the absorbance values of the respective samples after the reaction of each assay. As a negative control for the assays, the sample volume was replaced by 10% DMSO. For the preparation of reference standard curves, gallic acid, BHA, phloroglucinol, quercetin and Trolox (Sigma-Aldrich®, Brazil) were used and concentrations range (µg

mL^{-1}), standard-line equation (y = *a*x + *b*), regression coefficient (R^2) and conversion factor (CF) to gallic acid equivalent are shown in Table 1.

Table 1

Concentrations range (μg mL⁻¹), standard-line equation ($y = ax + b$) and regression coefficient (R^2) for the respective antioxidant assay and the reference standard gallic acid, BHA (butylated hydroxyanisole), phloroglucinol, quercetin and Trolox ($n = 3$). The respective conversion factor (CF) for each reference standard to gallic acid equivalent (GAE) is shown. To transform a GAE value to equivalent of BHA, phloroglucinol, quercetin or Trolox multiply the GAE value by conversion factor, or to transform a BHA, phloroglucinol, quercetin or Trolox equivalent value to GAE divide by the conversion factor

	Gallic acid	**BHA**	**Phloroglucinol**	**Quercetin**	**Trolox**
ABTS	0.025–1.250 μg mL^{-1}	1–5 μg mL^{-1}	0.25–1.75 μg mL^{-1}	1–5 μg mL^{-1}	1–5 μg mL^{-1}
	y = -2.0387x + 0.8685	y = -0.3809x + 0.7820	y = -1.1984x + 0.7040	y = -0.4555x + 0.7600	y = -0.3942x + 0.7735
	R^2 = 0.9995	R^2 = 0.9825	R^2 = 0.9830	R^2 = 0.9955	R^2 = 0.9979
	-	CF = 5.35	CF = 1.70	CF = 4.48	CF = 5.17
DPPH	0.5–3.0 μg mL^{-1}	2–12 μg mL^{-1}	20–100 μg mL^{-1}	2–8 μg mL^{-1}	2–10 μg mL^{-1}
	y = -1.134x + 0.8557	y = -0.1583x + 0.5890	y = -0.0199x + 0.7122	y = -0.2582x + 0.6224	y = -0.1794 + 0.7936
	R^2 = 0.9867	R^2 = 0.9209	R^2 = 0.9954	R^2 = 0.9493	R^2 = 0.9987
	-	CF = 7.16	CF = 56.98	CF = 4.39	CF = 6.32
FRAP	0.5–4.0 μg mL^{-1}	1.5–7.5 μg mL^{-1}	20–100 μg mL^{-1}	0.5–3.0 μg mL^{-1}	1.5–7.5 μg mL^{-1}
	y = 1.5794x − 0.0175	y = 0.7936x + 0.0008	y = 0.0409x − 0.0198	y = 1.4075x − 0.0193	y = 0.6844x − 0.0108
	R^2 = 0.9987	R^2 = 0.9990	R^2 = 0.9803	R^2 = 0.9987	R^2 = 0.9983
	-	CF = 1.99	CF = 38.62	CF = 1.12	CF = 2.31
Chelating	1–8 μg mL^{-1}	-	-	4–20 μg mL^{-1}	-
	y = -0.3804x + 1.0519	-	-	y = -0.1171x + 0.7729	-
	R^2 = 0.9929	-	-	R^2 = 0.9961	-
	-	-	-	CF = 3.25	-
Folin-Ciocalteu	2–20 μg mL^{-1}	15–90 μg mL^{-1}	20–60 μg mL^{-1}	2–20 μg mL^{-1}	20–100 μg mL^{-1}
	y = 0.2550x + 0.0194	y = 0.033x + 0.0474	y = 0.0418x + 0.029	y = 0.2067x + 0.0434	y = 0.0377x + 0.0579
	R^2 = 0.9967	R^2 = 0.9708	R^2 = 0.9901	R^2 = 0.9985	R^2 = 0.9872

	Gallic acid	BHA	Phloroglucinol	Quercetin	Trolox
	-	CF = 7.73	CF = 6.10	CF = 1.23	CF = 6.76

Results of antioxidant assays were expressed as gallic acid equivalent (GAE) relative to crude extract mass (CE) (mg GAE.g CE^{-1}). For the other standards, a conversion factor was calculated based on the gallic acid curve (Table 1). Results were also expressed as percentage of antioxidant activity and EC_{50}.The percentage of antioxidant activity (% AOX) for the ABTS radical scavenging, DPPH radical scavenging and metal chelating assays was determined according to the following formula:

% AOX = [(AbsCN - AbsS) / AbsCN] x 100

where: AbsCN - negative control absorbance; AbsS - absorbance of the sample.

The % AOX by the FRAP and Folin-Ciocalteu assays was determined considering as 100% of the antioxidant activity the absorbance value obtained at the maximum concentration of the gallic acid curve (1.25 μg mL^{-1}).

The EC_{50} was determined from the percentage values of the five concentrations evaluated with the GraphPad Prism® 6 software using a sigmoidal adjustment model (Chen et al. 2013).

2.3 Classification of total antioxidant potential

A total antioxidant capacity index was determined for each extract based on Seeram et al. (2008). With this index it was possible to classify the antioxidant potential of the extracts of the three species analyzed, considering, together, the result of the five assays. For each assay, the antioxidant activity value, represented as a standard equivalent, was considered as the extract score. The extract with the highest score was assigned an index number of 100 and the index number of the other extracts was determined as follows:

Index = (extract score / highest score) x100.

The total index of each extract was determined by the average index value of the five assays and was used to rank the antioxidant potential of the extracts in descending order.

2.4 Statistical analysis

Statistical analysis was performed using Statistica® 10.0 (StatSoft, Inc.) and the graphs were constructed with GraphPad Prism® 6.01 (GraphPad Software, Inc.). Data were analyzed for normality (Kolmogorov-Smirnov test) and homoscedasticity (Bartlett test), and subsequently submitted to one-way or two-way ANOVA, as appropriate. Newmann-Keuls *post-hoc* test was used to determine significant differences ($p < 0.05$).

To compare the percentage of antioxidant activity and the standard equivalent values by volume (μg GAE mL^{-1}) among the concentrations of the same extract, one-way ANOVA was used. To analyze the antioxidant potential, expressed as standard equivalent per crude extract mass (mg GAE g CE^{-1}) or EC_{50}, among the

three species and the extract types, two-way ANOVA was used. Tables summarizing ANOVA results are given as supplementary material (Table S1 and S2).

3. Results

The yield of the extracts is shown in Table 2. For the three species, the aqueous extract showed the highest yield, followed by the methanolic extract.

Table 2
Yield (mean ± SD; $n = 5$) of crude hexane, dichloromethane, ethyl acetate, methanolic and aqueous extracts in relation to the initial dry mass for the extraction of *Chnoospora minima*, *Padina gymnospora* and *Sargassum cymosum*. Different letters represent significant differences ($p < 0.05$)

	C. minima	***P. gymnospora***	***S. cymosum***
Initial dry mass for extraction	7.5 g	15 g	20 g
Hexane	0.43 ± 0.15%^{d}	0.30 ± 0.03%^{d}	0.20 ± 0.01%^{D}
Dichloromethane	1.01 ± 0.14%^{c}	0.23 ± 0.01%^{d}	0.34 ± 0.08%^{C}
Ethyl acetate	0.62 ± 0.08%^{d}	1.28 ± 0.18%^{c}	0.22 ± 0.03%^{D}
Methanolic	4.26 ± 0.57%^{b}	2.96 ± 0.32%^{b}	5.04 ± 0.22%^{B}
Aqueous	21.60 ± 1.61%^{a}	12.10 ± 0.43%^{a}	16.95 ± 0.87%^{A}

The results of the preliminary test, up to 400 μg mL^{-1} of crude extract, of antioxidant activity of methanolic and aqueous extracts of *C. minima*, *P. gymnospora* and *S. cymosum* are shown in Table 3. All extracts analyzed showed reaction in the different antioxidant assays. For *C. minima*, the reactivity of the aqueous extract in the five antioxidant assays was greater than or equal to the methanolic extract. The opposite situation was verified for *P. gymnospora*, in which the methanolic extract presented higher activity than the aqueous extract. For *S. cymosum*, antioxidant activity was similar for both extracts in the ABTS, DPPH and Folin-Ciocalteu assays, but for FRAP and metal chelating assays the activity of aqueous extract was higher than the methanolic extract (Table 3).

Table 3
Percentage of antioxidant activity (mean ± SD; *n* = 5) of methanolic and aqueous extracts of *Chnoospora minima*, *Padina gymnospora* and *Sargassum cymosum* in the ABTS, DPPH, Folin-Ciocalteu, FRAP and metal chelating assays at a concentration of 400 µg mL^{-1}

	C. minima		*P. gymnospora*		*S. cymosum*	
	Methanolic	Aqueous	Methanolic	Aqueous	Methanolic	Aqueous
ABTS	83.76 ± 12.36	93.10 ± 1.96	99.80 ± 0.07*	86.22 ± 7.72*	92.61 ± 3.46*	99.81 ± 0.26*
DPPH	16.24 ± 3.30	21.89 ± 10.40	91.34 ± 6.75	30.97 ± 4.45	57.31 ± 1.82	49.10 ± 3.52
Folin-Ciocalteu	17.04 ± 1.63	19.32 ± 1.45	198.63 ± 10.65	33.68 ± 2.36	49.78 ± 1.82	52.22 ± 1.86
FRAP	27.10 ± 8.07	39.00 ± 1.66	74.54 ± 3.65	48.53 ± 2.43	49.35 ± 2.70	89.26 ± 2.61
Metal chelating	10.91 ± 2.84	28.29 ± 9.66	25.45 ± 4.3	29.81 ± 2.77	13.71 ± 5.77	42.20 ± 6.82

*For the ABTS assay, methanolic and aqueous extracts of *P. gymnospora* and *S. cymosum* were tested at 200 µg mL^{-1}.

After the preliminary antioxidant test at a single crude extract concentration, subsequent analyzes were performed at various crude extract concentrations for all antioxidant assays, except for *C. minima* that showed low antioxidant activity at DPPH, FRAP, chelating and Folin-Ciocalteu assays (Table 3). In the ABTS radical scavenging assay, methanolic and aqueous extracts of *C. minima* (Fig. 1a), *P. gymnospora* (Fig. 1b) and *S. cymosum* (Fig. 1c) were tested at five different concentrations. For the three species, the increase of methanolic and aqueous extract concentration increased the antioxidant activity linearly ($R^2 > 0.94$). For *C. minima* (Fig. 1a), at the maximum concentration tested (400 µg mL^{-1}), activities of 64.94 ± 10.65% were obtained for the methanolic extract (equivalent to 0.96 ± 0.14 µg GAE mL^{-1}) and 86.98 ± 5.59% for the aqueous extract (equivalent to 1.25 ± 0.07 µg GAE mL^{-1}). For *P. gymnospora* (Fig. 1b) the maximum concentration of methanolic extract (50 µg mL^{-1}) reached activity of 85.34 ± 10.70% (1.21 ± 0.15 µg GAE mL^{-1}), and aqueous extract (250 µg mL^{-1}) reached activity of 63.21 ± 7.44% (0.90 ± 0.10 µg GAE mL^{-1}). In *S. cymosum* (Fig. 1c), the maximum concentration tested (200 µg mL^{-1}) presented activity of 66.94 ± 8.67% for the methanolic extract (0.92 ± 0.13 µg GAE mL^{-1}) and 73.56 ± 5.15% for the aqueous extract (1.02 ± 0.08 µg GAE mL^{-1}).

The DPPH radical scavenging assay was performed at different concentrations of methanolic and aqueous extracts for *P. gymnospora* and *S. cymosum* (Fig. 2). As the extract concentration increased, there was a linear increase in antioxidant activity ($R^2 > 0.95$). In *P. gymnospora* (Fig. 2a), the highest concentration tested for methanolic extract (400 µg mL^{-1}) showed activity of 78.31 ± 15.60% (1.94 ± 0.42 µg GAE mL^{-1}) and aqueous extract (1000 µg mL^{-1}) showed activity of 46.13 ± 5.58% (1.08 ± 0.15 µg GAE mL^{-1}). In *S. cymosum* (Fig. 2b), the maximum concentration tested (800 µg mL^{-1}) presented activity of 55.67 ± 4.46%

for the methanolic extract (1.33 ± 0.12 μg GAE mL^{-1}) and 42.67 ± 8.24% for the aqueous extract (0.99 ± 0.22 μg GAE mL^{-1}).

The FRAP assay showed a linear relationship (R^2 > 0.95) between increased antioxidant activity and increased extract concentration for *P. gymnospora* and *S. cymosum* (Fig. 3). In *P. gymnospora* (Fig. 3a) the highest concentrations of methanolic extract (400 μg mL^{-1}) and aqueous extract (800 μg mL^{-1}) showed activity of 74.79 ± 8.49% (2.18 ± 0.34 μg GAE mL^{-1}) and 74.95 ± 2.12% (2.22 ± 0.06 μg GAE mL^{-1}), respectively. For *S. cymosum* (Fig. 3b) the highest concentration of methanolic extract (800 μg mL^{-1}) showed activity of 67.73 ± 0.79% (1.99 ± 0.06 μg GAE mL^{-1}) and aqueous extract (400 μg mL^{-1}) showed the activity of 77.09 ± 1.01% (2.26 ± 0.07 μg GAE mL^{-1}).

For metal chelating activity (Fig. 4), only *S. cymosum* extracts were tested in several crude extract concentrations since low activity at this assay was registered for *C. minima* and *P. gymnospora* (Table 3). Increasing concentration of aqueous extract of *S. cymosum* there was increased linear antioxidant activity (R^2 = 0.9568). The maximum concentration tested for this extract (800 μg mL^{-1}) reached the activity of 84.22 ± 3.28% (7.74 ± 0.31 μg GAE mL^{-1}).

For the Folin-Ciocalteu assay (Fig. 5), the increased concentration of methanolic and aqueous extract of *P. gymnospora* and *S. cymosum* led to an increase in the content of total phenolic compounds (R^2 > 0.98). For *P. gymnospora* (Fig. 5a), the maximum concentration of methanolic extract (200 μg mL^{-1}) showed activity of 96.43 ± 10.89% (19.92 ± 2.28 μg GAE mL^{-1}) and aqueous extract (1000 μg mL^{-1}) presented activity of 61.90 ± 2.54% (12.70 ± 0.53 μg GAE mL^{-1}). In *S. cymosum* (Fig. 5b), the highest concentration of methanolic and aqueous extracts (800 μg mL^{-1}) showed activity of 75.18 ± 4.87% (14.57 ± 2.21 μg GAE mL^{-1}) and 80.24 ± 2.96% (16.53 ± 0.62 μg GAE mL^{-1}), respectively.

To compare the antioxidant potential between extracts and species, we used a standardized unit of GAE per amount in grams of crude extract (Fig. 6) and EC_{50} (Fig. 7), since both results better represent a standard unit and allow a more efficient comparison with the published literature.

For antioxidant activity values in mg GAE g CE^{-1} (Fig. 6), *C. minima* presented antioxidant potential only for the ABTS assay (Fig. 6a) and there was no significant difference between methanolic extract (0.25 ± 0.04 mg GAE g CE^{-1}) and the aqueous extract (0.29 ± 0.06 mg GAE g CE^{-1}). In *P. gymnospora* the methanolic extract showed highest antioxidant potential than aqueous extracts in the ABTS (24.28 ± 3.01 mg GAE g CE^{-1}; Fig. 6a), DPPH (4.84 ± 1.04 mg GAE g CE^{-1}; Fig. 6b), FRAP (6.45 ± 1.01 mg GAE g CE^{-1}; Fig. 6c) and Folin-Ciocalteu (99.61 ± 11.39 mg GAE g CE^{-1}; Fig. 6d) assays. For *S. cymosum* no significant differences were observed between the antioxidant potential of methanolic and aqueous extracts for the ABTS (Fig. 6a), DPPH (Fig. 6b) and Folin-Ciocalteu (Fig. 16d) assays; However, for the FRAP assay (Fig. 6c), the aqueous extract of *S. cymosum* (5.65 ± 0.18 mg GAE g CE^{-1}) had higher antioxidant potential than the methanolic extract (2.49 ± 0.07 mg GAE g CE^{-1}). For metal chelating assay only the aqueous extract of *S. cymosum*

was calculated for mg GAE g CE^{-1} unit, since lower activity was showed by the other species (Table 3), showing activity of 9.67 ± 0.38 mg GAE g CE^{-1}.

Among the extracts of the three species studied, methanolic extract of *P. gymnospora* showed the highest antioxidant activity values for the ABTS, DPPH, FRAP and Folin-Ciocalteu assays (Fig. 6).

The EC_{50} values are shown in Fig. 7, and it is important to keep in mind that lower EC_{50} values represent higher antioxidant activity. For *C. minima*, in the ABTS assay (Fig. 7a), the aqueous extract (175.24 ± 22.54 μg mL^{-1}) had higher antioxidant potential than the methanolic extract (226.77 ± 69.46 μg mL^{-1}). For *P. gymnospora*, methanolic extracts had higher antioxidant potential than aqueous extracts for ABTS (17.92 ± 2.75 μg mL^{-1}; Fig. 7a), DPPH (208.48 ± 123.31 μg mL^{-1}; Fig. 7b), FRAP (303.70 ± 43.87 μg mL^{-1}; Fig. 7c) and Folin-Ciocalteu (97.00 ± 10.45 μg mL^{-1}; Fig. 7d) assays. In *S. cymosum*, the methanolic and aqueous extracts showed no significant differences in the ABTS assay (Fig. 7a). For the same species, the methanolic extract presented higher antioxidant potential for DPPH assay (624.46 ± 78.06 μg mL^{-1}; Fig. 7b), and the aqueous extract presented higher potential for FRAP (347.91 ± 5.30 μg mL^{-1}; Fig. 7c) and Folin-Ciocalteu (415.49 ± 24.38 μg mL^{-1}; Fig. 7d) assays.

Comparing the EC_{50} values of the extracts of the three species studied (Fig. 7), methanolic extracts of *P. gymnospora* presented the highest antioxidant potential for the ABTS, DPPH, FRAP and Folin-Ciocalteu assays. For the metal chelating assay, only the aqueous extract of *S. cymosum* had antioxidant potential calculated as EC_{50} (326.23 ± 24.33 μg mL^{-1}).

The gallic acid standard had an EC_{50} of 0.79 ± 0.02 μg mL^{-1}, 1.24 ± 0.03 μg mL^{-1}, 1.62 ± 0.05 μg mL^{-1}, 5.55 ± 0.22 μg mL^{-1} and 10.26 ± 0.94 μg mL^{-1} for the ABTS, DPPH, FRAP, metal chelating and Folin-Ciocalteu assays, respectively.

The classification of total antioxidant potential of methanolic and aqueous extracts of the three species, considering the five antioxidant assays, is shown in Table 4. This index makes possible to rank improved efficiency of antioxidant capacity for each extract and species. The highest antioxidant potentials were observed for methanolic extracts of *P. gymnospora*, while methanolic extracts of *C. minima* presented the lowest antioxidant potential. Considering all calculated antioxidant index, the ranking of better species and extracts is *P. gymnospora* methanolic extract (MeOH) > *S. cymosum* aqueous extract > *P. gymnospora* aqueous extract > *S. cymosum* MeOH extract > *C. minima* aqueous extract > *C. minima* MeOH extract.

Table 4
Total antioxidant potential classification of methanolic and aqueous extracts of *Chnoospora minima*, *Padina gymnospora* and *Sargassum cymosum*

Species	Extract	ABTS index	DPPH index	FRAP index	Chelating index	Folin-Ciocalteu index	Average	Ranking
C. minima	MeOH	12.86	30.96	3.45	16.39	6.73	14.08	6
C. minima	Aqueous	12.09	19.83	2.90	64.62	7.93	21.48	5
P. gymnospora	MeOH	100.00	100.00	100.00	59.58	100.00	91.92	1
P. gymnospora	Aqueous	17.23	22.28	43.00	61.09	12.75	31.27	3
S. cymosum	MeOH	22.26	34.41	38.56	16.00	18.28	25.90	4
S. cymosum	Aqueous	20.94	25.46	87.67	100.00	20.75	50.96	2

4. Discussion

The evaluation of the antioxidant potential of seaweed extracts is an important tool for prospecting new sources of natural antioxidant substances.

The preliminary test, performed at a single concentration (400 µg mL^{-1}), was important to select the five most appropriate concentrations to test the antioxidant activity of the extracts in each assay.

The calculation of antioxidant potential index and the respective ranking among extracts and species is a valuable tool for looking and prospecting improved potential of new sources of natural antioxidant substances including different characteristic of antioxidant properties.

All extracts presented antioxidant activity in different degrees of action. Summarizing and comparing the response of the extracts in the five assays, the antioxidant potential followed the order: *P. gymnospora* methanolic > *S. cymosum* aqueous > *S. cymosum* methanolic > *P. gymnospora* aqueous > *C. minima* aqueous > *C. minima* methanolic.

The use of different assays is recommended for a more complete analysis of the extracts, as the substances have different mechanisms of antioxidant action (Frankel and Meyer 2000). Antioxidant substances may have primary or secondary mechanisms of action (Gordon 1990). In the primary mechanism there is free radical inhibition by hydrogen donation or electron transfer, interrupting oxidation reactions. Already the secondary mechanism reduces the process of initiation of oxidation and formation of free radicals. Thus, this study used five antioxidant assays, which evaluate different mechanisms of action. Moreover, they have reaction systems with different conditions, which may affect the reactivity of the substances present in crude extracts. For example, the ABTS, DPPH, FRAP and Folin-Ciocalteu assays evaluate the primary antioxidant mechanism of action, and the Fe^{2+} ion chelation assay involves the secondary antioxidant mechanism, as transition metals are capable of catalyzing free radical formation

reactions. In addition, the ABTS, DPPH, FRAP and Folin-Ciocalteu assays involve electron transfer reactions and may therefore be correlated. Among these assays, the same trend was observed in *P. gymnospora*, in which methanolic extract showed the highest activity for the three assays.

The Folin-Ciocalteu assay, besides analyzing the ability of the extract to transfer electrons, is widely used to quantify the concentration of total phenolic substances. Thus, some studies use the comparison between this and other tests to relate antioxidant activity with the content of total phenolic compounds (Gulcin 2020).

The DPPH assay is the most common assay for evaluating antioxidant potential and is based on the mechanisms of electron transfer and hydrogen atom transfer. Studies cite correlation between the Folin-Ciocalteu and DPPH assays, since the -OH group of phenolic compounds can transfer an electron and donate hydrogen (Fernando et al. 2016; Abirami et al. 2017). For *P. gymnospora*, the same trend was observed between these assays. The methanolic extract, which showed higher activity for the Folin-Ciocalteu assay, showed higher activity for the DPPH assay. This trend was also observed by Zubia et al. (2007) for the brown algae *Lobophora variegata* (J.V. Lamouroux) Womersley ex E.C. Oliveira, which among the 48 seaweed species analyzed, showed the highest activity by the DPPH assay and also the highest content of total phenolic compounds by the Folin-Ciocalteu assay.

In a study by Murugan and Iyer (2013), *P. gymnospora* methanolic extract showed higher activity in the DPPH assay than aqueous extract, however for the Folin-Ciocalteu assay the opposite result observed. According to the authors, in the Folin-Ciocalteu assay, other substances such as reducing sugars may have contributed to the greater activity of the aqueous extract.

For the Fe^{2+} ion chelation assay, only the aqueous extract of *S. cymosum* showed antioxidant potential. It is suggested that chelating activity may be related to the content of phenolic substances (Balanquit and Fuentes 2015), however for the Folin-Ciocalteu assay there was no significant difference between methanolic and aqueous extracts of *S. cymosum*. Studies analyzing the antioxidant potential of aqueous extracts of brown algae reported a low correlation between chelating activity and phenolic substances, suggesting that this antioxidant mechanism is related to the presence of sulfated polysaccharides (Wang et al. 2008; Wang et al. 2009; Praveen and Chakraborty 2013). On the other hand, according to Wang et al. (2016), the antioxidant activity of polysaccharides may also be related to the complex formed with phenolic compounds, especially phenolic acids. In the present work, the standard phloroglucinol, monomer pattern of phlorotannins, did not show metal chelating activity, indicating that this class of phenolic compound may present low reactivity in this assay. The reference standards gallic acid (phenolic acid) and quercetin (flavonoid) showed high metal chelating activity.

In the extract analyzed, phenolic compounds, especially phlorotannins, may be responsible for this antioxidant mechanism of action. However, as a crude extract, other substances such as acetogenins, reducing sugars and sulfated polysaccharides may contribute to antioxidant activity. Also, studies have suggested that the antioxidant activity of seaweeds may be attributed to a synergistic effect among different substances (Wang et al. 2009; Vega et al. 2020).

In general, the reactivity was higher for the ABTS, Folin-Ciocalteu and FRAP assays, compared to DPPH and Fe^{2+} ion chelator, indicating that the analyzed extracts present electron transfer as antioxidant mechanism of action.

The lower reactivity observed in the DPPH assay does not necessarily mean that the extracts had low antioxidant activity for this assay. The ethanolic extract of the brown algae *Padina concrecens* Thivy and *Cystoseira osmundacea* (Turner) C. Agardh showed high antioxidant activity (Tenorio-Rodriguez et al. 2017), with an EC_{50} of 50 µg mL^{-1} and 69 µg mL^{-1}, respectively. However, the other species analyzed presented EC_{50} higher than 400 µg mL^{-1}. Zubia et al. (2007) found higher activity for dichloromethane:methanol (1:1 *v/v*) extract of *L. variegata* (EC_{50} 320 µg mL^{-1}), while the other 47 species analyzed presented EC_{50} greater than 1000 µg mL^{-1}. Bianco et al. (2015) also found EC_{50} greater than 1000 µg mL^{-1} for methanolic extracts of 14 seaweed species, including *P. gymnospora* and *Sargassum vulgare* C. Agardh. The results show that *P. gymnospora* methanolic extract from this study showed high antioxidant activity for this assay, when compared to other studies on seaweed extracts, presenting potential as a natural source of antioxidant substances.

Padina gymnospora methanolic extract also showed a high content of phenolic compounds when compared to other studies with seaweeds. Methanolic extracts of seaweed species used in food had phenolic content ranging from 37.66 to 151.33 mg GAE g CE^{-1} (Cox et al. 2010). According to Machu et al. (2015), the phenolic content of nine seaweed food products ranged from 8.0 to 192.6 mg GAE g^{-1}. This demonstrates the potential of *P. gymnospora* methanolic extract and *S. cymosum* aqueous and methanolic extracts as a source of phenolic compounds, as they showed values of 99.61 mg GAE g CE^{-1}, 75.18 mg GAE g CE^{-1} and 80.24 mg GAE g CE^{-1}, respectively.

Finally, the species presented different antioxidant **reactivity** in the five analyzed assays, demonstrating the need to use more than one assay for a complete analysis of the antioxidant potential of seaweed extracts. The joint analysis of antioxidant activity in the five trials indicated that electron transfer is a mechanism of action of the extracts. *Padina gymnospora* and *S. cymosum* extracts showed antioxidant potential. *Padina gymonospora* methanolic extract presented, in general, the highest antioxidant potential, and the highest content of total phenolic compounds, indicating the potential of this species as a source of natural antioxidant compounds, which can be used in the conservation of food products. In addition, *P. gymospora* may have potential as a food or functional ingredient, and further studies on the composition and food safety of this species are required.

Declarations

Funding

The authors would like to thank the research funding agencies CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for supporting the postgraduate course. AMA thanks CNPq for the scholarship (Proc. 133009/2016-5). FCH

thanks FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo - Biota/Fapesp 2013/50731-1) for research grant and CNPq for research productivity grant (303937/2015-7).

Competing interests

The authors have no competing interests to declare that are relevant to the content of this article.

Availability of data and material

The generated datasets are available from the corresponding author on reasonable request.

Code availability

Not applicable.

Authors' contributions

All authors conceived and designed the experiments, analyzed, and interpreted the data, draft the manuscript and contributed with a critical revision of the article for important intellectual content and approved the final article.

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Figures

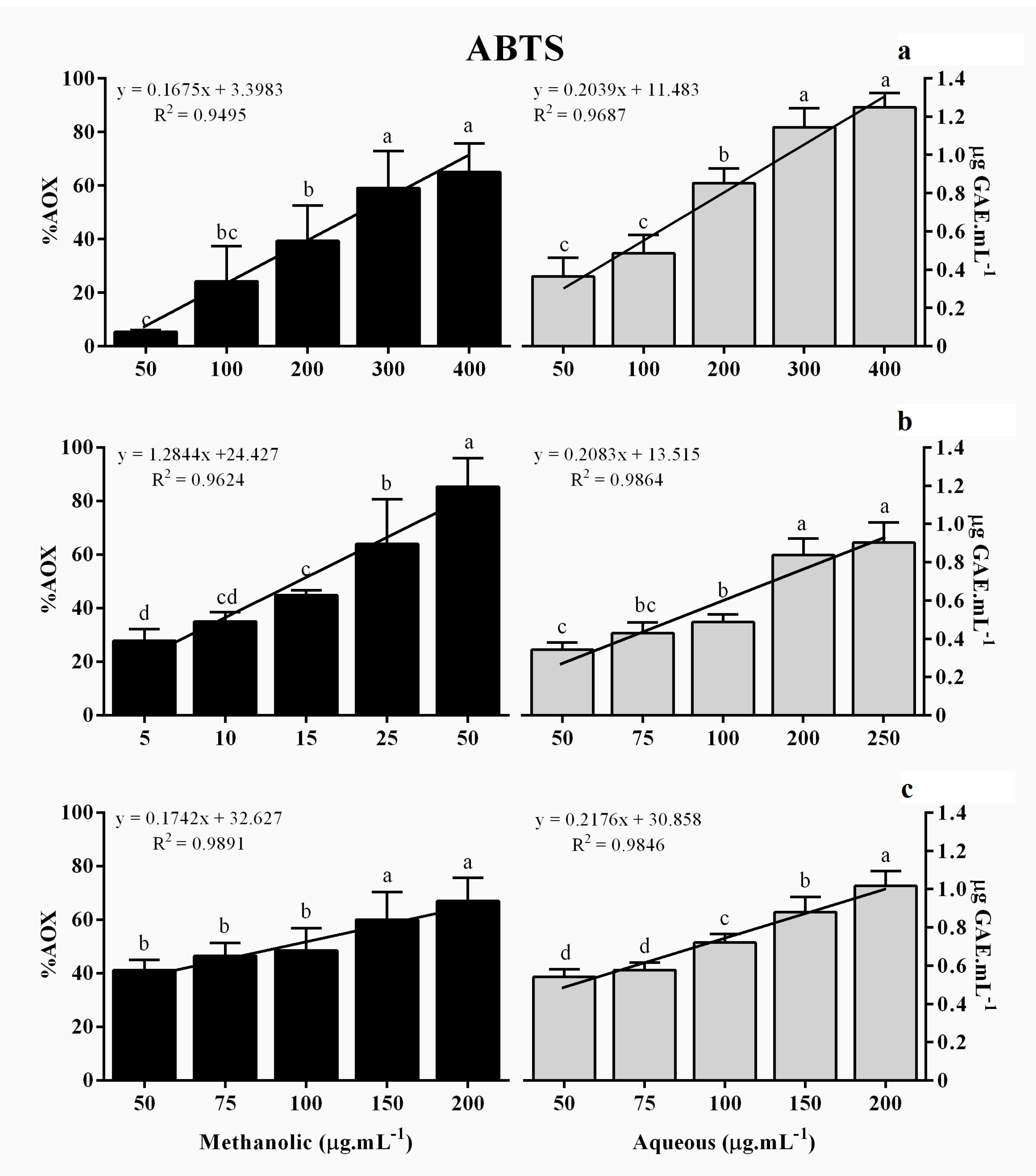

Figure 1

Antioxidant activity expressed as percentage (% AOX) and gallic acid equivalent (µg GAE mL^{-1}) for the ABTS radical scavenging assay (mean ± SD; n = 5) at different concentrations of methanolic and aqueous extracts from a) *Chnoospora minima*, b) *Padina gymnospora* and c) *Sargassum cymosum*. Different letters represent significant differences ($p < 0.05$)

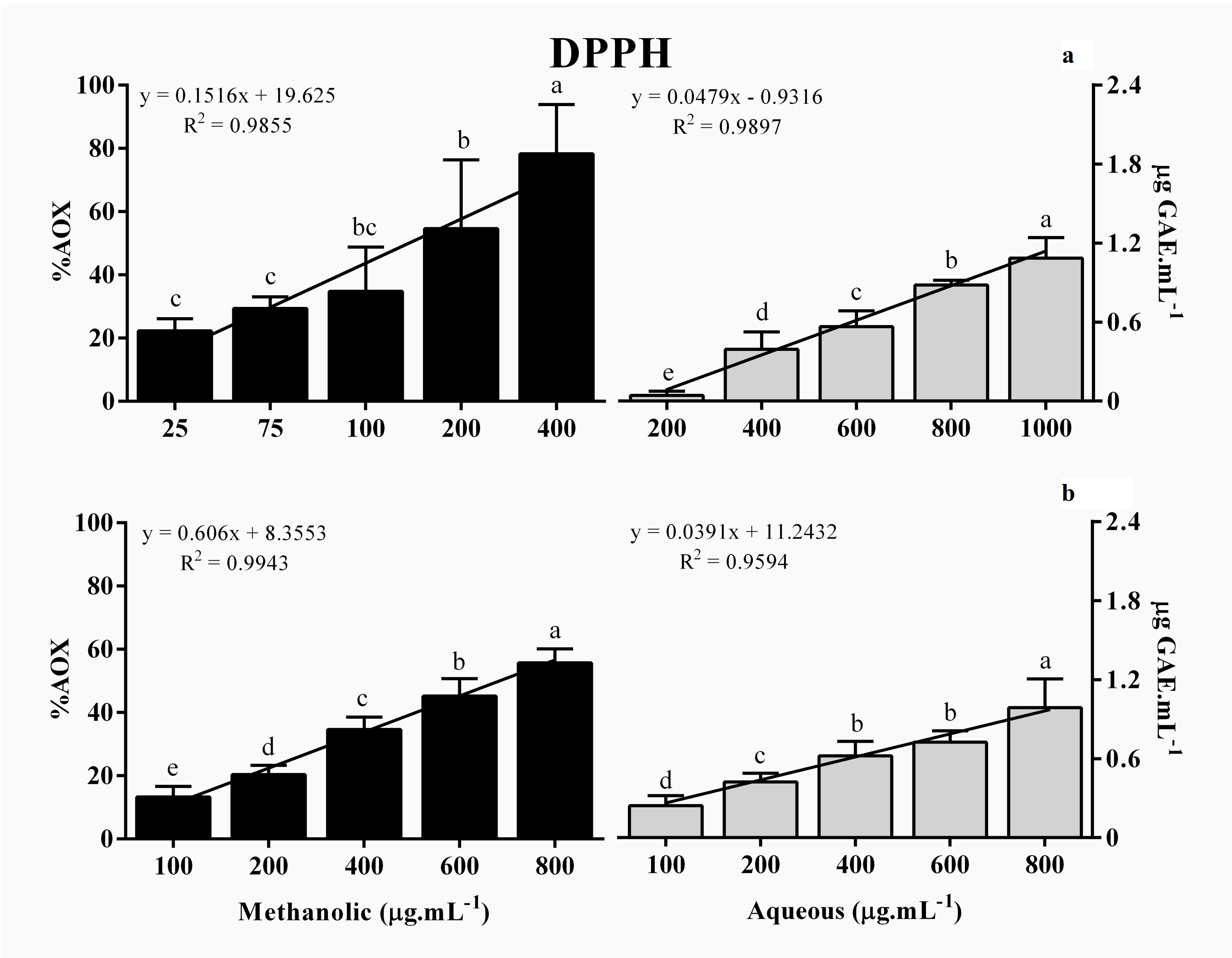

Figure 2

Antioxidant activity expressed as percentage (% AOX) and gallic acid equivalent (µg GAE mL^{-1}) for the DPPH radical scavenging assay (mean ± SD; n = 5) at different concentrations of methanolic and aqueous extracts from a) *Padina gymnospora* and b) *Sargassum cymosum*. Different letters represent significant differences (p < 0.05)

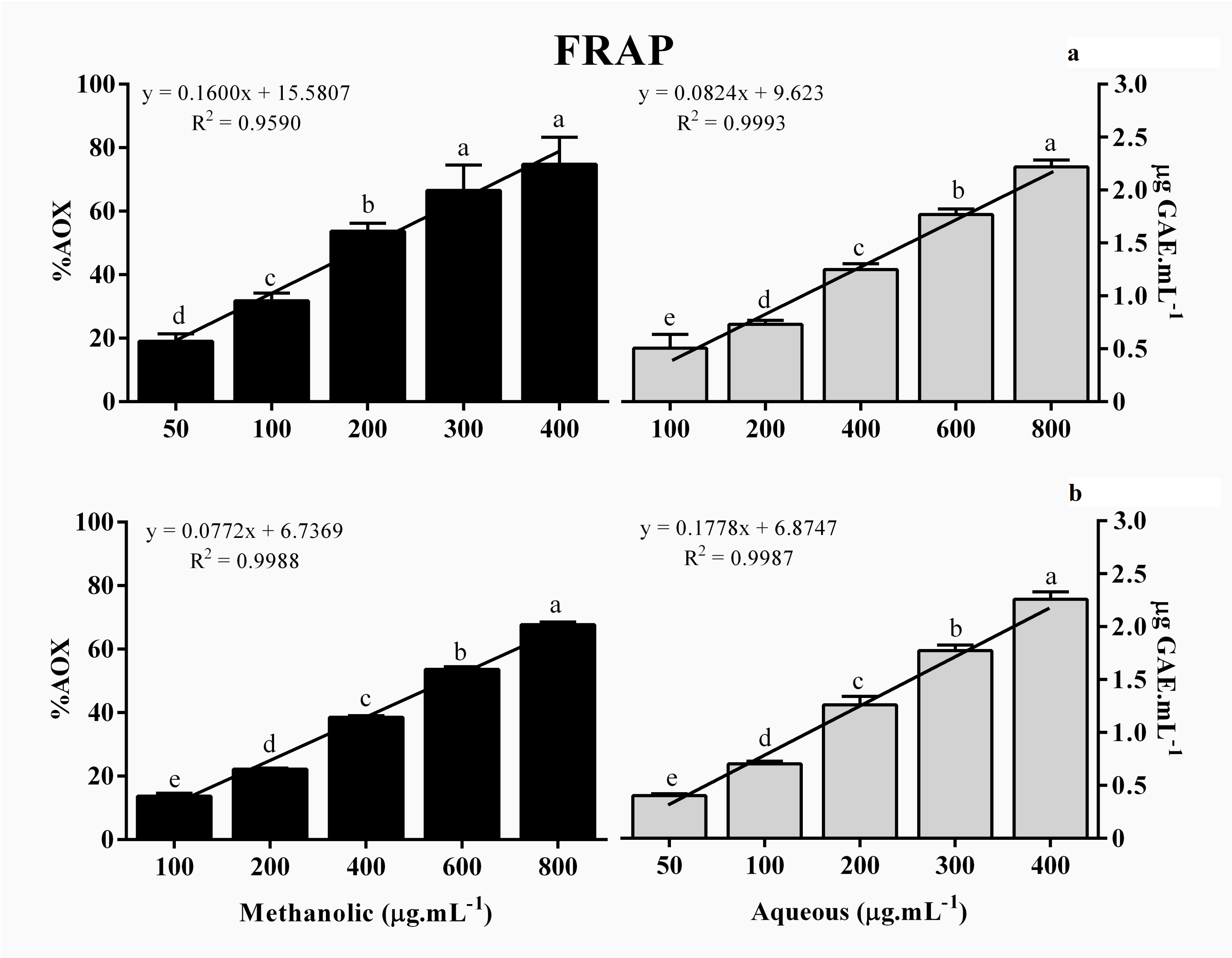

Figure 3

Antioxidant activity expressed as percentage (% AOX) and gallic acid equivalent (μg GAE mL^{-1}) for the FRAP assay (mean ± SD; $n = 5$) at different concentrations of methanolic and aqueous extracts from a) *Padina gymnospora* and b) *Sargassum cymosum.* Different letters represent significant differences ($p < 0.05$)

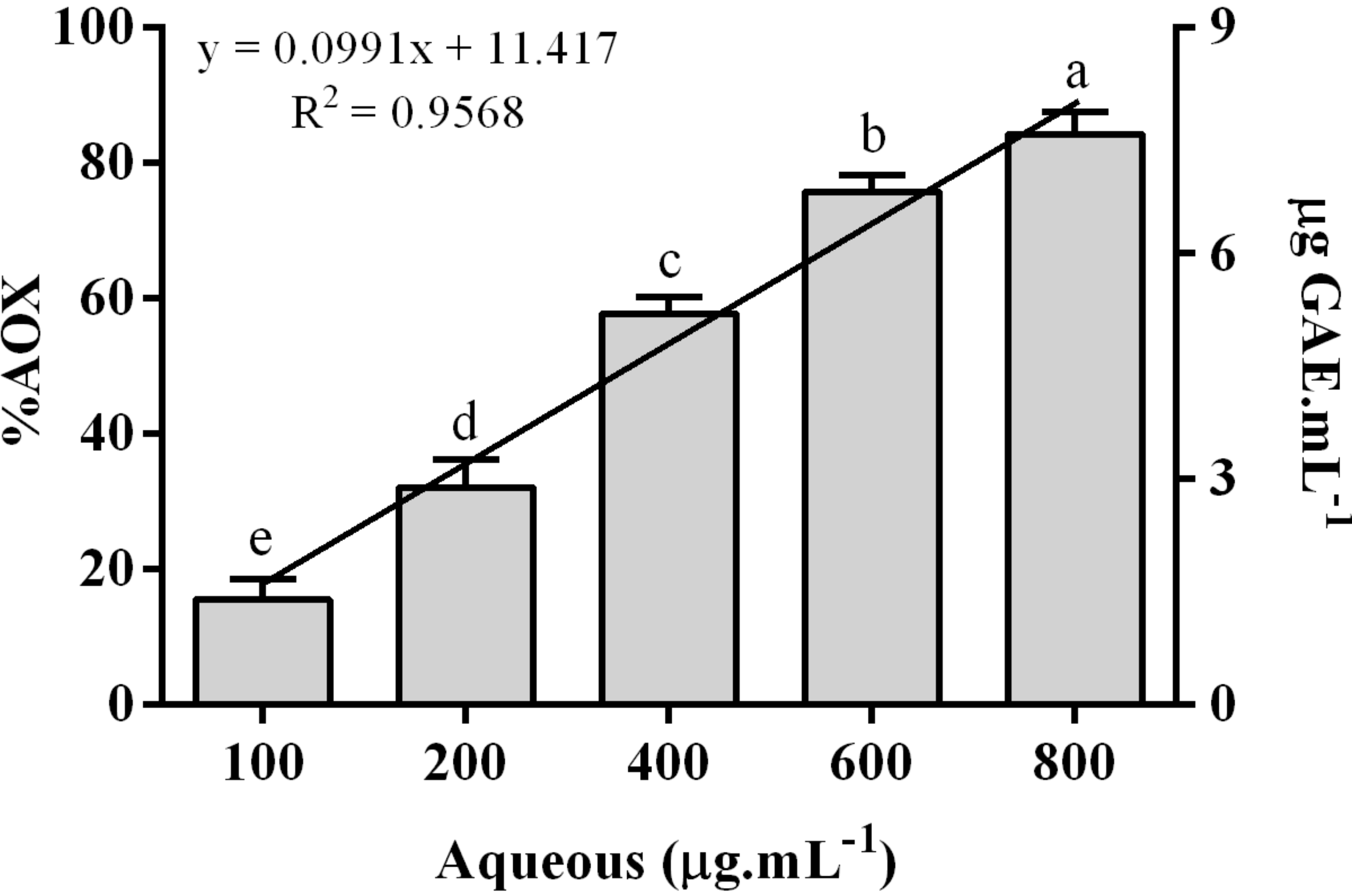

Figure 4

Antioxidant activity expressed as percentage (% AOX) and gallic acid equivalent (µg GAE mL^{-1}) for the metal chelating assay (mean ± SD; $n = 5$) at different concentrations of aqueous extracts from *Sargassum cymosum*. Different letters represent significant differences ($p < 0.05$)

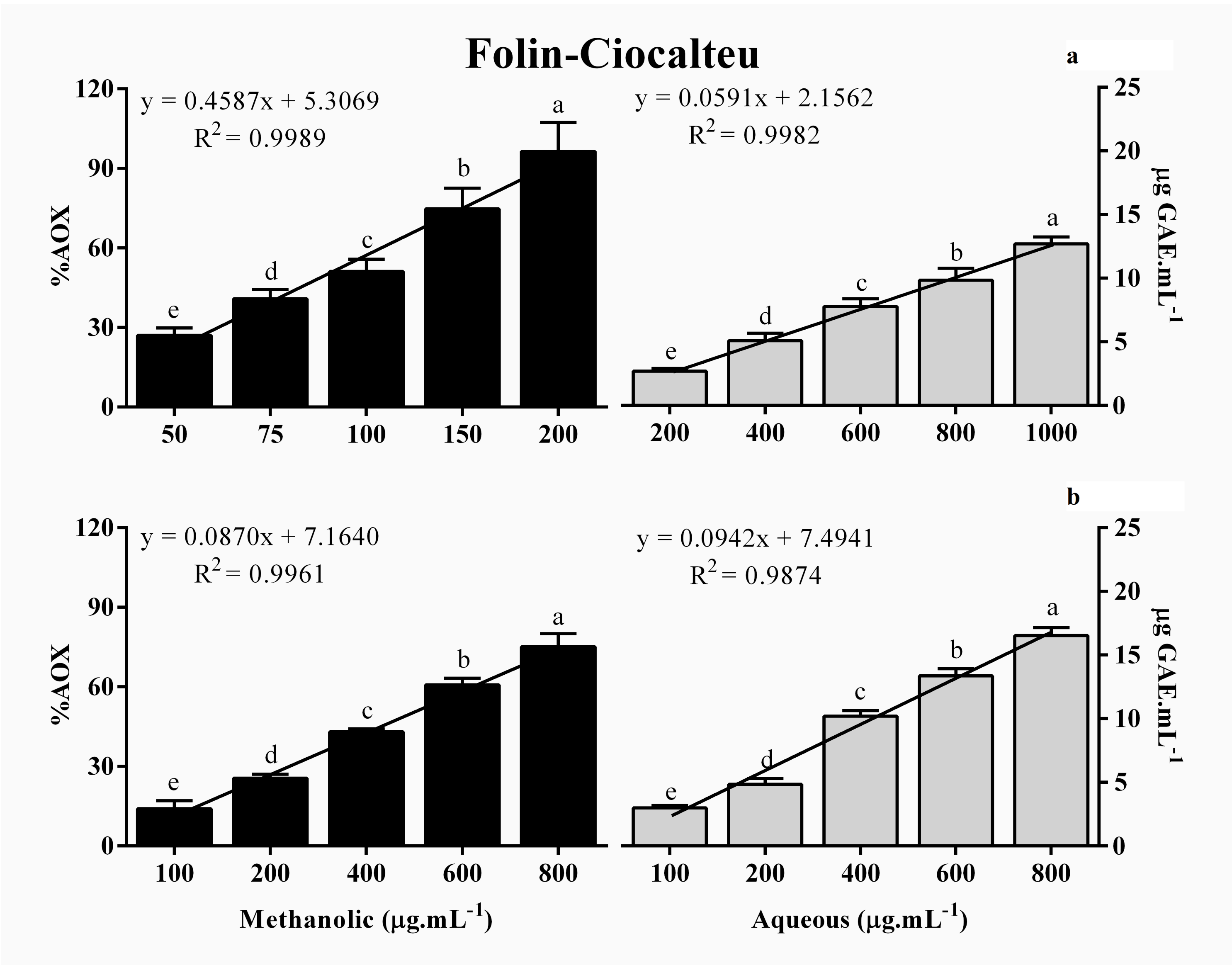

Figure 5

Total phenolic content expressed as percentage (% AOX) and gallic acid equivalent (µg GAE mL^{-1}), by the Folin-Ciocalteu assay (mean ± SD; $n = 5$), at different concentrations of methanolic and aqueous extracts from a) *Padina gymnospora* and b) *Sargassum cymosum*. Different letters represent significant differences ($p < 0.05$)

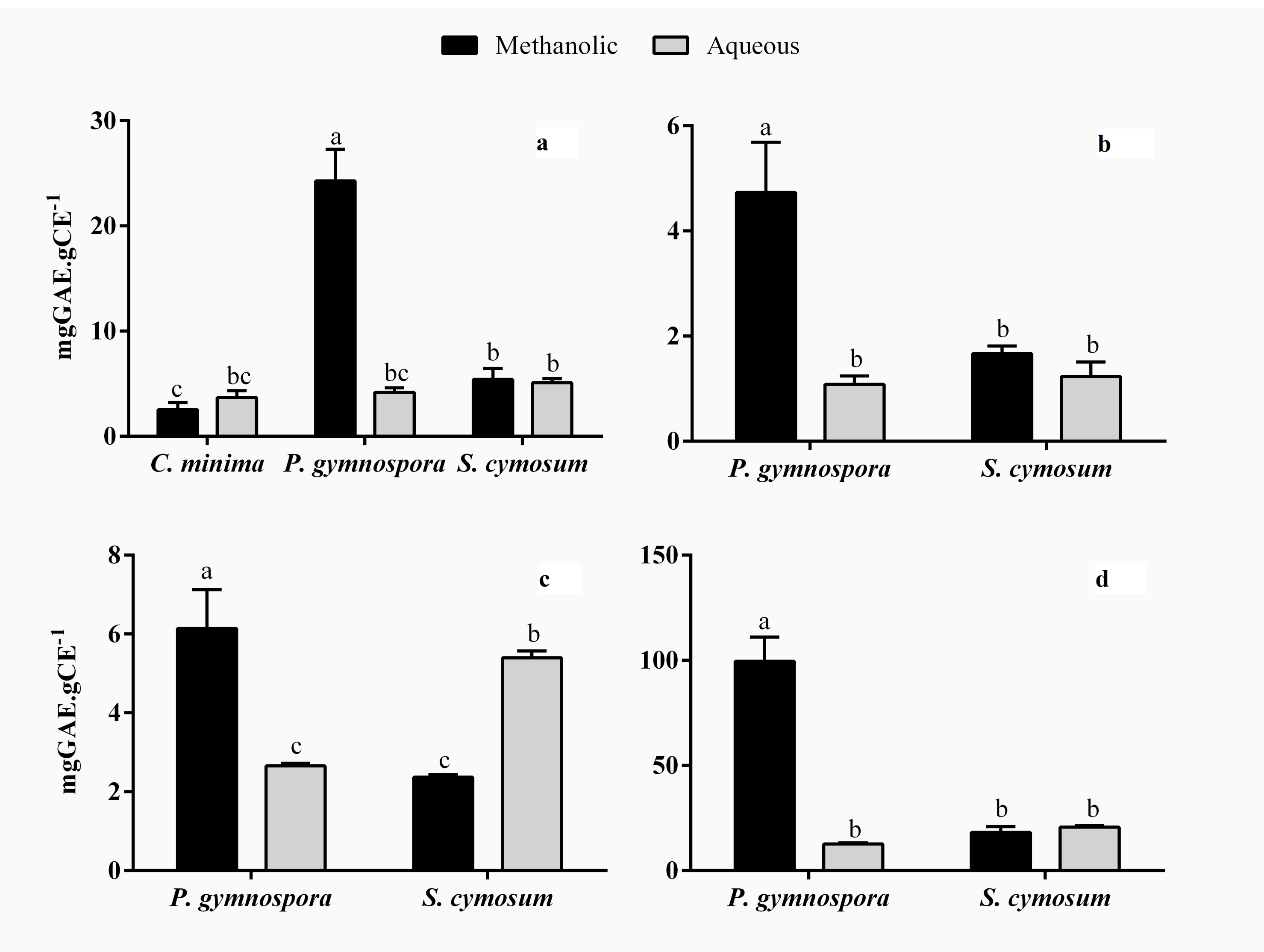

Figure 6

Comparison of antioxidant potential represented as equivalent of gallic acid (mg GAE g EB^{-1}; mean ± SD; *n* = 5) between extracts (methanolic and aqueous) and species, *Chnoospora minima*, *Padina gymnospora* and *Sargassum cymosum*. a) ABTS radical scavenging assay; b) DPPH radical scavenging assay; c) ferric reducing antioxidant power (FRAP) and d) total phenolic content by Folin-Ciocalteu assay. Different letters represent significant differences ($p < 0.05$)

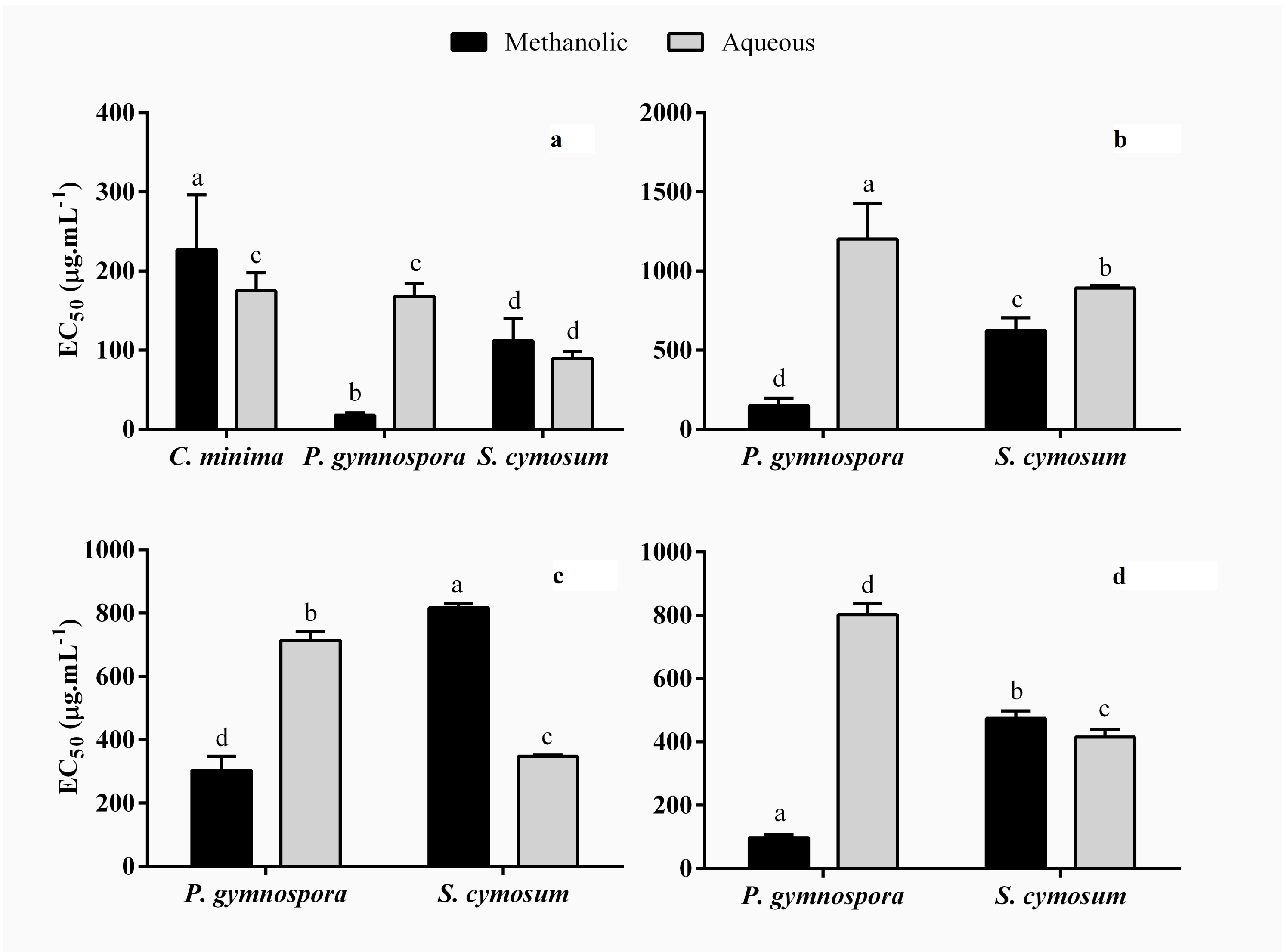

Figure 7

Comparison of antioxidant potential represented as EC_{50} (μg mL^{-1}; mean ± SD; *n* = 5) between extracts (methanolic and aqueous) and species, *Chnoospora minima*, *Padina gymnospora* and *Sargassum cymosum*. a) ABTS radical scavenging assay; b) DPPH radical scavenging assay; c) ferric reducing antioxidant power (FRAP) and d) total phenolic content by Folin-Ciocalteu assay. Different letters represent significant differences ($p < 0.05$)

Supplementary Files

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