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JULIANE BERNARDI

VARIAÇÃO DA ATIVIDADE ANTIOXIDANTE EM MACROALGAS RECIFAIS DE PERNAMBUCO

RECIFE, 2018

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Tese apresentada ao programa de Pós-graduação em Oceanografia da Universidade Federal de Pernambuco como requisito parcial para obtenção do título de Doutora em Oceanografia.

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Aos meus pais,
meu porto seguro

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“O que sabemos é uma gota; o que ignoramos é um oceano.”

Isaac Newton

RESUMO

Muitos estudos avaliam a atividade antioxidante em macroalgas marinhas devido ao aumento na busca por produtos naturais que previnam o estresse oxidativo, um dos principais causadores de doenças humanas, como câncer, disfunções cardiovasculares e enfermidades degenerativas. No entanto, a maioria dos trabalhos realiza um *screening* de forma pontual, visando encontrar uma ou mais espécies com potencial antioxidante, sem relacionar a produção de bioativos com os fatores ambientais. Desta forma, o objetivo deste trabalho é avaliar a atividade antioxidante de espécies de macroalgas tropicais representativas dos recifes de Pernambuco e relacionar esta bioatividade com fatores ambientais como: dessecação, radiação solar, batimento de ondas, salinidade, temperatura e pH. Para isso, foram amostradas espécies de macroalgas representativas, previamente selecionadas, nos recifes de Pernambuco durante a maré baixa. Os extratos brutos das algas foram obtidos com os solventes orgânicos diclorometano e metanol (2:1). A atividade antioxidante foi testada através de cinco métodos: DPPH, ABTS, FRAP, Folin-Ciocalteu e Quelante de metais, no Laboratório de Algas Marinhas (LAM) do Instituto de Biociências da USP. Todas as espécies apresentaram elevada atividade antioxidante, mesmo em baixas concentrações de extrato. Analisando três espécies dominantes do entre-marés inferior de recifes de Pernambuco, *Osmundaria obtusiloba* apresentou o maior potencial antioxidante, e sua atividade foi relacionada com compostos lipofílicos, como ácido palmítico. *Palisada perforata* foi amostrada em diferentes micro-hábitats recifais, visando comparar a produção de antioxidantes. As algas vivendo em ambiente exposto ao batimento de ondas, com elevada turbidez, e as algas crescendo no platô, sofrendo elevada dessecação, foram as que apresentaram as maiores atividades antioxidantes. Além disso, dois experimentos foram realizados com esta espécie para comparar a produção de antioxidantes com os fatores ambientais. Com base nos resultados obtidos, observamos que a dessecação é o fator mais estressante para estas algas, diminuindo a eficiência fotossintética e aumentando a atividade antioxidante, visando a proteção contra os danos oxidativos nas células. As variações de temperatura e salinidade não foram suficientes para causar estresse nestas algas. Concluímos então que as macroalgas tropicais apresentam elevada atividade antioxidante e que a dessecação é o principal fator influenciando a atividade antioxidante, e assim, a distribuição das algas nos recifes.

Palavras-chave: Antioxidantes. Eficiência fotossintética. Dano oxidativo. Dessecação. Recifes tropicais.

ABSTRACT

Many studies have evaluated the antioxidant activity of seaweeds due to increase in the search for natural products that prevent oxidative stress, one of the major causes of human deseases, as cancer, cardiovascular deseases and degenerative illness. However, most studies perform a punctual screening to find one or more species with antioxidant potential, not correlating the bioactive production with environmental factors. In this way, our main goal was to evaluate the antioxidant activity of tropical seaweed species that are representative on the reefs of Pernambuco coast and relate this activity with environmental factors, such as: desiccation, solar radiation, wave shock, salinity, temperature and pH. So, representative species of seaweeds were sampled at Pernambuco reefs during low tide. Organic crude extracts were made with dichloromethane and methanol (2:1). Antioxidante activity was measured through five methods: DPPH, ABTS, FRAP, Folin-Ciocalteu and metal chelating at Laboratorio de Algas Marinhas (LAM) from Instituto de Biociências at USP. All species showed high antioxidant activity, even in low extract concentrations. Analyzing three dominant species from low intertidal reefs of Pernambuco, *Osmundaria obtusiloba* presented the highest potential, and its activity was related with lipophilic compounds, as palmitic acid. *Palisada perforata* was sampled in different reef microhabitats, to compare the antioxidant production. Algae living in wave exposed environment, with high turbidity, and algae growing at plateau, suffering from desiccation, were the ones that had the highest activities. Besides, two laboratory experiments were performed with that species to compare the antioxidant production with environmental factors. Based on our results, we observed that desiccation is the most stressful factor to those algae, decreasing its photosynthetic efficiency and increasing its antioxidant activityin order to protect the cells agains oxidative damage. Temperature and salinity variations were not enough to cause stress to those algae. We concluded that tropical seaweeds present high antioxidant activity and that desiccation is the main factor influencing the antioxidant activity and, so, the distribution of algae on the reefs.

Keywords: Antioxidants. Desiccation. Oxidative damage. Photosynthetic efficiency. Tropical reefs.

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A Absorptância do talo

Abs Absorbance

ABTS 2,2’-azinobis(3-etilbenzotiazolina-6-ácido sulfônico)

ANOVA Análise de variância

ANVISA Agência Nacional de Vigilância Sanitária

AOX Atividade antioxidante

BHA butil-hidroxianisol

BHT butil-hidroxitolueno

Chelator Atividade quelante de metais

DO Dissolved oxygen

DPPH 2,2-difenil-1- picril-hidrazil

EC_{50} Concentração efetiva para 50% de atividade

ETR Taxa de transporte de elétrons

FAO Food and Agriculture Organization

FRAP Ferric Reducing Antioxidant Power

Fv/Fm Rendimento quântico máximo

GAE Gallic acid equivalent

MAAs Mycosporine-like aminoacids

MCA Metal chelating activity

n number of samples

NRS Nitrogen reactive species

OMS Organização Mundial da Saúde

PAM Pulso de amplitude modulada

PAR Radiação fotossinteticamente ativa

PCA Principal component analysis

PG propil galato

pH Potential of hydrogen

PS(I) Fotossistema I

PSII Fotossistema II

RDA Redundancy Analysis

ROS Reactive oxygen species

Sal Salinity

TBHQ terc-butil hidroquinona

Temp Temperature

TPC Total phenolic compounds

Turb Turbidity

UV Ultra-violeta

UV-vis Ultra-violeta/ visível

Y(II) Rendimento quântico efetivo

SUMÁRIO

1 INTRODUÇÃO GERAL

O atual conhecimento da diversidade de macroalgas marinhas do Brasil indica a ocorrência de 774 táxons infragenéricos, correspondendo a 482 Rhodophyta, 191 Chlorophyta e 101 Ochrophyta (Fujii et al. 2008). Segundo Horta et al. (2001), o litoral brasileiro pode ser dividido em duas regiões ficogeográficas: tropical e temperada quente, separada por uma zona de transição representada pelo estado do Espírito Santo. O litoral de Pernambuco está inserido na região tropical (Horta et al. 2001), correspondente à zona Ocidental proposta por Oliveira-Filho (1977) e é caracterizado por uma flora relativamente rica, estabelecida sobre os recifes de arenito incrustados por algas calcárias e corais, propícios para o crescimento de algas bentônicas. Pereira et al. (2002) listaram 301 táxons infragenéricos para o estado, distribuídos em 153 Rhodophyta, 105 Chlorophyta e 43 Ochrophyta.

1.1 AMBIENTE RECIFAL

Dentre os ecossistemas marinhos tropicais, o ambiente recifal caracteriza-se como um ecossistema extremamente rico, com enorme diversidade de nichos ecológicos, abrigando grande diversidade de espécies, com diversas relações interespecíficas (Sheppard et al., 2009). Os ambientes recifais correspondem a cerca de 15% do fundo marinho entre 0 e 30 metros de profundidade, distribuídos por todo o planeta (Villaça, 2002).

No Brasil, os recifes estão distribuídos em sua maior parte na região tropical nordestina e são constituídos por formações distintas, como corais, arenito, algas calcárias ou os resultantes da erosão de falésias (Maida e Ferreira 1997, Manso et al. 2006). Os recifes de Pernambuco constituem afloramentos marinhos que formam cordões paralelos à costa (Kempf 1970), com condições oceanográficas variáveis. Na costa do estado ocorrem extensas formações recifais constituídas por embasamentos arenítico e arenítico ferruginoso, que afloram na maré baixa ou

podem estar ligeiramente expostos acima do nível médio do mar, podendo em alguns locais, aparecer diretamente na face da praia (Dominguez et al., 1990; Pereira et al., 2002).

Diversos fatores bióticos e abióticos atuam sinergicamente influenciando a distribuição dos organismos no ambiente recifal. Por serem sésseis, as macroalgas marinhas são diretamente afetadas por esses fatores. A sua distribuição se dá entre o topo da zona entremarés até a máxima profundidade adequada para fotossíntese. Os principais fatores abióticos que afetam diretamente as macroalgas são a luz, temperatura, salinidade, hidrodinamismo e disponibilidade de nutrientes (Lobban e Harrison, 1994). Entre as interações biológicas estão a competição com outras plantas e animais sésseis, herbivoria e predação. Os fatores individuais de crescimento, morfologia e reprodução são consequências resultantes de todos estes fatores combinados. Outros parâmetros biológicos como idade, fenótipo e genótipo, bem como sua história evolutiva, podem afetar a resposta desses organismos ao meio abiótico (Lobban e Harrison, 1994).

1.2 ESTRESSE OXIDATIVO

A zona entre-marés, onde a maioria das macroalgas ocorre, é marcada pelos limites superior e inferior das marés, ou seja, é exposta na maré baixa e imersa na maré alta, sendo essa uma das razões para o estabelecimento de zonação da flora e da fauna (McNeill 2010). A distribuição vertical dos organismos nesse ecossistema coincide com os níveis horizontais da maré, formando três zonas discretas: supralitoral, mediolitoral e infralitoral. Estas zonas tornam-se altamente dinâmicas e sofrem rápidas mudanças nos fatores físicos com a flutuação da maré, além das mudanças climáticas causadas por variações meteorológicas sazonais (Davison e Pearson 1996).

As macroalgas de zonas entre-marés frequentemente vivenciam estresse severo como resultado da exposição periódica a uma gama de condições ambientais como flutuações de salinidade, dessecação, radiação intensa e temperaturas elevadas a cada mudança da maré. A severidade dessas condições é intensificada à medida que aumentamos a distância do mar. Direta ou indiretamente, estes estressores exercem considerável pressão sobre o balanço osmótico das células das macroalgas, levando à perturbação de várias funções fisiológicas em nível celular, eventualmente afetando a produtividade dos ecossistemas aquáticos (Davison e Pearson 1996).

Adaptação e aclimatação a tais estressores ambientais são, então, de grande importância em organismos bentônicos intermareais como as macroalgas. Apesar do elevado número de artigos publicados sobre a habilidade das macroalgas em tolerar condições ambientais adversas e variáveis, resultantes dos ciclos de marés, ainda existe debate sobre o mecanismo exato de tolerância ao estresse (Kumar et al. 2011).

Ao longo dos limites inferiores da zona entremarés, a distribuição e abundância relativa das macroalgas são principalmente controladas por fatores bióticos, como predação e competição intra e interespecífica, enquanto a distribuição nos limites superiores é determinada pela capacidade de tolerar fatores abióticos estressantes, como salinidade e dessecação (Kirst 1989). A salinidade da água do mar pode variar com mais intensidade nas regiões costeiras do que em mar aberto, devido à precipitação e proximidade de rios e efluentes, levando à diminuição da salinidade (estresse hiposalino), ou evaporação da água, aumentando a salinidade da água do mar (estresse hipersalino), principalmente em sistemas fechados, como poças de maré.

A tolerância à dessecação em macroalgas marinhas é considerada o principal fator responsável pelos padrões de zonação vertical na zona entremarés. Mais precisamente, é a extensão na qual o aparato fotossintético é capaz de recuperar-se da perda de água sobre a

reimersão que claramente distingue uma espécie tolerante à dessecação (habitando zonas superiores do litoral) de uma espécie sensível à dessecação (habitando zonas inferiores do litoral). Assim, o fator chave determinando sua distribuição vertical é a habilidade de suportar o estresse de dessecação (rápida recuperação durante reidratação) e não a de evitar a dessecação (habilidade de retenção de água) (Ji et al. 2005).

Fotossíntese e respiração são os processos fisiológicos mais importantes, já que estão diretamente relacionados com o potencial de um organismo fotossintetizante em crescer e competir com outros indivíduos e espécies por luz e outros recursos (Kumar et al. 2011). A recuperação da fotossíntese após emersão tem sido usada para avaliar a capacidade de adaptação de macroalgas intermareais (Dring e Brown 1982, Abe et al. 2001, Ji e Tanaka 2002, Ji et al. 2005).

Tanto o estresse salino quanto a dessecação resultam em dispersão de íons e eletrólitos da membrana celular, mudanças no pH, cristalização de soluto e desnaturação de proteínas, desencadeando alterações em uma série de processos fisiológicos, junto com a acumulação de espécies reativas de oxigênio (ROS, do inglês, *reactive oxygen species*) (Collen e Davison 1999 a,b, Karsten 2008, Bischof e Rautenberger 2012).

As ROS são geralmente produzidas como bio-produtos do metabolismo aeróbico normal, onde a excitação do O_2 e subsequente formação de oxigênio singleto, ou pela transferência de um, dois ou três elétrons para O_2 ou por protonação, que resulta na formação de radicais superóxidos ($O_2^{\circ -}$), peróxido de hidrogênio (H_2O_2) ou radicais hidroxila (OH°) (Bischof e Rautenberger 2012). Em altas concentrações, as ROS podem ser extremamente tóxicas, levando ao estresse oxidativo, o qual causa danos aos ácidos nucleicos, oxidação de proteínas, peroxidação lipídica nas membranas celulares, inibição e destruição do aparato fotossintético.

Neste contexto, durante aclimatação a alterações em condições ambientais, como flutuações de salinidade e estresse de dessecação, as macroalgas apresentam uma série de enzimas e antioxidantes não enzimáticos, tais como ascorbato, glutationa, tocoferol, carotenoides, flavonoides, os quais desempenham importante papel controlando os níveis de ROS nas células, mantendo o status redox e reparo celular, minimizando assim os riscos de efeitos deletérios do estresse oxidativo (Kumar et al. 2014).

1.3 ANTIOXIDANTES

Em termos gerais, um antioxidante pode ser considerado um agente que atrasa, previne ou remove os danos oxidativos de uma molécula alvo (Halliwell e Gutteridge 2007). O mecanismo antioxidante humano possui compostos endógenos e exógenos (Yoshihara et al. 2010). Existem antioxidantes apropriados para cada tipo de radical livre e a atuação desses compostos depende da espécie reativa gerada, como e onde foi gerada e qual o alvo do dano. Desta forma, é possível que um antioxidante proteja um sistema, mas falhe em outro.

Os compostos com ação antioxidante podem atuar de diferentes maneiras para a proteção do organismo contra os radicais livres. Um dos mecanismos de defesa consiste em impedir a sua formação, principalmente pela inibição de reações em cadeia com ferro e cobre. Outro mecanismo é baseado na interceptação dos radicais livres gerados pelo metabolismo celular ou por fontes exógenas, impedindo o ataque sobre as células alvo. Mais um mecanismo, associado com a capacidade de reparar lesões causadas por esses radicais através da remoção de danos na molécula de DNA e da reconstituição das membranas celulares danificadas, tem sido relatado. O aumento da síntese de enzimas antioxidantes em resposta a geração de radicais livres também é um mecanismo de defesa do organismo (Halliwell et al. 1995, Gálvez 2010).

Os antioxidantes sintetizados endogenamente na célula ou no líquido extracelular agem tanto enzimática quanto não enzimaticamente. Nos alimentos, principalmente nos vegetais, é encontrada uma grande variedade de compostos antioxidantes, como α-tocoferol, β-caroteno, ácido ascórbico e flavonoides (Barreiros et al. 2006, Gálvez 2010). Além dos antioxidantes naturais encontrados nos alimentos, antioxidantes sintéticos como butil-hidroxianisol (BHA), butil-hidroxitolueno (BHT), terc-butil hidroquinona (TBHQ) e propil galato (PG) têm importante papel na indústria alimentícia por retardar a oxidação lipídica dos alimentos e aumentar o tempo de prateleira de um produto (Andreo e Jorge 2006).

Os antioxidantes sintéticos apresentam uma estrutura fenólica que permite a doação de hidrogênio a um radical livre, regenerando a molécula de acilglicerol e interrompendo os mecanismos de oxidação por radicais livres. Entretanto, os derivados fenólicos transformam-se em radicais livres podendo se estabilizar ou promover reações de oxidação. Dessa forma, tais compostos representam riscos à saúde, normalmente associados ao seu consumo excessivo e podem ter efeito carcinogênico (Andreo e Jorge 2006, Dolatabadi e Kashanian 2010).

A utilização de antioxidantes sintéticos é regulamentada mundialmente pela *Food and Agriculture Organization* (FAO) e pela Organização Mundial da Saúde (OMS) e no Brasil, a Agência Nacional de Vigilância Sanitária (ANVISA) limita os teores de antioxidantes sintéticos nos alimentos em 0,01% de BHT ou PG e de 0,02% de BHA ou TBHQ, calculados sobre o teor de gordura (ANVISA 2012). Devido aos efeitos tóxicos e carcinogênicos causados por estas substâncias, a busca por antioxidantes naturais ganhou considerável atenção na última década, aliado à busca cada vez maior por alimentos naturais funcionais. Desta forma, têm aumentando a pesquisa por produtos naturais, sendo as algas marinhas uma das fontes mais ricas em antioxidantes (Dolatabadi e Kashanian 2010, Ngo et al. 2011). De forma geral, em uma recente busca bibliográfica feita por Pires (2017), quantificando os artigos pesquisados sobre

bioatividade em organismos diversos, a maioria dos trabalhos foi sobre atividade antioxidante (Figura 1).

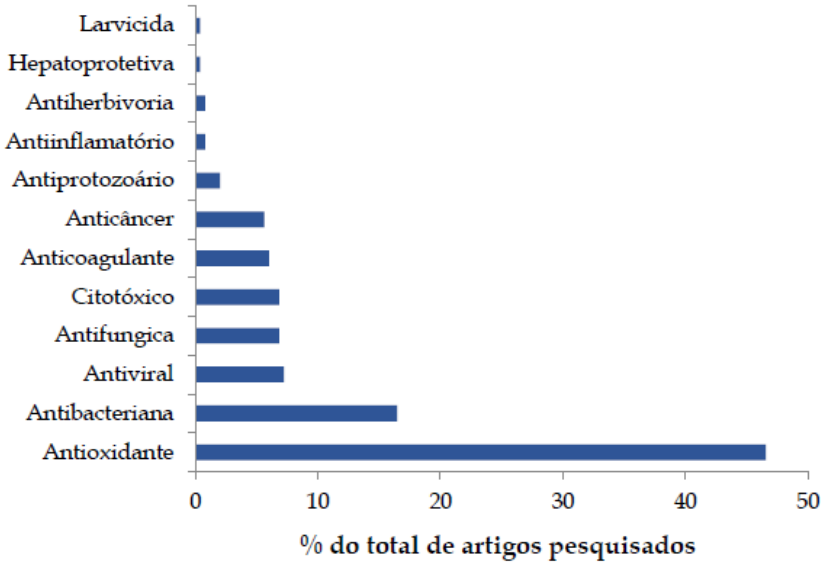
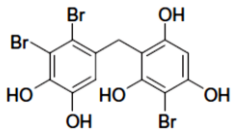
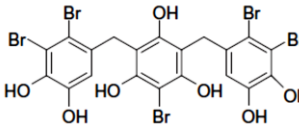
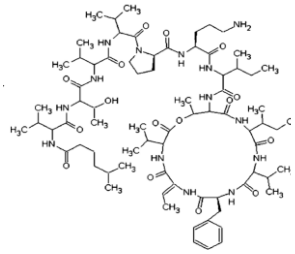


Figura 1 - Artigos pesquisados entre 2001 e 2014 com o tema bioatividade. Fonte: Pires (2017).

As macroalgas marinhas, por habitarem a zona entre-marés dos oceanos, estão sujeitas a uma combinação de fatores ambientais, como batimento de ondas, variação de temperatura e salinidade, radiação solar direta, exposição ao ar e principalmente, dessecação durante a maré baixa. A combinação destes fatores produz radicais livres e ROS, que podem causar danos em nível celular e até ao DNA. Desta forma, para sobreviver em ambientes tão estressantes, as macroalgas desenvolveram diversas estratégias de defesa, como a produção de antioxidantes. Alguns compostos com comprovada ação antioxidante, extraídos das espécies de macroalgas selecionadas para este estudo, estão listadas na Tabela 1, assim como o solvente utilizado na extração.

Tabela 1 - Atividade antioxidante relatada na literatura para as espécies de macroalgas utilizadas neste estudo, alguns compostos responsáveis pela atividade e o respectivo solvente utilizado para extração.

Espécies	**Principais compostos responsáveis pela atividade antioxidante**	**Solvente de extração**	**Atividade antioxidante**	**Local**	**Fonte**
Gracilaria caudata J. Agardh		Diclorometano/ metanol (2:1)	EC_{50} = 33,73 mg ml^{-1}	México	Zubia et al. (2007)
	Polissacarídeos sulfatados	Acetona	35%	Rio Grande do Norte, Brasil	Costa et al. (2010)
Gracilaria cearenses (A.B. Joly & Pinheiro) A.B. Joly & Pinheiro		Diclorometano/ metanol (1:1)	73,08%	Santa Catarina, Brasil	Martins et al. (2012)
Osmundaria obtusiloba (C. Agardh) R.E. Norris	Vidalol A 	10% metanol em clorofórmio	Antiinflamatória	Ilhas do Caribe	Wiemer et al. (1991)
		Diclorometano/ metanol (1:1)	Antioxidante 30,59%	Santa Catarina, Brasil	Martins et al. (2012)
	Vidalol B 				
Bryothamnion triquetrum (S.G. Gmelin) M. Howe		Diclorometano/ metanol (2:1)	EC_{50} = 12,89 mg ml^{-1}	México	Zubia et al. (2007)
		Água destilada	EC_{50} = 4,66 mg ml^{-1}	Cuba	Vidal et al. (2006)
Acanthophora spicifera (M. Vahl) Børgesen		Diclorometano/ metanol (2:1)	EC_{50} = 12,50 mg ml^{-1}	México	Zubia et al. (2007)
Chondracanthus acicularis (Roth) Fredericq		Diclorometano/ metanol (2:1)	EC_{50} = 0,53 mg ml^{-1}	Pernambuco, Brasil	Vasconcelos et al. (2017)
Amansia multifida J.V. Lamouroux		Diclorometano/ metanol (1:1)	72,76%	Santa Catarina, Brasil	Martins et al. (2012)
Cryptonemia seminervis (C. Agardh) J. Agardh		Diclorometano/ metanol (1:1)	80,05%	Santa Catarina, Brasil	Martins et al. (2012)
Palisada perforata (Bory) K.W. Nam		n-hexano, acetato de etila, metanol (1:6)	48%	Golfo da Pérsia	Pirian et al. (2017)

Dictyopteris delicatula J.V. Lamouroux		Metanol	30%	Índia	Vinayak et al. (2011)
	Heterofucanas	Acetona	23%	Rio Grande do Norte, Brasil	Magalhães et al. (2011)
	Polissacarídeos sulfatados	Acetona	25%	Rio Grande do Norte, Brasil	Costa et al. (2010)
Padina sp.		Diclorometano/ metanol (2:1)	EC_{50} = 3,45[a] mg ml^{-1}	México	Zubia et al. (2007)
		Diclorometano/ metanol (1:1)	28,32%[d]	Santa Catarina, Brasil	Martins et al. 2012)
	Heterofucanas	Acetona	EC_{50} = 0,24 mg ml^{-1}	Rio Grande do Norte, Brasil	Souza et al. (2007)
Sargassum sp.		Diclorometano/ metanol (2:1)	EC_{50} = 6,64[b] mg ml^{-1}	México	Zubia et al. (2007)
	Heterofucanas[c]	Acetona			Costa et al. (2011)
Caulerpa racemosa (Forssk.) J. Agardh	Compostos fenólicos	Etanol	30%[d]	China	Li et al. (2012)
Caulerpa cupressoides (Vahl) C. Agardh		Diclorometano/ metanol (2:1)	EC_{50} = 6,35 mg ml^{-1}	México	Zubia et al. (2007)
	Polissacarídeos sulfatados	Acetona	40%	Rio Grande do Norte, Brasil	Costa et al. (2012)
Bryopsis pennata J.V. Lamouroux	Kahalalida F 	Etanol	Anti-HIV	Havaí	Hamann e Scheuer (1993)

Os resultados em porcentagem estão na concentração de 1 mg ml^{-1} de extrato, avaliado em diferentes ensaios antioxidantes.
[a]*Padina gymnospora*
[a]*Sargassum ramifolium*
[c] *Sargassum filipendula*

A atividade antioxidante ganha importância uma vez que, mesmo tendo o O_2 como elemento fundamental para os organismos aeróbicos (Fleschin et al. 2000), nas reações biológicas, os radicais livres e outros derivados ativos do oxigênio são inevitavelmente co-produzidos. Esses radicais livres exercem papel fisiológico importante, mas também estão envolvidos em vários processos deletérios ao organismo humano, causando câncer, aterosclerose, *Diabetes mellitus*, artrite reumática, distrofia muscular, catarata, desordens neurológicas degenerativas e o envelhecimento (Nordberg e Arnér 2001).

Contra os efeitos das espécies reativas de oxigênio, todos os organismos aeróbicos desenvolveram mecanismos fisiológicos e biomoleculares. Entretanto, nas células de organismos fotossintetizantes esses mecanismos estão mais fortemente desenvolvidos devido aos tilacoides, que são alvo primário dos efeitos deletérios oxidativos, por conterem lipídios insaturados como componentes majoritários. É fato que as algas possuem numerosas substâncias antioxidantes, também para aguentar as rápidas variações de intensidade de luz e concentrações de O_2 e CO_2 ao longo da coluna d'água. Desta forma, elas podem representar uma importante fonte de antioxidantes naturais tanto para a indústria alimentícia quanto farmacêutica (Matsukawa et al. 1997).

As atividades antioxidantes têm sido atribuídas a várias reações e mecanismos: prevenção de iniciação de cadeias, ligação de catalisadores de íons de metais de transição, capacidade redutora, sequestrador de radical, etc. (Frankel e Meyer 2000; Huang et al. 2005). O mecanismo responsável pela atividade antioxidante nas macroalgas marinhas é diferente para cada método, por isso, diferentes ensaios antioxidantes e diferentes concentrações de extrato bruto foram testados, de acordo com a resposta de cada espécie ao estresse oxidativo.

2 HIPÓTESES

H_{1a}. Espécies de macroalgas marinhas recifais de Pernambuco que permanecem expostas ao ar e radiação UV durante a maré baixa possuem atividade antioxidante.

H_{1b}. A atividade antioxidante de *Palisada perforata* aumenta conforme aumentam a temperatura e salinidade da água do mar.

H_{1c}. A dessecação aumenta a atividade antioxidante de *Palisada perforata*.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Caracterizar a atividade antioxidante em espécies de macroalgas marinhas representativas dos recifes de Pernambuco e avaliar a variabilidade desta bioatividade sob diferentes situações ambientais estressantes.

3.2 OBJETIVOS ESPECÍFICOS

- Realizar um *screening* da atividade antioxidante nas espécies de macroalgas marinhas dos três grupos (Rhodophyta, Chlorophyta e Phaeophyceae) mais representativas, amostradas no ambiente recifal de duas praias de Pernambuco.
- Avaliar a atividade antioxidante das macroalgas através de cinco métodos espectrofotométricos amplamente utilizados.
- Relacionar a atividade antioxidante com a presença de classes de compostos químicos nos extratos das macroalgas, através do scan UV-visível.
- Analisar a correlação das respostas da atividade antioxidante com fatores ambientais.
- Comparar a atividade antioxidante de *Palisada perforata* amostrada em diferentes micro-hábitats recifais: lado exposto, platô, poça e lado protegido.
- Avaliar a atividade antioxidante em *Palisada perforata* em um experimento laboratorial sob diferentes condições de temperatura e salinidade.
- Avaliar a atividade antioxidante em *Palisada perforata* em um experimento laboratorial sob diferentes condições de dessecação.

4 METODOLOGIA GERAL

4.1 ÁREA DE ESTUDO

O litoral de Pernambuco possui, aproximadamente, 190 km de linha de costa, e está localizado na província biogeográfica do Atlântico Tropical Sul Ocidental (Spalding et al., 2007), na ecorregião do Nordeste brasileiro. O clima local é tropical úmido com temperaturas médias anuais entre 25ºC e 30ºC. As marés são classificadas como mesomarés semidiurnas, dominadas por ondas (Amaral et al., 2016). A precipitação média anual é em torno de 1720 mm, com precipitações abaixo de 100 mm, entre os meses de setembro e fevereiro (período seco) e acima de 100 mm, entre os meses de março a agosto (período chuvoso), sendo os meses de maio-junho-julho o trimestre mais úmido, e o mês de junho, comumente, o mais frio (FINEP/UFPE 1990).

Na costa do Estado ocorrem extensas formações recifais (*beachrocks*), corpos rochosos compostos basicamente de grãos de quartzo cimentados por carbonato de cálcio (Manso et al. 2006), dispostos paralelamente à linha de costa e que podem ter alguns quilômetros de comprimento e alguns metros de largura (Kempf, 1969; Pereira et al., 2002). São constituídos por embasamentos arenítico e arenítico ferruginoso que afloram na maré baixa ou podem estar ligeiramente expostos acima do nível médio do mar, podendo em alguns locais, aparecer diretamente na face da praia (Dominguez et al., 1990; Pereira et al., 2002). Os *beachrocks* ocorrem em faixas paralelas à linha de costa funcionando como um sistema natural de proteção e como substrato para macroalgas e diversos outros organismos marinhos (Guerra e Manso, 2004). Desta forma, esses recifes de arenito são de grande importância ecológica, além de formarem uma bela paisagem, atrativa ao turismo do Estado.

O presente trabalho foi realizado nas praias de Boa Viagem e Enseada dos Corais (Figura 2).

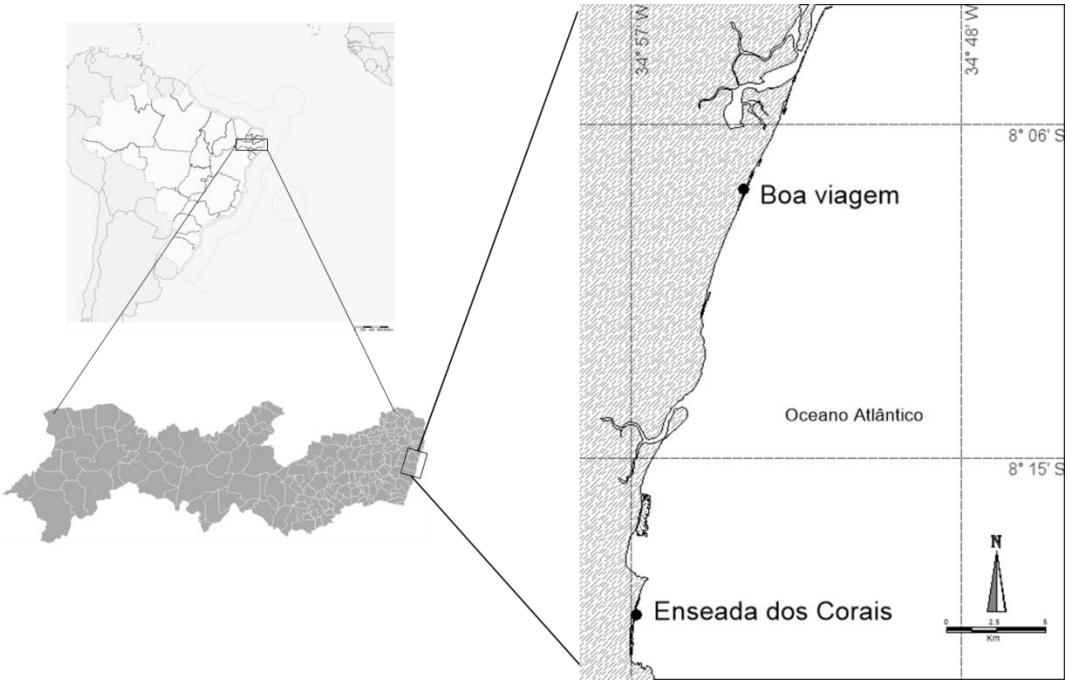


Figura 2 - Mapa do Brasil em destaque para o estado de Pernambuco. Em detalhe, a localização das duas praias amostradas, Boa Viagem e Enseada dos Corais.

O bairro de Boa viagem apresenta uma paisagem urbana caracterizada por uma longa faixa de edifícios, hotéis, centros comercias e empresariais. A área é bastante movimentada, com grande fluxo de visitantes e inúmeros pontos comerciais (Santos et al., 2006). Juntamente com a Praia do Pina, forma a orla metropolitana do Recife, com 57,48 ha e 8 km de extensão (Santos et al., 2006). O local de amostragem situa-se em frente ao hotel Grand Mercure na altura do número 4070 da Av. Boa Viagem (Figura sA), é um recife de arenito próximo à costa, o qual se apresenta emerso nas marés baixas de sizígia. A estrutura recifal apresenta pouca inclinação e um forte hidrodinamismo na face voltada ao mar aberto.

A praia de Enseada dos Corais (Figura 3B) tem cerca de 3 km de extensão, localizada na região metropolitana do Recife, no município do Cabo de Santo Agostinho, litoral sul de Pernambuco. Os recifes são largos e bem acidentados, formando poças e reentrâncias. A estrutura recifal amostrada fica em frente às casas de veraneio (entre as ruas 16 e 51) (8°19' 7,5" S e 34° 56' 53,2" W).

Figura 3 - Formações recifais amostradas em Pernambuco, Brasil. **A.** Praia de Boa Viagem. **B.** Praia de Enseada dos Corais.

Tomando como base o perfil transversal de um recife hipotético (Figura 4), foram amostrados quatro micro-hábitats distintos onde as macroalgas se desenvolvem, denominados, neste estudo, de lado protegido, poça, platô recifal e borda recifal. O lado protegido é a porção do recife voltada para a costa, protegida do batimento direto de ondas e da ação de correntes. A poça é caracterizada como uma depressão no platô recifal de até 1 m de diâmetro, a qual é inundada pela água do mar, permanecendo sempre submersa, mesmo durante a maré baixa. Na poça, as variações de salinidade, temperatura e pH podem ser extremas, devido à baixa circulação da água e elevada evaporação, fator estressante para as macroalgas. O platô recifal é a porção mais alta do recife que permanece exposta durante a maré baixa. Neste hábitat, as condições ambientais também podem ser estressantes, pois há elevada incidência solar e exposição ao ar, levando à dessecação das macroalgas. Por fim, a borda recifal é a porção frontal

do recife sujeito a elevado hidrodinamismo por batimento de ondas e ação de correntes, o que também pode ser estressante.

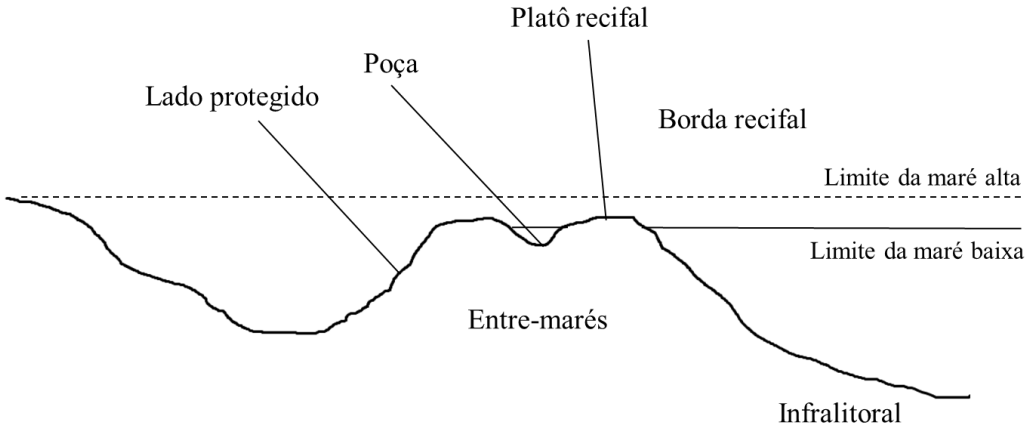


Figura 4 - Perfil transversal hipotético de um recife formando diferentes micro-hábitats durante a maré baixa.

4.2 DESENHO AMOSTRAL

Na primeira fase foram coletadas as espécies de macroalgas marinhas mais representativas (Vasconcelos et al. 2016) nos platôs, nos meses de janeiro e maio de 2015 . Para cada espécie, um extrato bruto foi feito com diclorometano/metanol (Bianco et al. 2013). Foi realizado o *screening* inicial da atividade antioxidante em diferentes concentrações.

A segunda fase consistiu na escolha de *Palisada perforata* para o estudo da variabilidade espacial da atividade antioxidante. A variabilidade espacial foi testada pela amostragem dos espécimes nos diferentes micro-hábitats recifais: lado exposto, platô, poça e lado protegido. Simultaneamente foram feitas amostragens de dados abióticos (n = 4) em cada micro-hábitat recifal para relacionar com as respostas antioxidantes.

Na terceira fase *P. perforata* foi amostrada, cultivada e submetida a dois experimentos de laboratório para caracterizar a variação da atividade antioxidante sob diferentes condições de temperatura, salinidade e dessecação, simulando os efeitos estressantes de um ciclo de maré.

4.3 ESCOLHA DAS ESPÉCIES E TRATAMENTO DAS AMOSTRAS

A escolha das espécies para a primeira fase levou em conta três critérios de elegibilidade, sendo eles: ampla distribuição geográfica, abundância relativamente significativa nos recifes de Pernambuco e a presença comprovada de algum tipo de atividade biológica com base na literatura. Desta forma, foram selecionadas oito espécies de Rhodophyta: *Gracilaria caudata* J. Agardh, *Gracilaria cearenses* (A.B. Joly & Pinheiro) A.B. Joly & Pinheiro, *Osmundaria obtusiloba* (C. Agardh) R.E. Norris, *Bryothamnion triquetrum* (S.G. Gmelin) M. Howe, *Acanthophora spicifera* (M. Vahl) Børgesen, *Chondracanthus acicularis* (Roth) Fredericq, *Amansia multifida* J.V. Lamouroux, *Cryptonemia seminervis* (C. Agardh) J. Agardh, três espécies de Phaeophyceae: *Dictyopteris delicatula* J.V. Lamouroux, *Padina* sp. e *Sargassum* sp. e três espécies de Chlorophyta: *Caulerpa racemosa* (Forssk.) J. Agardh, *Caulerpa cupressoides* (Vahl) C. Agardh e *Bryopsis pennata* J.V. Lamouroux (Figura 5). A proporção de representantes de cada grupo quanto a Ordens e Famílias se ajusta aproximadamente à observada em regiões tropicais. Além destas, a Rhodophyta *Palisada perforata* (Bory) K.W. Nam foi amostrada na terceira fase do projeto.

Em laboratório, as algas foram lavadas com água doce, limpas de epífitas e fauna associada, catalogadas e identificadas com auxílio de estereomicroscópio e microscópio. A identificação das espécies foi feita com base em literatura especializada e atualização nomenclatural proposta por Guiry e Guiry (2017) e Wynne (2017). Após a identificação, pelo menos um exemplar de cada espécie foi herborizado segundo as técnicas usuais em ficologia e depositado no Herbário Maria Eneyda P. K. Fidalgo do Instituto de Botânica de São Paulo. A biomassa restante foi congelada para posterior análise de antioxidantes.

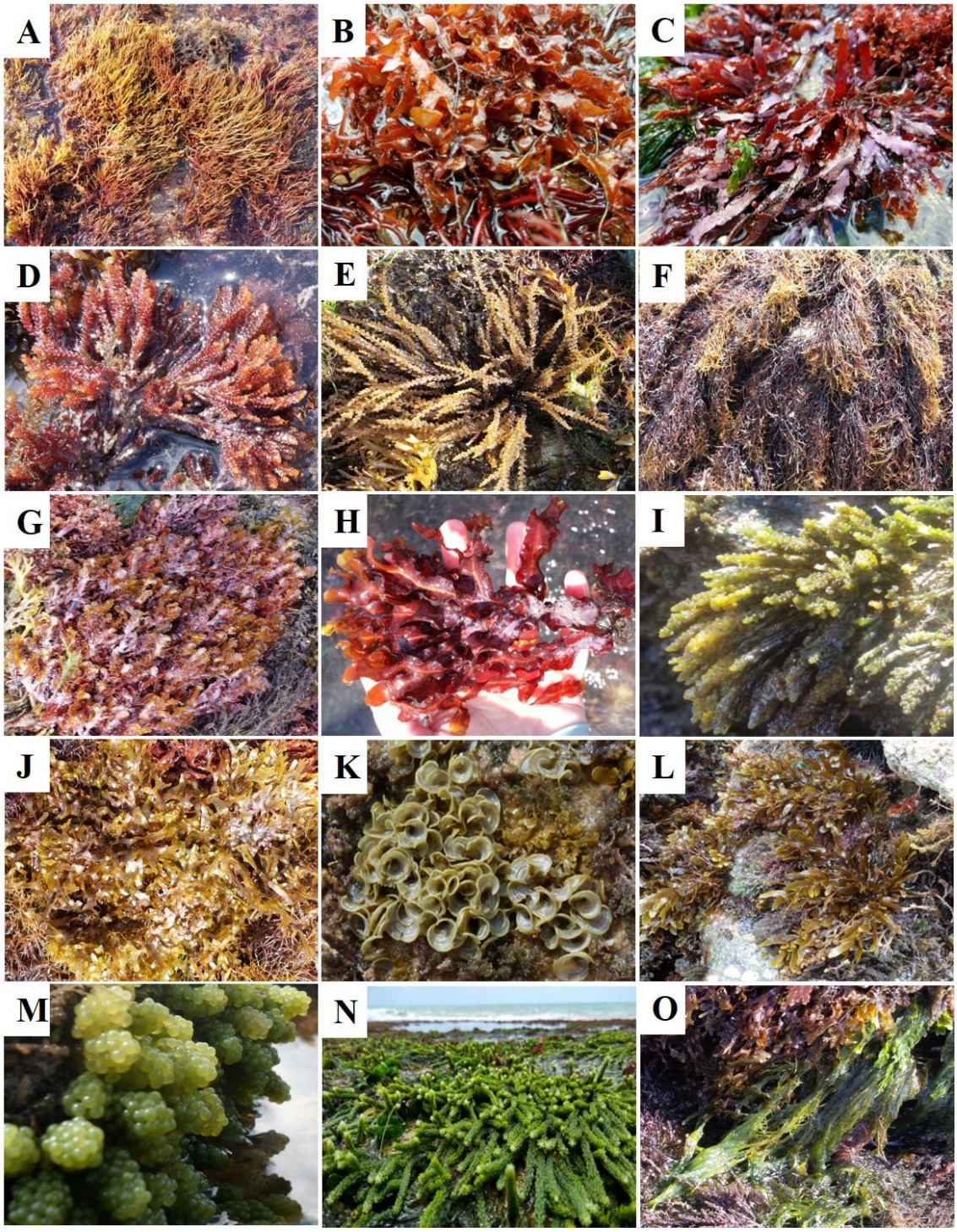


Figura 5 - Aspecto geral das macroalgas em campo. **A.** *Gracilaria caudata* **B.** *Gracilaria cearensis* **C.** *Osmundaria obtusiloba* **D.** *Bryothamnion triquetrum* **E.** *Acanthophora spicifera* **F.** *Chondracanthus acicularis* **G.** *Amansia multifida* **H.** *Cryptonemia seminervis* **I.** *Palisada perforata* **J.** *Dictyopteris delicatula* **K.** *Padina* sp. **L.** *Sargassum* sp. **M.** *Caulerpa racemosa* **N.** *Caulerpa cupressoides* **O.** *Bryopsis pennata.*

4.4 PREPARAÇÃO DOS EXTRATOS

Para a preparação dos extratos brutos, as algas foram descongeladas, lavadas com água doce, secas sob papel absorvente em temperatura ambiente, sem luz direta, trituradas em liquidificador até obter um pó, o qual foi acrescido dos solventes diclorometano e metanol (CH_2Cl_2/MeOH, 2:1), em quantidade suficiente para cobrir as algas, e vedado para evitar evaporação. O uso desses solventes na preparação dos extratos tem por finalidade selecionar, dentre todas as substâncias presentes nas macroalgas, aquelas com média polaridade ou apolares (lipofílicas), como, terpenóides e acetogeninas, já descritas na literatura como moléculas antioxidantes.

Após três dias de extração, os solventes foram filtrados e mais solventes foram adicionados às algas. Este processo foi repetido três vezes durante nove dias. No final do processo, os solventes filtrados foram acondicionados em um único balão volumétrico, para cada amostra, e evaporados completamente em evaporador rotativo (R-215 Buchi, Switzerland) a 40ºC e pressões entre 70 e 500 torr (Vacuubrand CVC 3000). Os extratos finais secos foram removidos dos balões por diluição com acetona e deixados para evaporar completamente em capela de exaustão.

4.5 ENSAIOS ANTIOXIDANTES

As atividades antioxidantes dos extratos de macroalgas recifais de Pernambuco foram caracterizadas por cinco ensaios bioquímicos complementares para suprimir a inabilidade de um simples teste unidimensional em representar com precisão as complexas interações *in vivo* entre antioxidantes (Frankel e Meyer 2000, Huang et al. 2005). Desta forma, os cinco ensaios utilizados foram: DPPH, ABTS, quelante de metais, Folin-Ciocalteu e FRAP.

O método DPPH é baseado na captura do radical DPPH (2,2-difenil-1- picril-hidrazil) por antioxidantes, produzindo um decréscimo da absorbância a 517 nm. No ensaio ocorre uma reação de oxi-redução, onde o DPPH, que apresenta coloração violeta, é reduzido (o elétron desemparelhado do N se emparelha com o elétron cedido pelo radical H de um oxidante), tornando a reação amarelada e ocorrendo a formação de DPPH-H, reduzido e estável (Figura 6).

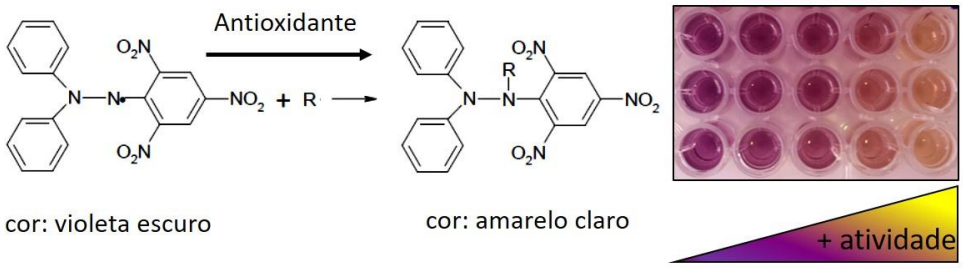


Figura 6 - Reação de oxi-redução com a adição de um agente antioxidante ao radical DPPH. Ilustração: Edy Sousa de Brito (Rufino et al. 2007a).

O ensaio antioxidante pela captura do radical livre ABTS (2,2'-azinobis(3-etilbenzotiazolina-6-ácido sulfônico)) é um dos mais utilizados para fazer um *screening* geral do potencial antioxidante. É baseado na captura do radical ABTS gerado através de uma reação eletroquímica ou enzimática (Figura 7). Esta metodologia permite medir o potencial antioxidante de substâncias de natureza hidrofílica e lipofílica (Rufino e Alves, 2007).

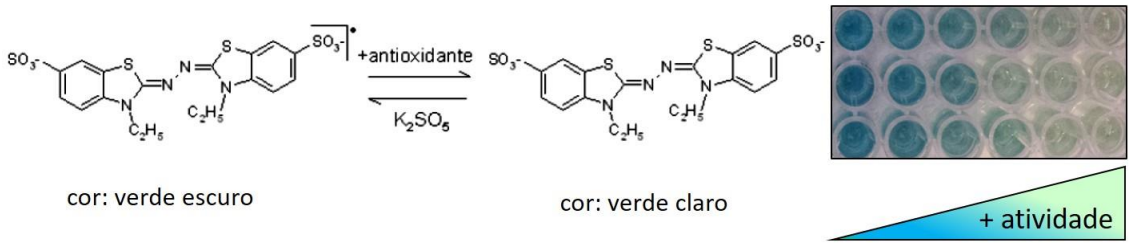


Figura 7 - Reação de estabilização do radical ABTS por um antioxidante e sua formação pelo persulfato de potássio. Ilustração: Edy Sousa de Brito (Rufino et al. 2007b).

O método quelante de metais é baseado na utilização do reagente ferrozina que é um composto amplamente utilizado na determinação de Fe^{2+}. Na presença de Fe^{2+}, a ferrozina forma um complexo de cor rosácea, cuja absorbância pode ser medida no comprimento de 562

nm (Figura 8). O método determina a quantidade remanescente de íons ferro não quelados na mistura de reação, que os agentes quelantes presentes no extrato testado não conseguiram quelar (Min et al., 2011).

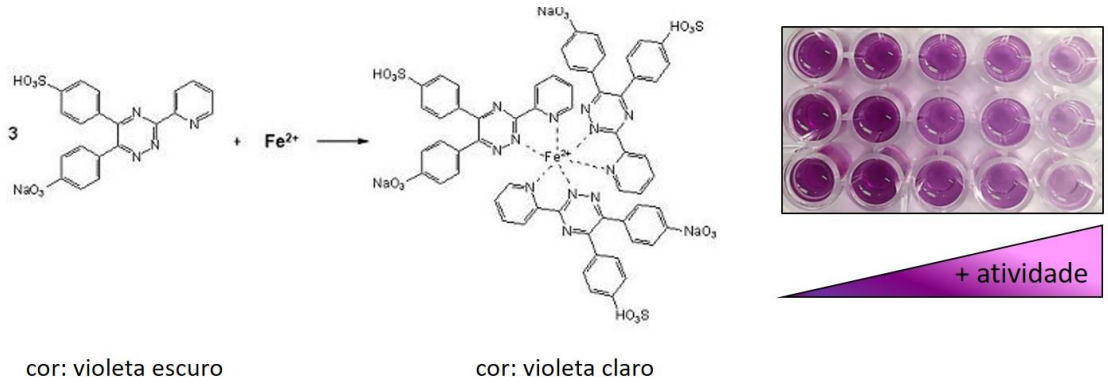


Figura 8 - Reação de redução de alguns metais com o ferro pelo ensaio quelante de metais. Fonte: Morescho et al. 2011.

O ensaio Folin-Ciocalteu é um método espectrofotométrico simples e baseia-se na propriedade redutora das substâncias fenólicas que reagem com o reagente de Folin-Ciocalteu em condições alcalinas. As substâncias fenólicas reagem com o reagente de Folin-Ciocalteu, uma mistura de ácido fosfotúngstico-fosfomolíbdico, apenas em condições alcalinas alcançadas com a adição de carbonato de sódio (Na_2CO_3), ocorrendo dissociação de um próton fenólico e levando à formação do ânion fenolato (Figura 9). O ânion mais propenso a doar elétrons e capaz de reduzir o reagente, ou seja, tem maior capacidade redutora, formando assim um complexo azul de molibdênio (Genovese et al. 2003; Huang et al. 2005).

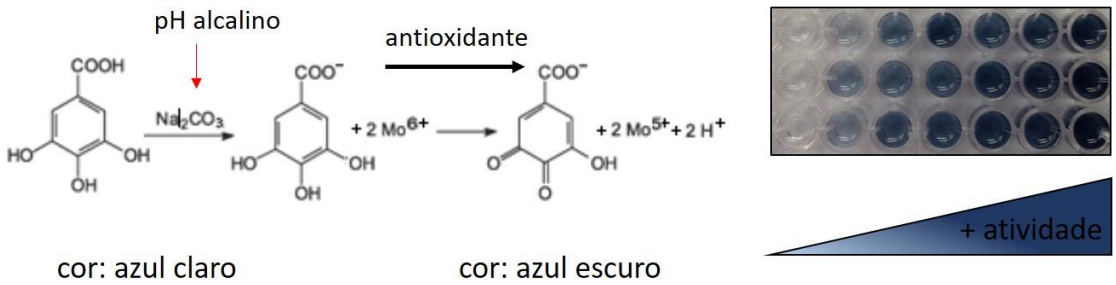


Figura 9 - Reação do ácido gálico com molibdênio, componente do reagente Folin-Ciocalteu. Fonte: Pires et al. (2017).

O método FRAP (Ferric Reducing Antioxidant Power) surgiu com uma alternativa para determinar a redução do ferro em fluidos biológicos e soluções aquosas de compostos puros. Em pH ácido, o complexo tripiridil hidrazina férrica (Fe^{3+} TPTZ) se reduz (produzindo intensa cor azul) que pode ser monitorada pela medida da absorbância no comprimento de onda de 595 nm (Figura 10). A reação é não específica e qualquer reação com baixo poder redutor, sob as condições de reação estabelecida, irá formar o íon ferroso (Fe^{2+}) a partir do íon férrico (Fe^{3+}). A mudança na absorbância está diretamente relacionada com o poder redutor total dos antioxidantes doadores de elétrons, presentes na mistura de reação (Tandon et al., 2008).

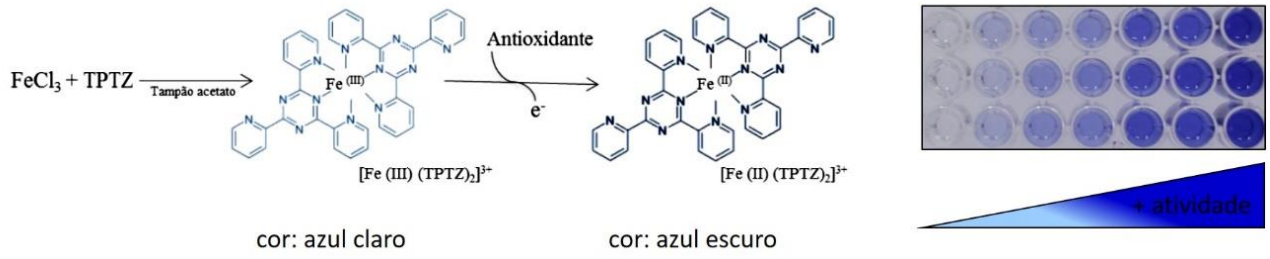


Figura 10 - Reação de redução do ferro pelo ensaio FRAP. Fonte: Urrea-Victoria et al. (2016).

4.6 PULSO DE AMPLITUDE MODULADA (PAM)

O uso do fluorômetro PAM em macroalgas é relativamente recente e tem se tornado uma ferramenta útil na avaliação da fotossíntese sob diferentes condições ambientais e no laboratório sob diferentes lâmpadas artificiais (Häder e Figueroa 1997). Quando a radiação atinge os organismos fotossintetizantes, em seus centros de reação do fotossistema II, a energia pode ser direcionada de três maneiras: absorvida pela clorofila e aproveitada para as reações químicas da célula, dissipada como calor ou outra forma de perda energética e, por fim, re-emitida em um comprimento de onda diferente (*e.g.* fluorescência). Por meio desta fluorescência da clorofila *a* dos organismos fotossintetizantes é possível estimar o funcionamento do aparato fotossintético.

A fluorescência da clorofila pode funcionar como um indicador dos diferentes níveis funcionais da fotossíntese como captura de fótons por pigmentos capturadores de luz, reações luminosas primárias, reações de transporte de elétrons do tilacoide, reações escuras enzimáticas do estroma e processos lentos regulatórios de *feedback* (Bückel e Wilhem 1993). Os princípios fotoquímicos incluem dois diferentes tipos de processos de desexcitação: (1) conversão de energia fotoquímica nos centros do fotossistema II (PSII) e (2) dissipação não-fotoquímica da energia de excitação nas antenas e níveis do centro de reação (Figueroa e Gómez 2001).

Algas aclimatadas ao escuro são usadas para determinar o rendimento quântico máximo ou ótimo (F_v/F_m) e sob luz actínica, o rendimento quântico efetivo (Y(II)). O parâmetro F_v representa a diferença entre o máximo e mínimo rendimento de flurorescência de uma amostra no escuro quando todos centros de reação do PSII estão completamente abertos. Quando a amostra é transferida para a luz actínica, o rendimento máximo diminui porque parte dos centros de reação foram reduzidos e o decaimento do elétron na reação escura da fotossíntese é parcialmente saturado (Figueroa e Gómez 2001).

A relação entre rendimento quântico efetivo da fluorescência e assimilação de carbono e/ou produção de oxigênio tem sido demostrada em plantas superiores, micro e macroalgas (Hanelt et al. 1994). Esta relação valida a fluorescência da clorofila como determinação indireta da fotossíntese e pode ser usada então para estimativa da produtividade primária. Para estimar o transporte de elétrons no aparato fotossintético, e portanto, da capacidade fotossintetizante, determinado pelas medidas de fluorescência, foi desenvolvida a seguinte expressão (Schreiber et al. 1986):

$$ETR = (Y(II))*I_{PAR}*A*0,5$$

onde Y(II) é o rendimento quântico efetivo em uma dada irradiância (I_{PAR}), A é a absorptância do talo da alga e 0,5 é o fator para algas vermelhas, da razão quanta/elétron (um

elétron requer absorção de dois quanta como dois fotossistemas estão envolvidos) (Schreiber e Neubauer 1990).

5 RESULTADOS

Os resultados obtidos estão apresentados na forma de artigos científicos, onde cada artigo corresponde a um capítulo da tese. Juntos, os resultados apresentam o status completo do potencial antioxidante de macroalgas recifais de Pernambuco.

A seção 6 trata de um *screening* geral buscando espécies de macroalgas dos recifes de Pernambuco mais promissoras quanto à atividade antioxidante.

A seção 7 analisa a atividade antioxidante de três espécies de macroalgas amostradas na porção inferior do recife entre-marés, sendo elas *Sargassum furcatum*, *Bryothamnion triquetrum* e *Osmundaria obtusiloba*.

A seção 8 compara a atividade antioxidante de *Palisada perforata* amostrada em quatro microhábitats recifais (poça, proetegido, platô e batido) e analisa a produção de antioxidantes e performance fotossintética desta mesma espécie sob diferentes condições de salinidade, temperatura e dessecação em experimentos de laboratório.

6 Screening for antioxidant capacity of tropical reef seaweeds: prospection for new natural antioxidants

Abstract

The screening of antioxidant capacity may be a useful, rapid and feasible tool to identify potential seaweed species for future prospecting approaches. For intertidal reefs of Pernambuco, Northeastern of Brazil, we report the first antioxidant activity screening of 11 abundant seaweeds, specifically dichloromethane:methanol extracts. Five different *in vitro* antioxidant assays (DPPH, ABTS, Chelator, FRAP and Folin-Ciocalteu assays) were chosen for evaluating the antioxidant ability. All studied species showed a dose-dependence antioxidant pattern and elevated capacity even at low extract concentration. Reef environments are stressful ecosystems which impose extreme abiotic conditions, resulting in adapted seaweeds that inhabit there with expressive competence to manage oxidative stress. The studied brown algae were the most prominent along with the green algae, enabling the possibility to reinforce the recommendation of further studies to prospect these species as sources of natural antioxidants for functional applications.

Keywords: antioxidant, free radicals, functional products, reactive oxygen species, tropical algae.

Resumo

O screening da capacidade antioxidante pode ser uma ferramenta útil, rápida e prática para identificar espécies de macroalgas marinhas potenciais para futuros estudos de prospecção. Apresentamos o primeiro screening da atividade antioxidante de 11 macroalgas marinhas representativas da região entre-marés de recifes de Pernambuco, Nordeste do Brasil, especificamente extraídas com diclorometano:metanol. Cinco diferentes ensaios in vitro (DPPH, ABTS, Quelante de metais, FRAP e Folin-Ciocalteu) foram escolhidos para avaliar a atividade antioxidante. Todas as espécies estudadas apresentaram um padrão antioxidante dose-dependente e elevada capacidade, mesmo em baixas concentrações de extrato. Ambientes recifais são ecossistemas estressantes, os quais impõem extremas condições abióticas, resultando em macroalgas marinhas adaptadas, com extrema competência para gerir o estresse oxidativo nesses ambientes. As macroalgas pardas estudadas foram as mais proeminentes junto com as verdes, reforçando a recomendação de estudos de prospecção destas espécies como fontes de antioxidantes naturais para aplicações funcionais.

Palavras-Chave: algas tropicais, antioxidante, espécies reativas de oxigênio, produtos funcionais, radicais livres.

INTRODUCTION

Among tropical marine ecosystems, the reef environments are characterized as extremely rich, holding great diversity of species (Sheppard *et al.,* 2009). The Pernambuco beach-rock reefs, located in the Northeastern Brazil, consist in marine outcrops parallel to the coastline (Kempf, 1970), with variable oceanographic conditions. In those reefs, seaweeds are often found in the intertidal zone, remaining emerged during low tides and immersed the rest of the time. As they are sessile, seaweeds are exposed to a combination of variable factors, such as desiccation, solar light, UV radiation, wave action, herbivory, among others, that may lead to the formation of free radicals and other agents that cause oxidative stress.

However, the lack of oxidative damage in seaweeds suggest that they may have diverse mechanism for protecting against oxidative damage, like production of antioxidant enzymes (superoxide dismutase, peroxidase, glutathione reductase, catalase) and antioxidant molecules (phlorotannins, ascorbic acid, tocopherols, carotenoids, phospholipids, chlorophyll related compounds, bromophenols, catechins, mycosporine-like amino acids, polysaccharides, etc.) (Fujimoto, 1990; Le Tutour *et al.*, 1998; Matsukawa *et al.*, 1997; Rupérez *et al.*, 2002; Yuan *et al.*, 2005).

Seaweeds that inhabit harsh environments, such as the tropical reefs, which are abundant in Pernambuco, can overcome excessive UV radiation and temperature conditions that result from tropical geographic position (Zubia *et al.*, 2007). Several authors have demonstrated that tropical seaweeds show higher amount of antioxidant substances than other species from increasing latitudes (Kelman *et al*., 2012, Li *et al*., 2012, Martins *et al*., 2012, Silva *et al*., 2012; Zubia *et al*., 2007). Despite that, only a few studies on the antioxidant potential of tropical seaweeds have been performed, enabling an appropriate scenario for the search of new candidate species as potential sources of natural antioxidant and functional bioproducts.

The Brazilian littoral has an extensive coastline with great biodiversity of seaweeds that present several bioactivities against human pathologies (*e.g.*, chronic inflammation, atherosclerosis, cancer and cardiovascular disorders) and ageing processes (Kohen and Nyska, 2002). In view of that, our main goal was to evaluate the antioxidant capacity of the crude extracts of 11 seaweed species for prospecting purpose as natural antioxidant candidates.

MATERIALS AND METHODS

Sampling and preparation of crude extracts

A total of 11 seaweed species, belonging to Phaeophyceae, Chlorophyta and Rhodophyta (Tab. 1), were collected along the Pernambuco coastline in Northeastern Brazil, at the reefs of Enseada dos Corais (8°18'44.7"S; 34°56'49.8"W) and Boa Viagem (8°07'30.8"S 34°53'45.9"W). The region is characterized by a tropical climate, with average temperatures ranging from 25°C to 30°C, and semidiurnal meso-tides (ranging between 2 and 4 m) dominated by waves (Amaral *et al*., 2016).

Approximately 500 g of each species were manually collected, transported to the laboratory and frozen at -20ºC. The material was washed with tap water to get rid of the excess of salt and sand, and it was also removed the associated fauna and epiphytes. Then, the material was air-dried at room temperature for about 72 h.

The dried seaweeds were ground to a fine powder and extracted for three days with a mixture of dichloromethane:methanol (2:1 v/v) with adequate solvent to cover and soak the material. The extraction procedure was repeated three times and all filtered extracts from the same species were gathered as a single extract and then evaporated in a rotavapor (R-215 Buchi, Switzerland) at 40ºC and pressures between 70 and 500 torr (Vacuubrand CVC 3000). The final

dried extracts were removed from the rotary flask by acetone dilution and left to completely evaporate in an exhaustion hood. The extract yield was calculated according to the formula: yield (%) = (dried extract weight x 100) / dried seaweed weight.

Antioxidant assays

The antioxidant capacity can act on the basis of the chemical reaction, therefore, the use of different antioxidant assays are valuable tools for a preliminary prospecting profile. The antioxidant analyses were performed in the crude extracts described above, dissolved in methanol, at different extract concentrations depending on the species. Results were expressed as percentage of antioxidant activity and EC_{50} (concentration which induces a response halfway between the baseline and the maximum at defined time reaction). Gallic acid (Sigma-Aldrich, Brazil) was used as standard. All antioxidant assays were performed in triplicate and the respective absorbance was read with a UV-vis microplate spectrophotometer (Epoch Biotek, USA).

DPPH radical scavenging activity

The DPPH (2,2-diphenyl-1-picryhydrazyl) antioxidant assay was performed according to Brand-Williams *et al*. (1995), as modified by Pires *et al*. (2017a). The method is based on DPPH radical capture by antioxidants, producing an absorbance decrease at 517 nm. Aliquots of 20 µl of extracts, or standard, (dissolved in methanol) were added to 280 µl of DPPH (32 µg ml^{-1}) (Sigma-Aldrich, Brazil) solution. After standing for 30 minutes in the dark at room temperature, the absorbance was read at 517 nm. The DPPH radical scavenging percentage was calculated through the following equation: $[(Abs_{DPPH} - Abs_{sample}) / Abs_{DPPH}] \times 100$, where

Abs_{DPPH} is the control absorbance of DPPH, and Abs_{sample} is the absorbance of sample in DPPH reactive solution. For DPPH assay was also calculated the EC_{50} index as the activity of the tested crude extracts that enabled their estimation.

ABTS radical scavenging activity

The antioxidant assay by ABTS (2,2–azinobis (3-ethylbenzthiazoline-6-sulfonic acid) free radical scavenging is one of the most used methods and measures the antioxidant potential of hydrophilic and lipophilic substances (Rufino *et al*., 2007). The method used was described by Rufino *et al*. (2007) and modified by Torres *et al.* (2017). Aliquots of 20 µl of extract, or standard, were added to 280 µl of ABTS radical. Absorbance was read at 734 nm after 20 minutes of incubation in the dark. ABTS radical scavenging percentage was calculated by the following formula: $[(Abs_{ABTS} - Abs_{sample})/Abs_{ABTS}] \times 100$, where Abs_{ABTS} is the ABTS's control absorbance of ABTS, and Abs_{sample} is the absorbance of the sample in ABTS reactive solution.

Metal chelating activity (Chelator)

The metal chelating activity (Chelator) determines the quantity of non-chelated iron ions in the reaction mixture remaining after chelation. The method was performed as described by Dinis *et al*. (1994) and modified by Harb *et al*. (2016). The Solutions were composed of 250 µl of acetate 10%, 15 µl of ammonium sulfate, and 20 µl of extract, with standard or methanol as blank. After 15 minutes, 15 µl of ferrozine solution were added. The microplates were homogenized and incubated in the dark for 10 minutes, followed by shaking at 100 rpm for further reading at 562 nm. The percentage of chelating potential was calculated using the

following formula: [($Abs_{Chelator}$ − $Abs_{Chelator}$)/$Abs_{Chelator}$] x 100, where $Abs_{Chelator}$ is the control absorbance of the reaction mixture, and Abs_{sample} is the absorbance in the Chelator reactive solution.

Ferric reducing antioxidant power (FRAP)

The ability of seaweed to act as a reducer of substances was tested through the FRAP assay, as described by Benzie and Strain (1996) and modified by Urrea-Victoria *et al*. (2016). Change of absorbance is directly related to the reducing power of the electron donors present in the reaction mixture (Ferreira *et al*., 2007). The microplates were assembled in the dark, mixing 15 µl of ultrapure water with 20 µl of sample, with standard or methanol as blank, and 265 µl of FRAP reagent (25 ml of buffer acetate 0.3 M, 2.5 ml of ferric tripyridyl hydrazine solution 10 mM and 2.5 ml of a ferric chloride aqueous solution 20 mM; Sigma-Aldrich, Brazil). Absorbance was read after 30 minutes of incubation at 595 nm. FRAP percentage was calculated as (Abs_{sample} x 100)/Abs_{FRAP}, where Abs_{sample} is the absorbance of the sample in the reaction mixture, and Abs_{FRAP} is the maximum absorbance obtained by gallic acid standard, indicating the maximum antioxidant activity.

Folin-Ciocalteu assay

This assay was described by Singleton and Rossi (1965) and Waterman and Mole (1994) and then modified by Pires *et al*. (2017b). It is based on the reductive property of total phenolic compounds that react with Folin-Ciocalteu reagent under alkaline conditions. The reaction mixture was composed of 200 µl of ultrapure water, 20 µl of sample, with standard or methanol

as blank, 20 µl of Folin-Ciocalteu (1 N) reagent and 60 µl of Na_2CO_3 (10 mg ml^{-1} of ultrapure water), followed by incubation in the dark for 30 minutes and subsequent reading at 760 nm. Folin-Ciocalteu percentage was calculated as follows: (Abs_{sample} x 100)/$Abs_{Folin\text{-}Ciocalteu}$, where Abs_{sample} is the absorbance of sample in the reaction mixture, and $Abs_{Folin\text{-}Ciocalteu}$ is the maximum absorbance obtained by gallic acid standard, indicating the maximum antioxidant activity.

Environmental parameters

Temperature (Temp), salinity (Sal), pH, dissolved oxygen (DO) and turbidity (Turb) were registered from each sampling site using a multiparameter probe (Horiba, USA) for correlating possible relationships between the antioxidant activity and abiotic parameters in a Redundancy Analysis.

Statistical analysis

The antioxidant activity percentages obtained for DPPH, ABTS, Chelator, FRAP and Folin-Ciocalteu assays with different extract concentrations were compared with one-way analysis of variance (ANOVA), prior transformation of the percentages achieve the statistical assumptions. Significant differences were determined by Tukey's post-hoc test at 95% significance level ($p < 0.05$), comparing the same extract concentration between species tested for each method. The results were expressed as mean ± standard deviation (n = 3). All analyses were performed with Statistica v 10. A Redundancy Analysis (RDA) was performed in Excel

with XLSTAT (2014.5.03) to correlate the response of antioxidant activity with the environmental data (temperature, salinity, DO, pH and turbidity) as explanatory variables.

RESULTS

Eleven representative species of seaweed were collected from the Pernambuco reefs: 3 Phaeophyceae, 3 Chlorophyta and 5 Rhodophyta (Tab. 1). The extract yield of each species was variable, ranging between 0.39% for *Palisada perforata* and 10.63% for *Caulerpa cupressoides* (Tab. 1). All crude extract showed antioxidant activity for the different tests analyzed (Tab. 2), exhibiting a positive reactivity for the selected methodologies.

Table 1 - Summary of the collected species, indicating the local and date, in Pernambuco State, Northeastern Brazil. The extract yield (%) for each species is also included.

Species	Extract yield	Sampling site	Sampling date
Phaeophyceae			
Padina tetrastomatica Hauck	3.76%	Enseada dos Corais	05/17/2015
Padina gymnospora (Kützing) Sonder	6.29%	Boa Viagem	05/18/2015
Dictyopteris delicatula J.V. Lamouroux	4.05%	Enseada dos Corais	05/17/2015
Chlorophyta			
Caulerpa racemosa (Forsskål) J. Agardh	4.79%	Enseada dos Corais	05/17/2015
Caulerpa cupressoides (Vahl) C. Agardh	10.63%	Enseada dos Corais	05/17/2015
Bryopsis pennata J.V. Lamouroux	7.12%	Boa Viagem	05/18/2015
Rhodophyta			
Acanthophora spicifera (M. Vahl) Børgesen	3.28%	Boa Viagem	05/18/2015
Chondracanthus acicularis (Roth) Fredericq	2.21%	Boa Viagem	05/18/2015
Palisada perforata (Bory) K.W. Nam	0.39%	Enseada dos Corais	04/27/2017

Gracilaria cearenses (A.B. Joly & Pinheiro) A.B. Joly & Pinheiro	5.69%	Boa Viagem	05/18/2015
Gracilaria caudata J. Agardh	1.26%	Enseada dos Corais	05/17/2015

All species at different assays presented a dose-dependence activity. Specifically, for DPPH assay, the EC_{50} index was calculated and is shown in Fig. 1. A lower EC_{50} indicates better antioxidant activity, and then the studied species of Phaeophyceae and Chlorophyta presented better antioxidant activity by the DPPH scavenging assay.

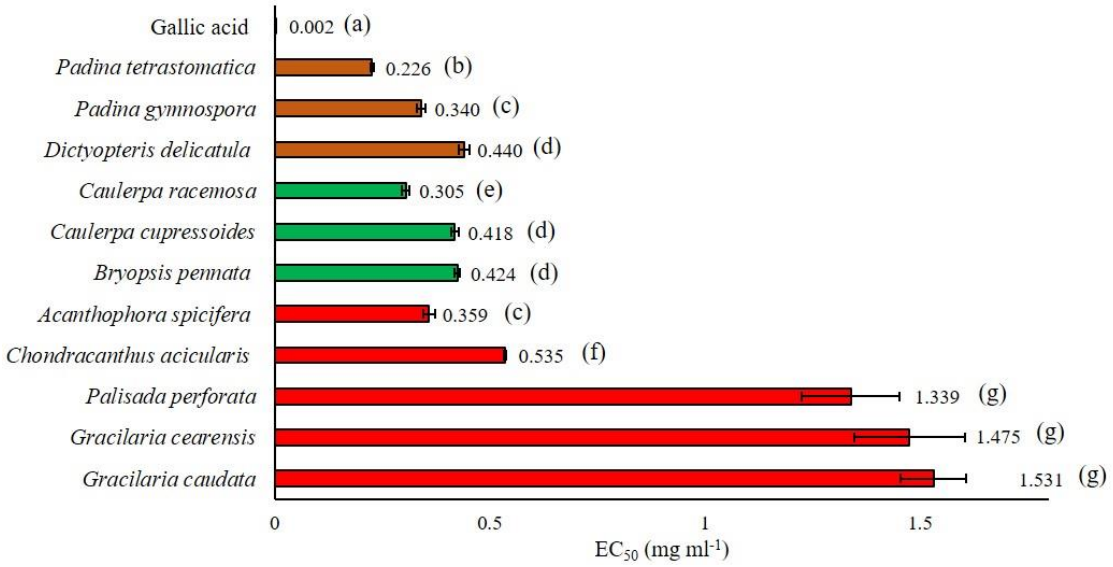


Figure 1 - DPPH radical scavenging activity expressed in oxidation index EC_{50} given in mg ml^{-1} (mean ± SD, n = 3) for tropical seaweeds from Pernambuco reefs and standard gallic acid. Bars represent standard deviations. Brown bars indicate Phaeophyceae species, green bars indicate Chlorophyta species and red bars indicate Rhodophyta species. Significant differences are indicated by different letters as determined by Tukey HSD test (p < 0.05).

The species showed different levels of antioxidant activity for the DPPH assay, evidencing a potential characteristic of the species (Tab. 2). Nevertheless, brown algae showed the highest percentage of antioxidant activity among the studied seaweeds, with a mean value of 182.53% for DPPH assay at 1 mg mL^{-1} of extract concentration, followed by the green algae, with 142.00%. Red algae had the lowest mean of antioxidant activity, with a value of 67.14% for DPPH.

Among the brown algae, *P. tetrastomatica* exhibited the highest activity at DPPH (251.51±3.56%) and ABTS (102.39±0.23%) assays (Tab. 2), with a very low EC_{50} (Fig. 1), which was statistically different from all others ($p < 0.05$). The green algae *C. racemosa* exhibited the second highest antioxidant activity at DPPH (163.48±1.97%) and ABTS (87.54±0.09%) assays (Tab. 2), with a low EC_{50}, differing from the other species ($p < 0.05$). For the red algae, *A. spicifera* showed the best antioxidant results at DPPH (126.96±0.95%) and Chelator (74.90±5.84%) assays, with a low EC_{50}, similar to a brown species, *P. gymnospora* ($p > 0.05$) (Fig. 1).

The influence of environmental parameters (temperature, salinity, pH, DO and turbidity) on the antioxidant activities of seaweeds was assessed through a redundancy analysis (Fig. 2). For the species collected in Boa Viagem beach (Fig. 2A), pH, DO and turbidity seem to be the main parameters related to activities of the red species *A. spicifera* and *C. acicularis*, while *P. gymnospora* antioxidant potential may be mostly affected by salinity. In Enseada dos Corais beach (Fig. 2B), pH, salinity and temperature had greater influence on the activities of both *Caulerpa* species, *G. caudata* and *D. delicatula*. This last one was more affected by increase of temperature.

Table 2 - Antioxidant activity (%) of gallic acid, used as standard, and tropical seaweeds from Pernambuco reefs (Northeastern Brazil) at different standard or extract concentrations denoted in brackets. Data are expressed as mean ± SD (n = 3).

	DPPH	ABTS	Chelator	FRAP	Folin-Ciocalteu
	[3 µg mL^{-1}]	[1.75 µg mL^{-1}]	[8 µg mL^{-1}]	[6 µg mL^{-1}]	[12 µg mL^{-1}]
Gallic acid	72.69±2.07	75.52±3.14	59.75±1.80	100.00±6.44	100.00±2.60

	DPPH				ABTS			Chelators	FRAP	Folin-Ciocalteu	
	[1 mg mL^{-1}]	[0.6 mg mL^{-1}]	[0.4 mg mL^{-1}]	[0.2 mg mL^{-1}]	[0.6 mg mL^{-1}]	[0.4 mg mL^{-1}]	[0.2 mg mL^{-1}]	[0.6 mg mL^{-1}]	[0.2 mg mL^{-1}]	[0.2 mg mL^{-1}]	[0.15 mg mL^{-1}]
Phaeophyceae											
P. tetrastomatica	251.51±3.56	114.84±1.51	118.05±14.83	22.91±1.40	-	102.39±0.23	57.83±0.14	8.90±6.33	26.35±0.74	31.80±1.76	24.31±3.57
P. gymnospora	153.87±0.59	89.99±1.26	59.81±0.10	15.38±3.09	-	77.70±0.19	57.33±3.03	41.39±11.80	69.42±2.40	32.70±3.53	18.14±11.22
D. delicatula	142.21±2.95	55.12±0.76	42.27±0.52	20.40±2.80	62.34±0.62	51.38±0.38	-	30.62±3.50	44.84±1.46	66.04±2.30	38.25±9.03
Chlorophyta											
C. racemosa	163.48±1.97	65.04±3.40	71.41±0.33	21.19±2.61	-	87.54±0.09	40.64±0.78	37.94±7.89	30.69±0.65	86.12±6.50	65.61±3.76
C. cupressoides	133.51±2.20	50.04±0.61	49.95±0.92	17.03±0.59	74.02±2.37	44.00±1.96	-	28.52±9.55	20.73±0.82	37.28±1.64	52.78±6.42
B. pennata	129.01±5.45	86.77±1.97	40.80±1.62	11.75±1.64	-	65.96±1.01	53.87±0.80	21.29±7.26	53.89±3.44	60.10±6.99	30.80±11.04
Rhodophyta											
A. spicifera	126.96±0.95	75.72±0.52	56.65±1.38	14.58±3.31	62.59±2.63	52.94±0.44	44.25±3.20	74.90±5.84	53.35±1.62	33.48±2.47	20.62±8.18
C. acicularis	89.04±0.42	48.33±0.24	33.91±1.19	14.75±0.85	60.75±3.07	45.00±1.51	47.33±1.16	42.39±2.90	32.29±1.79	42.43±7.76	23.61±4.69
P. perforata	44.37±2.44	27.73±1.38	18.15±0.28	15.67±0.67	84.22±1.26	68.72±1.05	-	47.95±15.10	65.36±6.14*	39.41±5.48*	
G. cearensis	35.22±1.41	32.65±1.35	11.04±0.37	6.64±2.89	31.79±2.11	28.02±0.21	29.67±0.83	37.17±4.51	29.14±0.24	39.19±3.65	24.31±4.88
G. caudata	40.00±0.55	28.25±2.34	28.89±0.88	10.28±1.24	36.00±2.94	27.34±0.44	-	34.39±6.26	9.34±0.28	30.55±7.25	18.46±4.22

*Results for 0.4 mg mL^{-1} extract concentration.

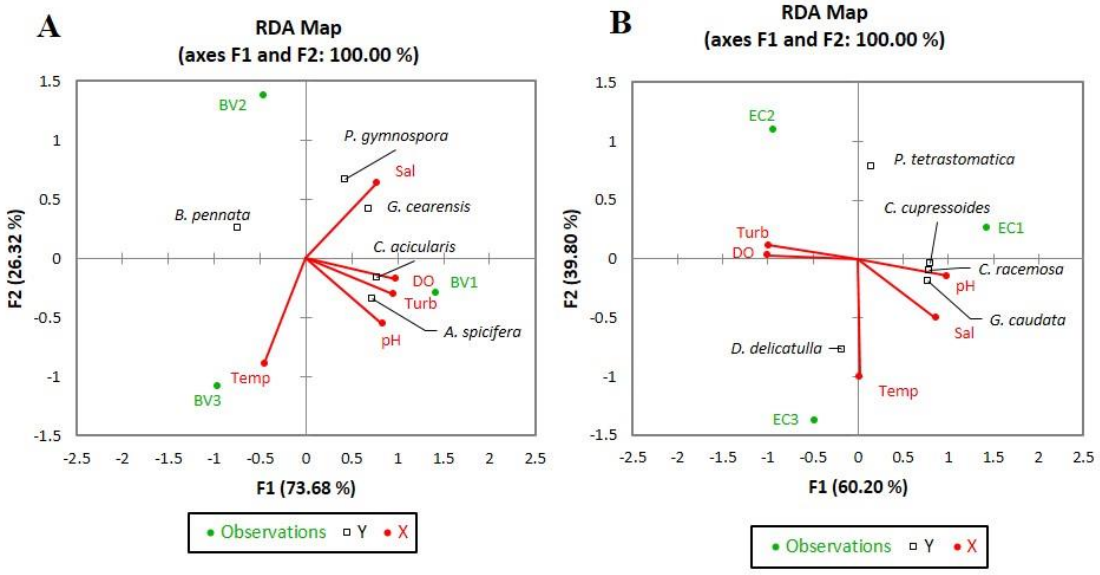


Figure 2 - Redundancy analysis for the influence of abiotic parameters on the antioxidant activity of seaweed species collected from the reefs of (A) Boa Viagem beach and (B) Enseada dos Corais beach, in Pernambuco. Observations: replicates (n = 3) made in Boa Viagem (BV) and Enseada dos Corais (EC). Y: analyzed species. X: abiotic parameters [Temperature (Temp), salinity (Sal), pH, dissolved oxygen (DO) and turbidity (Turb)].

DISCUSSION

All sampled seaweeds showed antioxidant activity through the analyzed assays. Those seaweeds inhabit harsh environments in tropical regions and, in Pernambuco, the reefs have extremely stressful conditions. When tide level lowers, seaweeds get exposed to air and direct UV radiation, leading to desiccation. When tide level rises, they must cope with osmotic variation, which leads to oxidative stress (Burritt *et al*., 2002; Collén and Davison, 1999a,b; Contreras *et al*., 2005; 2009; Kumar *et al*., 2010,2011). This condition of desiccation and osmotic variation may be the main abiotic factor regulating the production of antioxidants on seaweeds.

The analyzed seaweeds were sampled at the intertidal zone of the reefs during low spring tides, so, it´s possible that their high antioxidant activity is related to the desiccation stress. Among them, the brown species showed higher potential. Screening 30 Hawaiian seaweed species, Kelman *et al*. (2012) found *Turbinaria ornata* (Turner) J. Agardh, a brown alga, to be

the most active, and identified fucoxanthin as the major bioactive antioxidant. Bioprospecting studies that test several seaweeds usually find that brown species tend to have higher antioxidant potential (Fujimoto and Kaneda, 1980; 1984; Kelman *et al*., 2012; Le Tutour, 1990; Matsukawa *et al*., 1997). Fujimoto and Kaneda (1984) studied the antioxidant potential of 36 seaweeds and confirmed that brown species had higher antioxidant activity. Le Tutour *et al*. (1990) analyzed the polar extracts of seven seaweeds from the French coast and obtained similar results.

Zubia *et al*. (2007) found antioxidant potential in 48 tropical seaweeds from Mexico, highlighting the brown species *Lobophora variegata* (J.V. Lamouroux) Womersley ex E.C. Oliveira as the most active, with very low oxidation index EC_{50} (0.32±0.01 mg mL^{-1}). We found even lower oxidation index EC_{50} (0.226 mg mL^{-1}) for *P. tetrastomatica*. Studying this same species, Vinayak *et al*. (2011) found high antioxidant activity and low cytotoxicity for its methanolic extracts, suggesting that *P. tetrastomatica* could be employed in functional food and cosmetic industries for preventing the oxidative stress.

The high activity of brown algae was positively correlated to phenolic contents (Connan *et al*., 2006; Pedersen, 1984). Jiménez-Escrig *et al.* (2001) showed that antioxidant activity measured by the DPPH assay was closely related to the phenolic content of brown algae. Studies also show that phenolic compounds may have anti-inflammatory, antitumor and antiviral activities, with positive effects on human health (Novoa *et al*. 2011; Thomas and Kim, 2011; Wijesingher and Jeon, 2012).

Green species analyzed also showed great antioxidant potential. *Caulerpa racemosa*, that had the higher activity, can be found at the top of the reefs, at extremely dry habitats, during low tides. It´s high antioxidant activity found here, and *e.g*. by Cavas and Yurdakoc (2005) and Li *et al.* (2012), may explain its capacity to occupy such harsh environments. In fact, *C. racemosa* is an invasive species in the Mediterranean (Chisholm *et al*., 2000; Verlaque *et al*.,

2003) due to its efficient chemical defense strategy, for the production of the phytotoxic caulerpenyne (Raniello *et al*., 2007), for instance.

The low antioxidant activity reported here by the red species it's not in accordance with the literature. Guaratini *et al*. (2012) found high antioxidant activity in carotenoids and fatty acids isolated from several *Gracilaria* species of Brazil. Great activity was also found for sulfated polysaccharides from *Gracilaria* spp. by Souza *et al*. (2012). The antioxidant activity registered for this genus is often related to water soluble compounds, like pigments and hydrocolloids. Nonetheless, the methodology we used favors finding relatively lipophilic compounds because of the very nonpolar solvent used. This could explain the low activity we found for red species, that are rich in those hydrophilic contents and that probably these components would be in low quantity in our extract due to the fact of being an extract with components mostly nonpolar.

This is the first study to comprehensively evaluate the antioxidant potential of tropical seaweeds from Pernambuco reefs. Even in low concentrations, we found that these seaweeds still showed antioxidant potential, featuring the brown alga *P. tetrastomatica* as a source of natural antioxidants that could be employed in cosmetic, nutraceutical, pharmacological and other industries.

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7 Antioxidant activity of three seaweeds from tropical reefs of Brazil: potential sources for bioprospecting

Abstract

We report the first screening for antioxidant activity (AOX) of three dominant seaweeds from low intertidal reefs of Brazil. *Sargassum furcatum, Bryothamnion triquetrum* and *Osmundaria obtusiloba* were extracted with dichloromethane and methanol (2:1), and AOX was measured by five UV-Vis microplate spectrophotometric methods: DPPH (2,2-diphenyl-1-picryhydrazyl) and ABTS (2,2–azinobis (3-ethylbenzthiazoline-6-sulfonic acid)) free radical scavenging, metal chelating capacity, total phenolic compounds using reducing power by Folin-Ciocalteu and FRAP (ferric reducing antioxidant power). All studied species showed high AOX, even at very low extract concentrations. We can attribute these results to the stressful environment in which these seaweeds live, high hydrodynamics, air exposure during low spring tides, intense solar radiation and other factors that may contribute to oxidative stress, featuring *O. obtusiloba* as a new source of natural antioxidants.

Keywords: antioxidant assays, fatty acids, oxidative stress, tropical reefs, *Osmundaria*.

Resumo

Reportamos o primeiro *screening* da atividade antioxidante (AOX) de três macroalgas dominantes do intermareal inferior de recifes do Brasil. *Sargassum furcatum, Bryothamnion triquetrum* e *Osmundaria obtusiloba* foram extraídas com diclorometano e metanol (2:1), e a AOX foi medida por cinco métodos espectrofotométricos em microplacas de UV-vis: sequestro dos radicais livres DPPH ((2,2-diphenil-1-picrihidrazil) e ABTS (2,2–azinobis (3-ethilbenzthiazoline-6-ácido sulfônico)), capacidade quelante de metais, poder de redução de compostos fenólicos totais usando Folin-Ciocalteu e FRAP (poder antioxidante de redução do ferro). Todas as espécies estudadas apresentaram elevada AOX, mesmo em concentrações de extrato muito baixas. Podemos então atribuir estes resultados ao ambiente estressante no qual estas algas vivem, com elevado hidrodinamismo, exposição ao ar durante as marés baixas, radiação solar intensa e outros fatores que podem contribuir para o estresse oxidativo, evidenciando *O. obtusiloba* como nova fonte de antioxidantes naturais.

Palavras-chave: Ensaios antioxidantes, estresse oxidativo, recifes tropicais, *Osmundaria*.

INTRODUCTION

In all living organisms, reactive oxygen species (ROS) and nitrogen reactive species (NRS) are normally produced with important roles in cellular signaling and homeostasis. Under stressing conditions (e.g., UV radiation, heat exposure, grazing pressure, wounding) the levels of reactive species can increase dramatically, acting as cascade triggers for defense systems which under extreme situations can cause irreversible oxidative damage to all cellular components, including proteins, amino acids, lipids and nucleic acids (Murphy 1983; Vass 1997; Bischof et al. 2006; Karsten 2008). Thus, oxidative stress has been associated with several diseases, such as cancer, hypertension, diabetes, atherosclerosis, and neurological and inflammatory disorders (Reaven and Witzum 1996; Aruoma 1999; O' Sullivan et al. 2011; Tierney et al. 2013; Boisvert et al. 2015; Paloczi et al. 2017; Poprac et al. 2017) and aging processes (Kohen and Nyska 2002; Höhn et al. 2017; Zhang et al. 2017).

In addition to damaging cellular components, ROS can also degrade oils and fats in foods, which causes odors and rancid flavor, decreasing food quality and nutritional value (Ngo et al. 2012; Tierney et al. 2013; Jacobsen and Sørensen 2015). To increase shelf life and prevent food from oxidation, synthetic antioxidants, such as BHA (butylated hydroxyanisol) and BHT (butylated hydroxytoluene), are used as food additives (O'Sullivan et al. 2011). Notwithstanding, collateral effects and carcinogenic outcomes have been reported in the literature (Ito et al. 1986; Nakagawa et al. 1994; Altuntaş and Değer 2017; Laganà et al. 2017). Consequently, alternatives to artificial antioxidants are increasingly demanded. Accordingly, more and more species of seaweeds have been studied as promising sources of healthy and bioactive compounds.

Seaweeds are commonly consumed in Asian countries in the form of roll sushi, snacks, spices, condiments, pasta, soups and other dishes, and they are also employed in the phycocolloid industry as food additives (e.g., agar, carrageenan, and alginate) (Lahaye 2001;

Rioux and Turgeon 2015). Recently, the market for seaweeds has considerably expanded into the pharmaceutical and para-pharmaceutical sectors, owing to their exceptional richness in bioactive compounds, including antimicrobial, anti-inflammatory and antitumor properties (Smit 2004; Holdt and Kraan 2011; Centella et al. 2017). Furthermore, because of their low lipid content, high concentration in polysaccharides, natural minerals, polyunsaturated fatty acids, and vitamins, as well as richness in bioactive molecules, seaweeds are known to be a source of functional food (Smit 2004; Ganesan et al. 2008; Bajpai 2017; Díaz et al. 2017; Wells et al. 2017).

The absence of structural damage in seaweeds during their constant exposure to air-water intertidal habitat implies that these organisms are able to produce antioxidants to protect themselves against oxidation. They have developed a complex defense system against reactive species, producing pigments (Fujimoto and Kaneda 1980), enzymes (Ahn et al. 2004), mycosporine-like amino acids (Sinha et al. 2000; Torres et al. 2016), phenolic compounds (Matanjun et al. 2008) and other antioxidant substances (Rocha et al. 2007; Maschek and Baker 2008). Studies reporting the chemical defense of seaweeds suggest a wide variation in the production of substances associated with environmental factors as temperature (e.g., Abrahamsson et al. 2003), depth (e.g., Amade and Lemeé 1998), salinity (e.g., Schmidt et al. 2015), solar incidence (e.g., Bouzon et al. 2012), grazing pressure (e.g., Paul and Puglisi 2004; Amsler and Fairhead 2006), seasonality (e.g., Amade and Lemeé 1998; Maréchal at al. 2004) and geographic distribution (e.g., Le Lann et al. 2013). However, little is known about the ecological role of these substances.

Seaweeds inhabiting harsh environments, such as the tropical reefs, which are abundant in northeastern Brazil, can overcome excessive UV radiation and high temperature (Zubia et al. 2007). Several authors have demonstrated that tropical seaweeds show higher antioxidant substances than other species from increasing latitudes (Zubia et al. 2007; Kelman et al. 2012;

Li et al. 2012; Martins et al. 2012; Silva et al. 2012), enabling the study of tropical seaweeds for bioprospecting natural sources of bioactive compounds. Despite that, only a few studies on the antioxidant potential of tropical northeastern Brazilian seaweeds have been performed.

The Brazilian littoral has an extensive coastline with great biodiversity of marine algae still unexplored. The red seaweeds *Bryothamnion triquetrum* (S.G. Gmelin) and *Osmundaria obtusiloba* (C. Agardh) R.E. Norris and the brown seaweed *Sargassum furcatum* Kützing are abundant along the Pernambuco coast throughout the year and dominate the low intertidal reef zone. These species were chosen for the present study because they inhabit that portion of the reef which experiences higher wave action, and they are exposed to air during low tides, resulting in a combination of elevated hydrodynamics, air exposure and UV radiation incidence that could lead to the induction of oxidative stress. Therefore, this study aimed to evaluate the potential of these three species for further bioprospecting by assessing the antioxidant activity of their crude extracts.

MATERIALS AND METHODS

Sampling

In 2015, approximately 500 g of *S. furcatum*, *B. triquetrum* and *O. obtusiloba* were sampled at low intertidal zones of the reefs of Enseada dos Corais Beach (8°18'44.7"S; 34°56'49.8"W), at Pernambuco cost in northeastern Brazil. This region is located in the biogeographic province of Southwestern Tropical Atlantic (Spalding et al. 2007). Local climate is tropical humid with average temperatures ranging from 25°C to 30°C, and semidiurnal meso tides dominated by waves (Amaral et al. 2016).

Seaweeds were immediately frozen at -20ºC and after defrosting, epiphytes were removed from seaweed material which was further washed to remove salt, sand and associated fauna. They were dried over absorbent paper at room temperature for about 72 h.

Preparation of crude extracts

Dried seaweeds were ground to a powder, and a mixture of dichloromethane and methanol (2:1 v/v), enough to cover the amount of algae, was added, sealed and kept at room temperature for a three-day extraction process. After that period, the solvent was filtered, and more solvent was added to the remaining seaweed. This procedure was repeated three times over the course of nine days. All extracts were filtered, and the extracts from the same species were gathered as a single extract and then evaporated in a rotavapor (R-215 Buchi, Switzerland), on water bath (40ºC), with pressures between 70 and 500 torr (Vacuubrand CVC 3000). The final extracts were removed from the rotary flask by acetone dilution, placed in penicillin bottles, and left to completely evaporate in an exhaustion hood. Extract yield was calculated according to the following formula: yield (%) = (extract weight x 100)/dried seaweed weight. For antioxidant analyses, the crude algal extracts were dissolved in methanol at a concentration of 3 mg ml^{-1}.

Antioxidant assays

The antioxidant activities of extracts from the three studied species were comprehensively characterized by five complementary biochemical methods to accurately determine differences AOX, given the variable responses of organisms to oxidative stress (Frankel and Meyer 2000; Huang et al. 2005). The mechanism responsible for AOX is different

for each method used. Therefore, different antioxidant assays and extract concentrations (0.025, 0.0375, 0.05, 0.075, 0.1, 0.15, 0.2, 0.4, 0.6 and 1 mg ml^{-1}) were analyzed, according to the responses of each species to oxidative stress, that is why concentrations were different among species. The EC_{50} values were calculated using GraphPad Prism, v 7.03, for each species with different extract concentrations and for each method that reached 50% of AOX.

All antioxidant assays were performed in triplicate, and the absorbance was read with a UV-vis (Ultraviolet-visible) microplate spectrophotometer (Epoch Biotek, USA). Results are expressed as AOX percentage and gallic acid equivalent (µg ml^{-1} GAE) calculated from the standard curve. The commercial pattern used on all assays was gallic acid (Sigma-Aldrich, Brazil).

DPPH radical scavenging activity

The DPPH (2,2-diphenyl-1-picryhydrazyl) antioxidant assay was performed according to Brand-Williams et al. (1995), as modified by Pires et al. (2017a). The method is based on DPPH radical capture by antioxidants, producing an absorbance decrease at 517 nm. Aliquots of 20 µl of extracts, or standard, (dissolved in methanol) were added to 280 µl of DPPH (32 µg ml^{-1}) (Sigma-Aldrich, Brazil) solution. After standing for 30 minutes in the dark at room temperature, the absorbance was read at 517 nm. The inhibition percentage was calculated through the following equation: % AOX DPPH = $[(Abs_{DPPH} - Abs_{sample})/Abs_{DPPH}] \times 100$, where Abs_{DPPH} is the control absorbance of DPPH, and Abs_{sample} is the absorbance of sample in DPPH reactive solution.

ABTS radical scavenging activity

The antioxidant assay by ABTS (2,2–azinobis (3-ethylbenzthiazoline-6-sulfonic acid) free radical scavenging is one of the most used and allows us to measure the antioxidant potential of hydrophilic and lipophilic substances (Rufino et al. 2007). The method used was described by Rufino et al. (2007) and modified by Torres et al. (2017). Aliquots of 20 µl of extract, or standard, were added to 280 µl of ABTS radical. Absorbance was read at 734 nm after 20 minutes of incubation in the dark. ABTS radical scavenging percentage was calculated by the following formula: % AOX ABTS = $[(Abs_{ABTS} - Abs_{sample})/Abs_{ABTS}] \times 100$, where Abs_{ABTS} is the ABTS's control absorbance of ABTS, and Abs_{sample} is absorbance of the sample in ABTS reactive solution.

Metal chelating activity (MCA)

The MCA method, as described by Dinis et al. (1994) and modified by Harb et al. (2016), determines the quantity of non-chelated iron ions in the reaction mixture remaining after chelation. Solutions were composed of 250 µl of acetate 10%, 15 µl of ammonium sulfate, and 20 µl of extract, with standard or methanol as blank. After 15 minutes, 15 µl of ferrozine solution were added. The microplates were homogenized and incubated in the dark for 10 minutes, followed by shaking at 100 rpm for further reading at 562 nm. MCA percentage was calculated using the following formula: % AOX MCA = $[(Abs_{MCA} - Abs_{sample})/Abs_{MCA}] \times 100$, where Abs_{MCA} is the absorbance of acetate, ammonium sulfate, methanol and ferrozine reaction mixture, and Abs_{sample} is the absorbance of all reaction reagents with the sample.

Folin-Ciocalteu assay

This assay was described by Singleton and Rossi (1965) and Waterman and Mole (1994) and then modified by Pires et al. (2017b). It is based on the reductive property of total phenolic compounds (TPC) that react with Folin-Ciocalteu reagent under alkaline conditions. The reaction mixture was composed of 200 µl of ultrapure water, 20 µl of sample, with standard or methanol as blank, 20 µl of Folin-Ciocalteu (1 N) reagent and 60 µl of Na_2CO_3 (10 mg ml^{-1} of ultrapure water), followed by incubation in the dark for 30 minutes and subsequent reading at 760 nm. AOX percentage was calculated as follows: % AOX Folin-Ciocalteu = (Abs_{sample} x 100)/$Abs_{Folin\text{-}Ciocalteu,}$ where Abs_{sample} is the absorbance of sample in the reaction mixture, and $Abs_{Folin\text{-}Ciocalteu}$ is the maximum absorbance obtained by gallic acid standard, indicating the maximum AOX.

Ferric reducing antioxidant power (FRAP)

The ability of seaweed to act as a reducer of substances was tested through the FRAP assay, as described by Benzie and Strain (1996) and modified by Urrea-Victoria et al. (2016). Change of absorbance is directly related to the reducing power of the electron donors present in the reaction mixture (Ferreira et al. 2007). The microplates were assembled in the dark, mixing 15 µl of ultrapure water with 20 µl of sample, with standard or methanol as blank, and 265 µl of FRAP reagent (25 ml of buffer acetate 0.3 M, 2.5 ml of ferric tripyridyl hydrazine solution 10 mM and 2.5 ml of a ferric chloride aqueous solution 20 mM; Sigma-Aldrich, Brazil). Absorbance was read after 30 minutes of incubation at 595 nm. AOX percentage was calculated as % AOX FRAP = (Abs_{sample} x 100)/Abs_{FRAP}, where Abs_{sample} is absorbance of the

sample in the reaction mixture, and Abs_{FRAP} is the maximum absorbance obtained by gallic acid standard, indicating the maximum AOX.

UV-vis absorption spectrum

In order to evaluate the general absorbance spectrum of the samples and recognize UV-vis absorbing compounds, an aliquot of 100 µl of each sample at the concentration of 100 µg ml^{-1} was read by using a microplate UV-vis spectrophotometer (Epoch Biotek, USA) at wavelengths from 200 to 750 nm. The area under each band was calculated based on the trapezium estimate method.

Statistical analyses

The AOX percentage and EC_{50} values obtained for DPPH, ABTS, MCA, Folin-Ciocalteu and FRAP assays with different extract concentrations were compared with one-way analysis of variance (ANOVA). Significant differences were determined by Tukey's post-hoc test at 95% significance level ($p < 0.05$), comparing different extract concentrations tested for each method and between species. The results were expressed as mean ± standard deviation (n = 3). All analyses were performed with Statistica, v 10.

RESULTS

All seaweed species presented AOX at the five spectrophotometric methods. Using the DPPH assay, the AOX generally increased with extract concentration for all species (Fig. 1). The highest AOX (137.58 ± 0.95% and 5.77 ± 0.04 µg ml^{-1} of GAE) was recorded in *Sargassum*

furcatum at the highest extract concentration (1 mg ml^{-1}), and it was statistically different from other species and concentrations ($p < 0.05$, Tukey's test). *Osmundaria obtusiloba* had high AOX (111.82 ± 1.74% and 2.02 ± 0.01 µg ml^{-1} GAE), with 80% less extract than *S. furcatum*. *Bryothamnion triquetrum* showed a maximum AOX of 87.81 ± 0.65% and 3.63 ± 0.02 µg ml^{-1} GAE at the highest concentration tested (1 mg ml^{-1}), and it had lower AOX than *S. furcatum* and *O. obtusiloba* (Fig. 1).

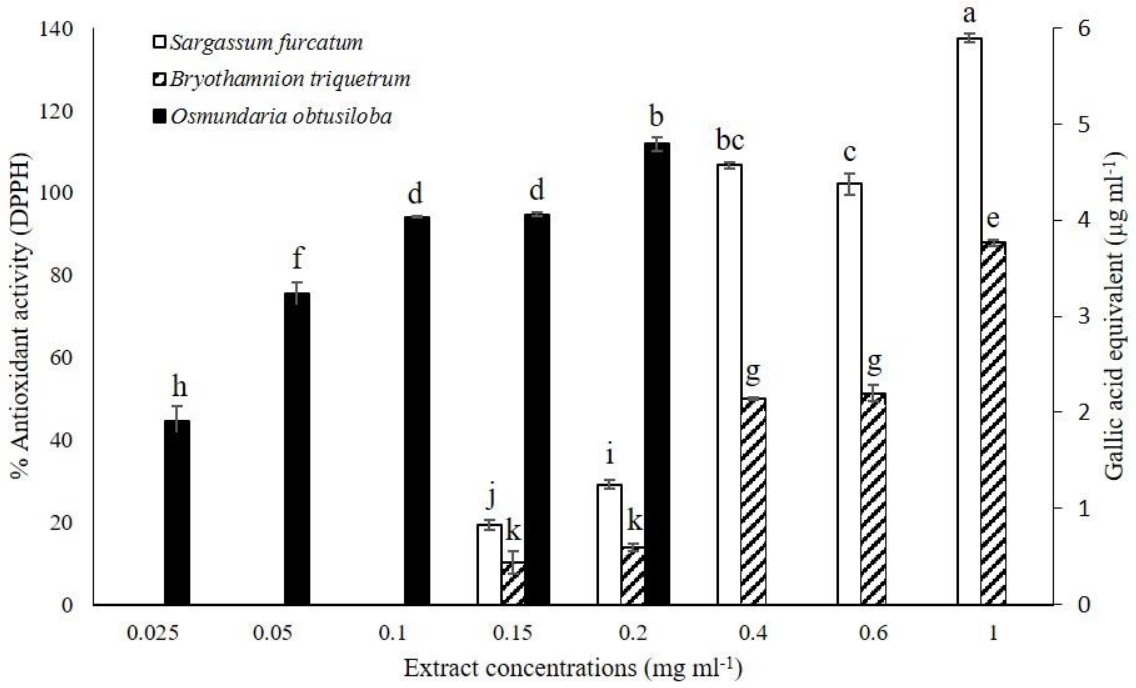

Fig. 1 - Antioxidant and gallic acid equivalent activities through DPPH assay of the three tropical seaweeds from Pernambuco reefs tested under different extract concentrations (mg ml^{-1}). Results are presented as mean ± standard deviation (n = 3). Different letters represent significant differences ($p < 0.05$) among concentrations and species

Results of AOX for the ABTS radical scavenging assay are presented in Fig. 2. *Osmundaria obtusiloba* had the highest AOX, with 101.33 ± 0.2% (2.25 ± 0.00 µg ml^{-1} GAE) and 92.46 ± 0.2% (2.03 ± 0.06 µg ml^{-1} GAE) of AOX at concentrations of 0.1 and 0.05 µg ml^{-1}, respectively, showing statistical difference from the other species ($p < 0.05$, Tukey's test). The lowest concentration tested for this species, 0.025 mg ml^{-1}, showed higher AOX than the lowest concentration tested (0.2 mg ml^{-1}) for *S. furcatum* and *B. triquetrum* (Fig. 2). *Sargassum*

furcatum and *B. triquetrum* had approximately 30% less AOX than *O. obtusiloba* at their highest tested concentration (0.6 mg ml^{-1}), which was six times higher than the highest concentration tested for *O. obtusiloba*.

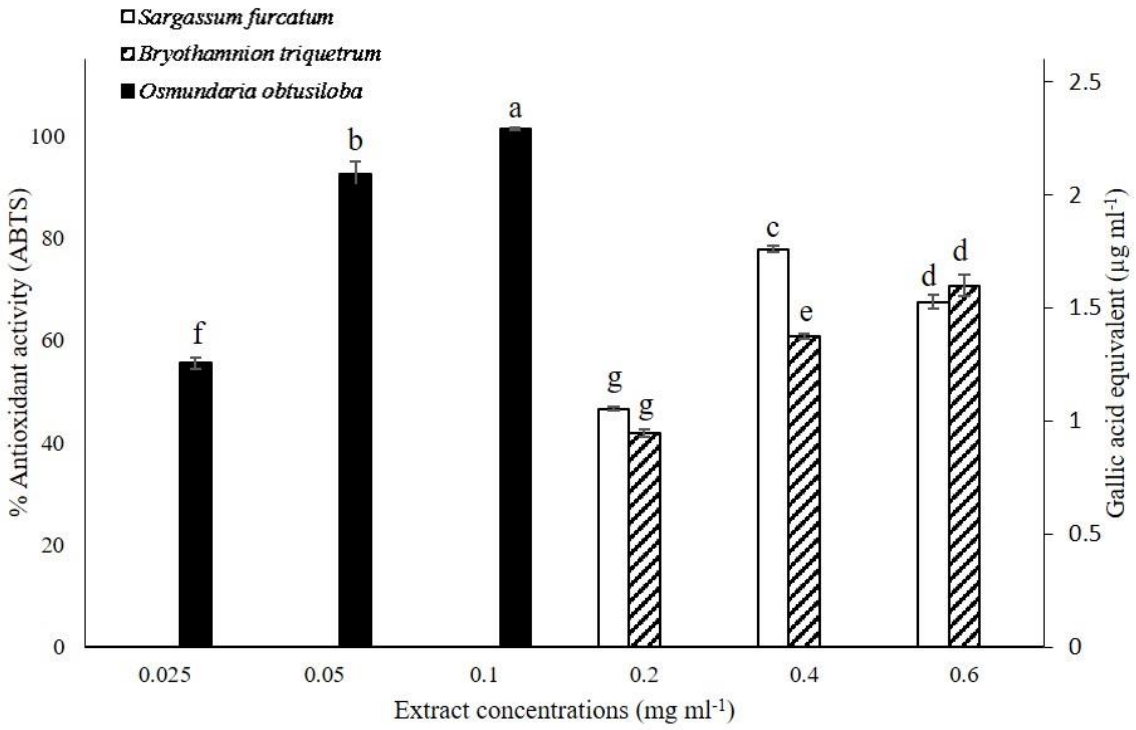

Fig. 2 - Antioxidant and gallic acid equivalent activities through ABTS assay of the three tropical seaweeds from Pernambuco reefs tested under different extract concentrations (mg ml^{-1}). Results are presented as mean ± standard deviation (n = 3). Different letters represent significant differences ($p < 0.05$) among concentrations and species

For MCA (Fig. 3), at the highest concentration tested, all species had similar AOX (around 60% and 8 µg ml^{-1} GAE), with no statistical differences ($p > 0.05$, Tukey's test), although *O. obtusiloba* reached that value with 6 times less extract concentration than the other two species.

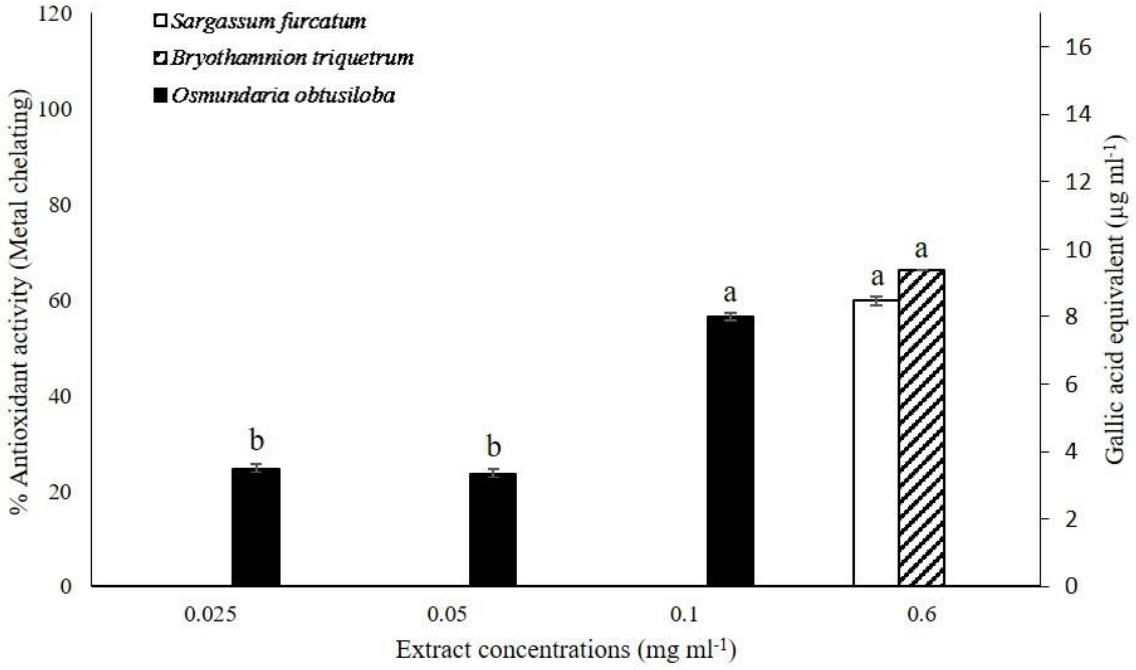

Fig. 3 - Antioxidant and gallic acid equivalent activities through metal chelating assay of the three tropical seaweeds from Pernambuco reefs tested under different extract concentrations (mg ml^{-1}). Results are presented as mean ± standard deviation (n = 3). Different letters represent significant differences ($p < 0.05$) among concentrations and species

Maximum AOX was recorded at Folin-Ciocalteu assay in *O. obtusiloba* (128.15 ± 4.39% and 15.74 ± 0.08 µg ml^{-1} GAE) at the highest concentration tested (0.2 mg ml^{-1}) (Fig. 4). Based on its high AOX values at concentrations of 0.15 and 0.2 $mg.ml^{-1}$, *O. obtusiloba* statistically differed ($p < 0.05$, Tukey's test) from the other two species at the same concentrations. *Bryothamnion triquetrum* showed a maximum AOX of 50.29 ± 4.50% (6.40 ± 0.57 of µg ml^{-1} GAE) at the highest concentration tested (0.4 mg ml^{-1}), while *S. furcatum* reached maximum AOX of 36.34 ± 5.80% (4.09 ± 0.74 µg ml^{-1} GAE) at the concentration of 0.2 mg ml^{-1}.

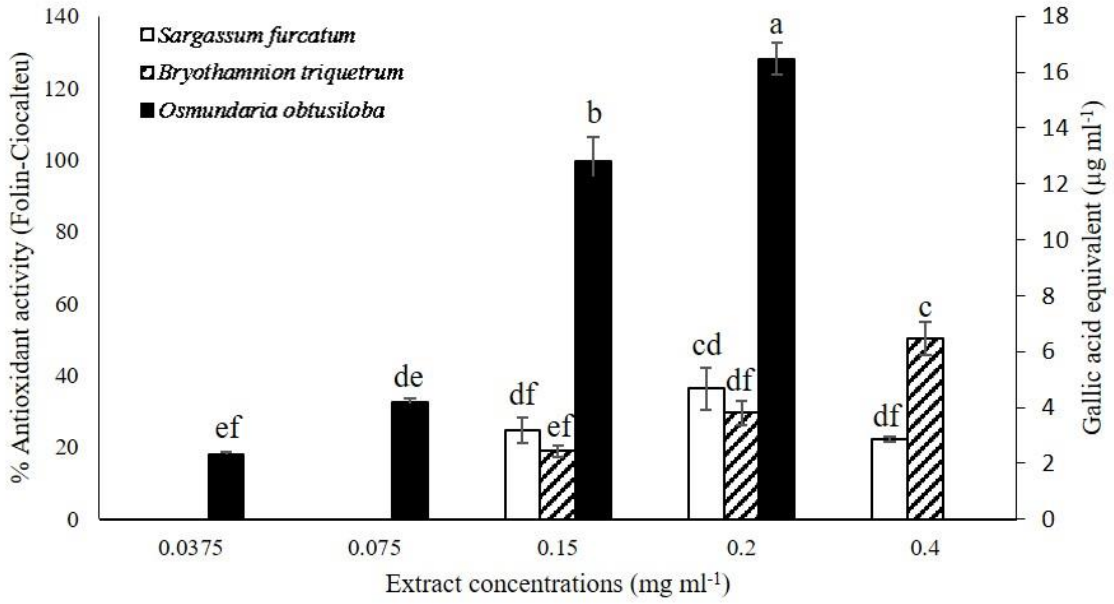

Fig. 4 - Antioxidant and gallic acid equivalent activities through Folin-Ciocalteu assay of the three tropical seaweeds from Pernambuco reefs tested under different extract concentrations (mg ml^{-1}). Results are presented as mean ± standard deviation (n = 3). Different letters represent significant differences ($p < 0.05$) among concentrations and species

The Folin-Ciocalteu method measures AOX though the quantification of TPC in the extract. The results of the three species studied and a summary of similar species are shown in Table 1 for comparison. As also shown in Table 1, *O. obtusiloba* had the highest phenolic content at extract concentration of 0.15 mg ml^{-1}. The maximum phenolic content for *S. furcatum* was obtained at 0.2 mg ml^{-1} extract concentration and for *B. triquetrum* at 0.4 mg ml^{-1} extract concentration.

Table 1 - Total phenolic contents using Folin-Ciocalteu method compared to results obtained by other authors for some tropical seaweed species. Data are expressed as gallic acid equivalent (mg GAE g^{-1})

Species	Algae extract concentration (mg ml^{-1})	Total phenolic compounds (mg GAE g^{-1})	Site	Reference
Sargassum furcatum	0.1500	17.42 ± 2.99	Brazil	Present study
Sargassum furcatum	0.2000	20.46 ± 3.73	Brazil	Present study
Sargassum furcatum	0.4000	7.07 ± 0.23	Brazil	Present study
Sargassum vulgare	1.0000	252.65 ± 30.18	Brazil	Martins et al. (2012)
Sargassum variable	-	14.85 ± 0.09	India	Vinayak et al. (2011)
Sargassum polycystum	-	45.16 ± 3.01	India	Matanjun et al. (2008)
Bryothamnion triquetrum	0.1500	12.43 ± 1.38	Brazil	Present study
Bryothamnion triquetrum	0.2000	12.09 ± 3.73	Brazil	Present study
Bryothamnion triquetrum	0.4000	16.01 ± 1.43	Brazil	Present study
Bryothamnion triquetrum	-	8.05	Cuba	Novoa et al. (2001)
Osmundaria obtusiloba	0.0375	61.42 ± 4.88	Brazil	Present study
Osmundaria obtusiloba	0.0750	57.67 ± 6.38	Brazil	Present study
Osmundaria obtusiloba	0.1500	81.41 ± 5.99	Brazil	Present study
Osmundaria obtusiloba	0.2000	78.71 ± 2.42	Brazil	Present study
*Osmundaria obtusiloba**	1.0000	569.33 ± 36.10	Brazil	Martins et al. (2012)
*Osmundaria obtusiloba**	1.0000	30.54	Brazil	Alencar et al. (2016)
Amansia sp.	1.0000	128.60 ± 10.50	Brazil	Martins et al. (2012)
Amansia multifida	-	45.40 ± 2.99	Brazil	Alencar et al. (2014)

- Data not shown

Using the FRAP assay (Fig. 5), the best result was obtained by *O. obtusiloba*, with values between 82.00 ± 1.55% (7.10 ± 0.16 µg ml^{-1} GAE) and 94.35 ± 1.32% (8.38 ± 0.13 µg ml^{-1} GAE) of AOX for 0.15 and 0.2 mg ml^{-1} extract concentrations, respectively. At 0.15 mg ml^{-1} extract concentration, *B. triquetrum* and *S. furcatum* obtained low values of AOX with 11.67 ± 1.07% and 21.58 ± 1.60%, respectively. At 0.2 mg ml^{-1} extract concentration, these last two species presented similar AOX ($p > 0.05$, Tukey's test), approximately 35%.

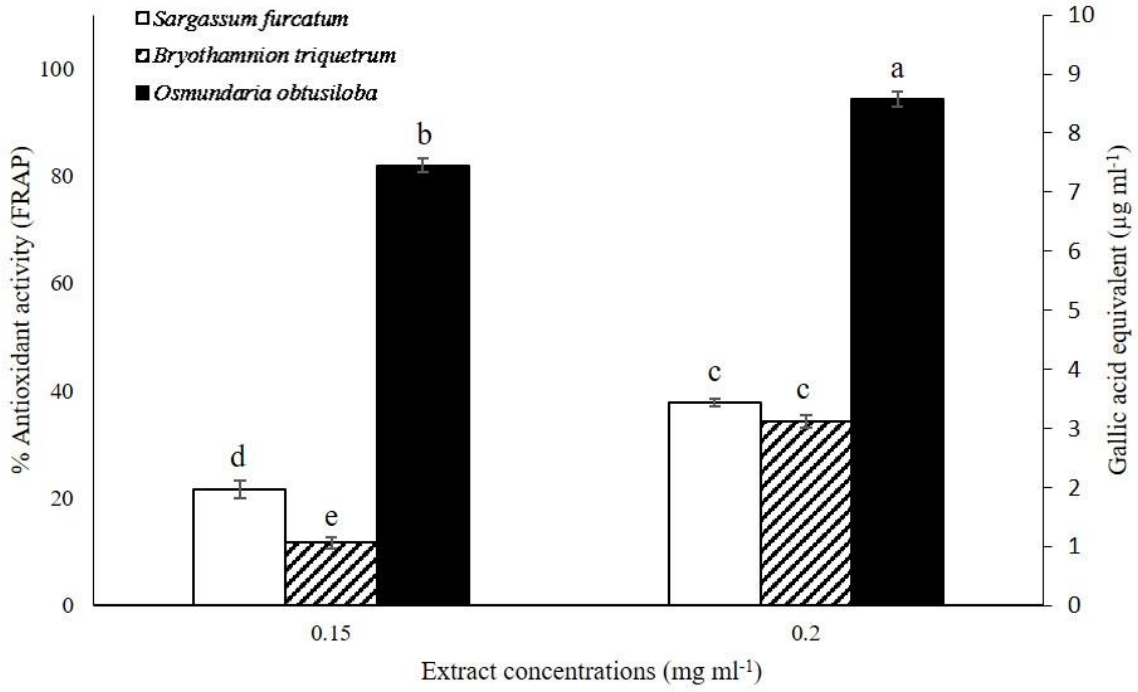

Fig. 5 - Antioxidant and gallic acid equivalent activities through FRAP assay of the three tropical seaweeds from Pernambuco reefs tested under different extract concentrations (mg ml^{-1}). Results are presented as mean ± standard deviation (n = 3). Different letters represent significant differences ($p < 0.05$) among concentrations and species

The EC_{50} values were also calculated and presented in Table 2. *Osmundaria obtusiloba* presented the lowest EC_{50} values when compared to the other two species for DPPH and ABTS assays, with statistical differences among them ($p < 0.05$). Based on its low EC_{50} values, *O. obtusiloba* showed very strong AOX. For both DPPH and ABTS assays, the EC_{50} values for *S. furcatum* and *B. triquetrum* were approximately 10 times higher than that of *O. obtusiloba*, meaning that those species require 10 times more seaweed extract to reach the antioxidant potential of *O. obtusiloba*. Similarly, EC_{50} values for the gallic acid standard were approximately 20 to 30 times lower than those for *O. obtusiloba* when tested by DPPH and ABTS assays, respectively.

Table 2 - Extract yield calculated for each species and EC_{50} values calculated for antioxidant activity by the DPPH, ABTS, metal chelating, Folin-Ciocalteu and FRAP assays. EC_{50} values are presented as mean ± standard deviation of extract concentration (mg ml^{-1})

		EC_{50} (mg ml^{-1})				
	Extract yield (%)	DPPH	ABTS	Metal chelating	Folin-Ciocalteu	FRAP
Sargassum furcatum	1.71	0.461 ± 0.006	0.266 ± 0.009	*	**	**
Bryothamnion triquetrum	2.08	0.357 ± 0.008	0.370 ± 0.034	*	**	**
Osmundaria obtusiloba	2.29	0.041 ± 0.003	0.031 ± 0.000	0.100 ± 0.028	0.128 ± 0.010	0.106 ± 0.010
Gallic acid	-	0.002 ± 0.000	0.001 ± 0.000	0.006 ± 0.000	0.005 ± 0.000	0.003 ± 0.000

*Species tested with only one concentration of crude extract (0.6 mg ml^{-1}); not able to calculate EC_{50}
**Extracts did not reach 50% of activity; not able to calculate EC_{50}

For the MCA, it was not possible to calculate the EC_{50} for *B. triquetrum* and *S. furcatum* because they were tested in a single extract concentration (0.6 mg ml^{-1}). *Osmundaria obtusiloba* had 16 times higher EC_{50} values when compared to the gallic acid standard.

As a consequence of the low AOX results, it was not possible to calculate EC_{50} values for *B. triquetrum* and *S. furcatum* tested by both Folin-Ciocalteu and FRAP assays. When compared with the gallic acid standard, *O. obtusiloba* showed EC_{50} values 25 and 35 times higher in the Folin-Ciocalteu and FRAP assays, respectively.

The absorption spectrum are presented in Fig. 6A-B for the three studied species. Fig. 6C shows the area under the curve calculated for each wavelength band identified as maximal peaks.

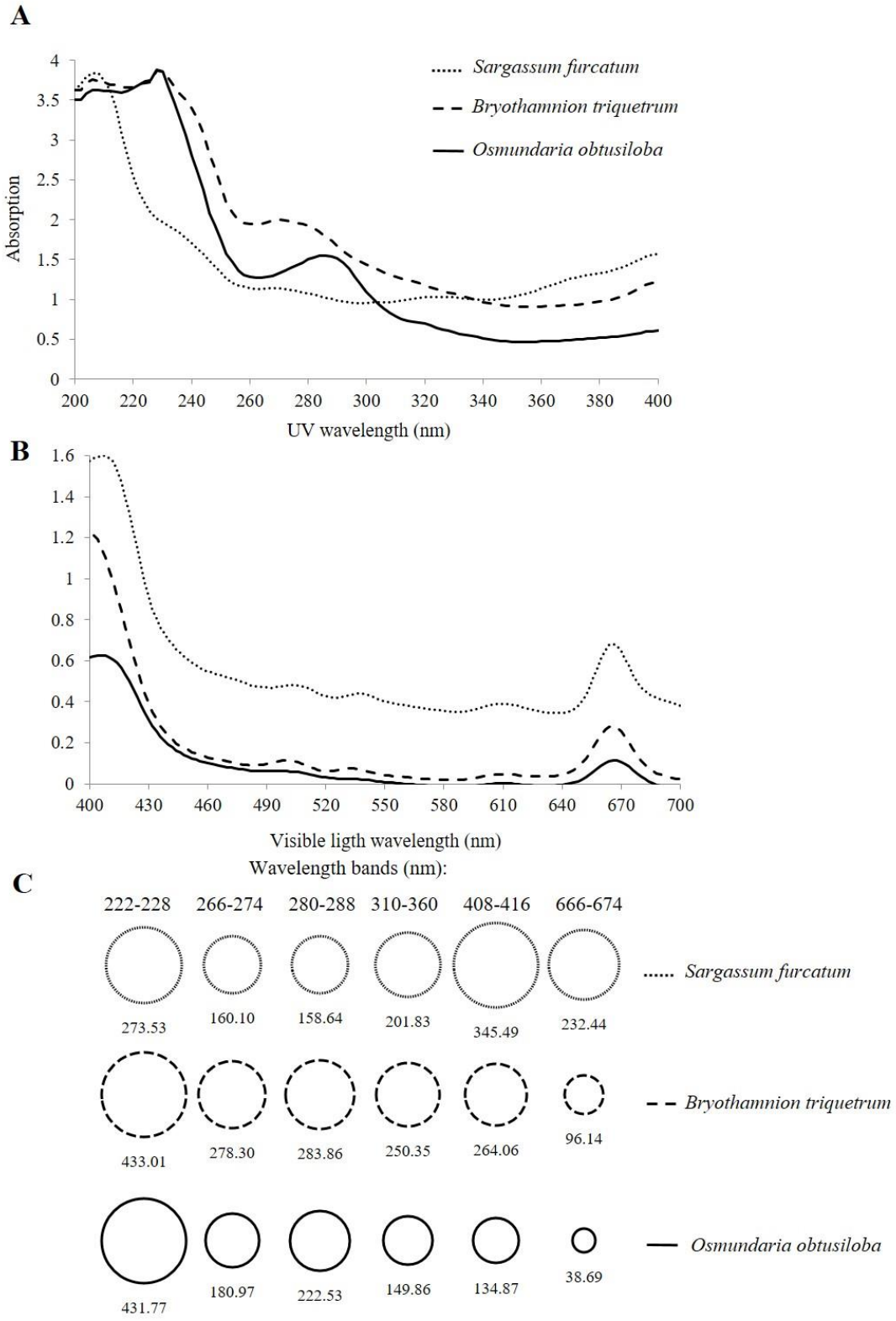

Fig. 6 - Absorption spectrum and area under the curve of the main wavelength bands of tropical seaweeds from Pernambuco reefs at wavelengths from 200 to 400 nm (UV spectrum) (A) and from 400 to 700 nm (visible light spectrum) (B), and the area under the curve for specific wavelength bands (C)

The UV spectrum showed different profiles for the three studied species (Fig. 6A), with six wavelength bands identified. The first band (222 – 228 nm) could indicate the presence of acetogenins in the seaweed extracts, with larger amounts found for the red species *B. triquetrum* and *O. obtusiloba* (Fig. 6C). The following band (266 – 274 nm), at smaller magnitude for the three species, may indicate the involvement of phenolic compounds in the extracts. We could also identify another band in the extract profiles, between 280 and 288 nm, likely indicating the presence of proteins. Mycosporine-like amino acids (MAAs) have absorption bands between 310 and 360 nm, which, although present in the extracts, did not show prominent peaks.

The visible light spectrum (400 to 700 nm), as presented in Fig. 6B, shows two major bands (408 – 416 nm and 666 – 674 nm) that are representative of chlorophyll-*a* in the extracts. The absorption profile for the three species was similar, varying only in quantitative magnitude. *Sargassum furcatum* showed a greater amount of chlorophyll-a compared to either *B. triquetrum* or *O. obtusiloba* at both bands (Fig. 6C). Carotenoids have absorption bands around 442 and 472 nm, which, although present in the extracts, did not show prominent peaks.

DISCUSSION

All species showed AOX activity. The analyzed seaweeds dominate reef environments with high hydrodynamics, air exposure during low spring tides, intense UV radiation and other factors that may contribute to oxidative stress. These environmental features could explain why all studied species showed high AOX at very low extract concentrations.

Using the DPPH assay, *S. furcatum* had greater than 100% of AOX at the highest concentration tested. This high antioxidant potential for *S. furcatum* may be related to the presence of phlorotannins, a class of phenolic compounds that absorb UV radiation and behave like antioxidants and mainly found in brown seaweeds (Ragan and Glombitza 1986; Pavia et

al. 1997; Amsler and Fairhead 2006). Studies also show that phenolic compounds may have anti-inflammatory, antitumor and antiviral activities, with positive effects on human health (Novoa et al. 2011; Thomas and Kim 2011; Wijesingher and Jeon 2012).

Bioprospecting studies that test several seaweeds usually find that brown species tend to have higher antioxidant potential (Fujimoto and Kaneda 1980; 1984; Le Tutour 1990; Matsukawa et al. 1997; Kelman et al. 2012). Fujimoto and Kaneda (1984) studied the antioxidant potential of 36 seaweeds and confirmed that brown species had higher AOX. Le Tutour et al. (1990) analyzed the polar extracts of seven seaweeds from the French coast and obtained similar results. Recently, Kelman et al. (2012) performed an AOX screening for 30 seaweed species from Hawaii, and, once again, a brown species (*Turbinaria ornata*) had the best result through FRAP assay [10.27 ± 0.40 μM μg^{-1}].

In Phaeophyceae the highest phlorotannin levels are found in Fucales (e.g., *Sargassum*) and Dictyotales, with 20 and 30% dry weight, respectively (Ragan and Glombitza 1986; Targett et al. 1995). Although *S. furcatum* is a brown seaweed species, it presented lower phenolic content than that reported by Martins et al. (2012) for *S. vulgare* and by Matanjun et al. (2008) for *S. polycystum* (Table 1). These results evidence the variability of antioxidant potential among species of the same genus. Besides abiotic factors, algal phenolic levels are also influenced by herbivory (Pavia and Toth 2000) and inter-individual as well as intra-individual variation (Ilvessalo and Tuomi 1989). Thallus size (Denton et al. 1990), stage in life cycle (Van Alstyne et al. 2001), type (Targett and Arnold 1998), age (Pedersen 1984) and reproductive status (Ragan and Jensen 1978) also play important roles in the accumulation of phlorotannins.

Sargassum furcatum was not the species with the highest AOX; nor did it have the highest content of TPC among the three species tested. This might be explained by the extraction method used. Most authors have utilized polar solvent (e.g., methanol) (Matanjun et al. 2008; Vinayak et al. 2011) and we used a mixture of dichloromethane and methanol, making

the extracts less polar. That difference in polarity may result in the extraction of distinct molecules, which could explain the lower potential of *S. furcatum*. Indeed, *O. obtusiloba*, a red seaweed species, had the highest levels of TPC and the highest AOX. The positive correlation between AOX and TPC means that the phenolic contents may be an important antioxidant defense mechanism in these seaweeds. However, we could not assert that the AOX of this species is completely related to the presence of phenolic substances since a complementary antioxidant could be present. For red seaweeds, other bioactive substances, different than phenolic compounds, can also be important as efficient scavengers of free radicals, such as vitamin E, provitamin A, carotenoids and sulfated polysaccharides (Pires et al. 2008, Sousa et al. 2008, Pires-Cavalcante et al. 2011).

Martins et al. (2012) found higher AOX for red seaweeds (e.g., 80% of AOX at 1 mg ml^{-1} extract concentration of *Cryptonemia seminervis*) and higher TPC (see Table 2) compared to brown species. They also found 64% of AOX (0.1 mg ml^{-1} extract concentration) for *O. obtusiloba* through DPPH assay, which was lower than our result for the same species. Recently, Alencar et al. (2016) found 99.47±0.22% of AOX through DPPH assay for *O. obtusiloba* collected in northeastern Brazil; however, they used a 1 mg ml^{-1} extract concentration to reach that value, while we obtained over 100% of AOX with 5 times less extract concentration. That could be explained by the nonpolar characteristic of our extracts, which may have resulted in the extraction of such nonpolar compounds as glycolipids, phospholipids, steroids, terpenes, fatty acids, carotenoids and tocopherols (Sousa et al. 2008; Guaratini et al. 2012; Alencar et al. 2016) responsible for the elevated AOX.

The UV-vis absorption spectrum, while basic, may be useful in bioprospecting studies because it provides a general profile of the presence of potential chemical classes in the seaweed extracts, which helps in the search for bioactive compounds. Besides, it is possible to relate the antioxidant potential obtained by the studied seaweeds according to the class of bioactive

compounds determined by UV-vis absorption spectrum, even if such determination is preliminary.

Osmundaria and *Amansia* belong to the Amansiae tribe (Rhodomelaceae), and the high AOX found for these seaweeds in our study and by Martins et al. (2012), respectively, may be related to similar compounds, such as MAAs. Another red seaweed species tested was *Bryothamnion triquetrum*, which also had UV absorption bands characteristic of MAAs. Among the algal groups, MAAs are mainly found in red algae (Carreto et al. 2005; Karsten 2008; Yuan et al. 2009).

Besides acting as sunscreens in aquatic organisms, it is suggested that MAAs may act as antioxidants (Dunlap and Yamamoto 1995; Andreguetti et al. 2003), protecting the cells against oxidative damage caused by reactive species. A great number of skin care products with MAAs are being tested on the basis of their efficiency as a natural UV blocker, and they are extensively used in sunscreen formulas (Cardozo et al. 2007).

Carotenoids represent another class of substances present in all species. These pigments are found in all algal groups and act both as light-harvesting complex and antioxidant to reduce oxidative stress caused by air and light exposure (von Elbe and Schwartz 1996; Olson 1999). The antioxidant properties of algal carotenoids play an important role in preventing human pathologies derived from oxidative stress (Maschek and Baker 2008).

Using all five assays, we also found that AOX was dose-dependent, meaning that under higher extract concentration, greater AOX activity was found. Ganesan et al. (2008) evaluated the potential of three red seaweeds and reported that the AOX of all species was dose-dependent, as it increased with the increasing extract concentration.

To sum up, this represents the first study of its kind to comprehensively evaluate the antioxidant potential of tropical seaweeds from Brazilian reefs. The absorption spectrum indicated different UV-vis absorbing compounds for the analyzed species, providing very

useful results from the perspective of bioprospecting, in particular by elucidating the chemical composition of the respective crude extracts. Even in low concentrations, we found that these seaweeds still showed antioxidant potential, featuring the red alga *O. obtusiloba* as a new source of natural antioxidants that could be employed in cosmetic, nutraceutical, pharmacological and other applications.

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8 Environmental stress tolerance and antioxidant response of the red seaweed *Palisada perforata* from a tropical reef

Abstract

We investigated the antioxidant activity of *Palisada perforata* collected in four different reef microhabitats and compared it with the antioxidant activity of this same species cultivated in laboratory under stressful conditions like those found in the reefs. We also measured algal photosynthetic performance through chlorophyll fluorescence parameters. *P. perforata* is more affected by desiccation stress than by temperature or salinity variations. This is in agreement with the observed at the reefs, were algae found growing at plateau, and thus, exposed to air and UV radiation during low tides, had higher antioxidant activity to protect themselves from oxidative damage. The capacity to produce antioxidants to protect its photosynthetic apparatus make *P. perforata* an adapted seaweed to inhabit harsh environments as the intertidal reefs of tropical regions.

Key-words: Desiccation, intertidal, *Palisada*, oxidative damage.

Resumo

Investigamos a atividade antioxidante de *Palisada perforata* amostrada em quatro diferentes micro-hábitas recifais a comparamos com a atividade antioxidante desta mesma espécie cultivada sob condições estressantes que elas encontram nesses micro-hábitats. Medimos também a sua performance fotossintética por parâmetros de fluorescência da clorofila. *P. perforata* é mais afetada pelo estresse de dessecação do que pelas variações na temperatura e salinidade. Isto está de acordo com o que foi observado nos recifes, onde as algas encontradas crescendo no platô, e assim, expostas ao ar e radiação UV durante as marés baixas, tiveram maior atividade antioxidante para se protegerem do dano oxidativo. A capacidade de produzir antioxidantes para proteger seu aparato fotossintético torna *P. perforata* uma macroalga adaptada a viver em ambientes severos como os recifes entremareais de regiões tropicais.

Palavras-chave: Dessecação, dano oxidativo, entre-marés, *Palisada*.

INTRODUCTION

Tropical reefs hold great biodiversity of seaweeds, which occupy a basal position in marine food webs (Lobban and Harrison, 1994). The reef intertidal regions are extremely dynamic and stressful habitats for seaweeds, since algae have to cope with a constantly changes in environmental characteristics due to tidal fluctuations. As a result, twice a day, they are exposed to a wide range of environmental stresses including high light, rapid fluctuations in temperature, osmotic stress and desiccation. Also, in tropical regions, some factors can be intensified due to higher UV radiation, increasing both the temperature and salinity of seawater.

Exposure of seaweeds during low tidal emersions demands the alga to prepare not only for desiccation but also for subsequent rehydration and eventual cellular damage (Burritt et al., 2002). Extended desiccation may cause a decline in photosynthesis rate while interrupting the electron flow between photosystem (PS) I and II (Heber et al., 2010; Gao et al., 2011). Additionally, fluctuating and dynamic environmental conditions in the intertidal zone trigger the accumulation of reactive oxygen species (ROS) (Collén and Davison, 1999a,b; Kumar et al., 2010,2011) that, if not buffered, result in an oxidative stress condition.

Few reports highlight the induction of ROS and activation of the antioxidant system in seaweeds in response to desiccation (Collén and Davison, 1999a,b; Kumar et al., 2011; López-Cristoffanini et al., 2013). Independent studies investigating algal responses to fluctuating environmental conditions show alterations in photosynthetic performance (Fv/Fm), cellular morphology and ontogeny (e.g. Abe et al., 2001; Varela et al., 2006; Kumar et al.,2011; Contreras-Porcia et al., 2012; Gao and Wang, 2012). However, integrative studies addressing cellular responses to desiccation are lacking.

In reefs seaweeds are distributed in zones, according to their abilities to withstand environmental stresses. Vertical zonation is a distribution pattern of intertidal organisms that

have been extensively investigated (Lubchenco 1980, Underwood e Chapmane 1996, Rodil et al. 2006). However, some seaweeds are able to colonize different zones in the intertidal region and an ability to tolerate desiccation is a prerequisite for their survival.

In Pernambuco coast, one of the few seaweed species that occur at different portions of the reef, subjected to different environmental stresses, is the Rhodophyta *Palisada perforata*, which is distributed worldwide (Guiry and Guiry, 2017) and dominates algae communities in Brazilian tropical reefs (Vasconcelos et al. 2016). *Palisada perforata* occurs from low to high intertidal regions of the studied reef, being exposed to different conditions in each microhabitat. How it manages to dominate this hostile environment, while other species present a distribution in zones due to their adaptations, is our main question here.

In this way, we hypothesize that this species must have an antioxidant defense system that allows it to cope with desiccation and fluctuations in seawater temperature and salinity. We investigated the antioxidant activity of *P. perforata* collected in four different reef microhabitats and compared it with the antioxidant activity of this same species cultivated under stressful conditions found in those microhabitats.

MATERIALS AND METHODS

Sampling and reef characteristics

Samplings were made during low spring tide (April 2017) at a sandstone reef of Enseada dos Corais beach (8°18'44.7"S; 34°56'49.8"W), Pernambuco state, on the NE coast of Brazil. This region is characterized by a tropical climate, with average temperatures ranging from 25°C to 30°C, and semidiurnal meso tides dominated by waves (Amaral et al., 2016).

The reef is composed by several features, due to its topology, that serve as microhabitats for the seaweed communities. Based on previous observations made by Cocentino et al. (2014), we considered four distinct reef microhabitats, formed during low spring tides, which were named here as sheltered, tide pool, plateau and exposed. The sheltered side is the reef portion facing the coast, protected from direct wave shock, currents and UV radiation. The tide pool is characterized by a depression on the reef plateau, which is flooded by seawater, remaining immersed even during low tides. Because it is exposed to UV radiation and there is no water circulation, the temperature and salinity of the tide pool can drastically increase due to evaporation. The plateau is the highest portion of the reef that remains emerged during low tides and hence exposed to UV radiation and air, leading to desiccation of seaweeds. At last, the exposed side is the portion of the reef facing the ocean, subjected to high hydrodynamics caused by wave shock and currents.

Palisada perforata specimens (about 500 g) were sampled at each microhabitat and immediately frozen at -20ºC for posterior analysis. Simultaneously, environmental data (temperature, salinity, dissolved oxygen (DO), pH and turbidity) were obtained at the microhabitats sheltered, tide pool and exposed (n = 4) with a multi-parameter probe (Horiba, U-50). The plateau was excluded because it had not enough water content to place the probe.

Preparation of crude extracts

After defrosting, seaweed material was washed to remove salt, sand and associated fauna and air dried at room temperature for about 72 h. Dried seaweeds were ground to a powder, extracted with a nonpolar solution of dichloromethane and methanol (2:1 v/v) during 15 days and then evaporated in a rotavapor (R-215 Buchi, Switzerland). For antioxidant analyses, the crude algal extracts were dissolved in methanol at a concentration of 3 mg ml^{-1}.

Antioxidant assays

The mechanism responsible for antioxidant activity (AOX) is different for each method used, so different antioxidant assays were performed. All antioxidant assays were done in triplicate, and the absorbance was read with a UV-vis (Ultraviolet-visible) microplate spectrophotometer (Epoch Biotek, USA). Results are expressed as AOX percentage and gallic acid equivalent (μg ml^{-1} GAE) calculated from the standard curve. The commercial pattern used on all assays was gallic acid (Sigma-Aldrich, Brazil).

The DPPH (2,2-diphenyl-1-picryhydrazyl) radical scavenging activity was performed according to Brand-Williams et al. (1995), as modified by Pires et al. (2017a). The inhibition percentage was calculated through the following equation: % AOX DPPH = [(Abs_{DPPH} – Abs_{sample})/Abs_{DPPH}] x 100, where Abs_{DPPH} is the control absorbance of DPPH, and Abs_{sample} is the absorbance of sample in DPPH reactive solution.

The antioxidant assay by ABTS (2,2–azinobis (3-ethylbenzthiazoline-6-sulfonic acid) free radical scavenging was described by Rufino et al. (2007) and modified by Torres et al. (2017). ABTS radical scavenging percentage was calculated by the following formula: % AOX ABTS = [(Abs_{ABTS} – Abs_{sample})/Abs_{ABTS}] x 100, where Abs_{ABTS} is the ABTS's control absorbance of ABTS, and Abs_{sample} is absorbance of the sample in ABTS reactive solution.

The metal chelating activity (Chelators) method was described by Dinis et al. (1994) and modified by Harb et al. (2016). Chelators percentage was calculated using the following formula: % AOX Chelators = [($Abs_{Chelators}$ – Abs_{sample})/$Abs_{Chelators}$] x 100, where $Abs_{Chelators}$ is the absorbance of reaction mixture, and Abs_{sample} is the absorbance of all reaction reagents with the sample.

The Folin-Ciocalteu assay was described by Singleton and Rossi (1965) and Waterman and Mole (1994) and then modified by Pires et al. (2017b). AOX percentage was calculated as

follows: % AOX Folin-Ciocalteu = (Abs_{sample} x 100)/$Abs_{Folin\text{-}Ciocalteu}$, where Abs_{sample} is the absorbance of sample in the reaction mixture, and $Abs_{Folin\text{-}Ciocalteu}$ is the maximum absorbance obtained by gallic acid standard, indicating the maximum AOX.

The ferric reducing antioxidant power (FRAP) assay was described by Benzie and Strain (1996) and modified by Urrea-Victoria et al. (2016). AOX percentage was calculated as % AOX FRAP = (Abs_{sample} x 100)/Abs_{FRAP}, where Abs_{sample} is absorbance of the sample in the reaction mixture, and Abs_{FRAP} is the maximum absorbance obtained by gallic acid standard, indicating the maximum AOX.

Experimental setup

Specimens were sampled at the reef plateau of Enseada dos Corais beach and kept in plastic bottles with seawater. Once in the laboratory, the fronds were cleaned, immersed in sterilized seawater (salinity 30) enriched with von Stosch solution, as recommended by Ursi and Plastino (2001) modified from Edwards (1970), and acclimated in a culture room for 14 days at 25 ± 1°C, 14h:10h light:dark photoperiod, 250 ± 5 μmol photon m^{-2} s^{-1} irradiance and 30 min intermittent aeration.

Temperature x salinity in vitro experiment

The first in vitro experiment consisted in a simulation of seawater temperature and salinity variation that seaweeds may find in the different reef microhabitats. We tested five temperatures (20, 25, 30, 35 and 40°C) and, for each temperature, two salinities (30 and 40). For every replicate (n = 4), 200 mg of *P. perforata* apical parts were placed in flasks containing 100 mL of enriched seawater and maintained in experimental conditions during six days. After

one day of experiment, we observed that at 40°C, and both salinities, the fronds had lost their color, so such treatment was interrupted and fronds were immediately frozen by immersion in liquid nitrogen and kept at -80°C until analysis.

Desiccation in vitro experiment

The second experiment simulated the desiccation effect experienced by seaweeds during tidal fluctuations. For each replicate (n = 4), 200 mg of *P. perforata* apical parts were placed in flasks containing 100 mL of enriched seawater or in petri dishes without water and kept at the same conditions as the acclimation period. For dehydrated treatment, fronds were placed in petri dishes, without water, during 2h, simulating the effect of low tide. Control treatment was obtained by fronds immersed in seawater during 2h (control D). A subset of dehydrated fronds was re-hydrated in enriched seawater, during 2h, to characterize the recovery from oxidative stress caused by desiccation. Control fronds were kept immersed in enriched seawater during 4h (control R).

Photosynthetic responses

At the end of both experiments, the photosynthetic responses of *P. perforata* fronds were assessed by a portable fluorometer (PAM 2500, Waltz, Germany). The maximum quantum yield (Fv/Fm = (Fm – Fo/Fm), which indicates the ratio of variable to maximal fluorescence of chlorophyll *a* of photosystem II (PSII), was measured in fronds previously kept in the darkness for 15 min.

The effective quantum yield (Y(II) = (Fm'- Fo')/Fm') was calculated according to Schreiber et al (1986) where Fo is fluorescence at steady state, Fm is the maximum fluorescence

after the incidence of a light saturating pulse in dark adapted samples, where reaction centers are closed, Fm' is the maximum fluorescence after the incidence of a light saturating pulse in light adapted samples, where reaction centers are opened, Fv is the variable fluorescence (Fm – Fo), Ft is the transient fluorescence.

Electron transport rate (ETR) was estimated through P-I (photosynthesis – irradiance) curves under different photosynthetically active radiations (PAR) and calculated as ETR = Y(II) x PAR x A x 0.15; where A is the thallus absorptance (Mercado et al. 1996); 0.15 factor is the chlorophyll *a* associated to PSII for red seaweeds (Figueroa et al. 2003).

Extraction

After photosynthetic analysis, the fronds were immediately frozen and then macerated in liquid nitrogen to obtain small fragments, suspended in methanol at 450 mg ml^{-1} concentration for 3h in the dark, at room temperature and then centrifuged at 14000 rpm for 15 minutes at room temperature. The supernatants obtained were analyzed at 30 mg ml^{-1} concentration. The antioxidant activity of those extracts was measured through five assays already described here.

Statistical analysis

To test for changes between the factors levels, univariate and multivariate analyzes were performed, both using Software Statistica v 10. One-way ANOVA was tested when only one interest factor occurred (reef microhabitats and desiccation level) and factorial ANOVA was performed when there was more than one factor (temperature and salinity). ANOVA assumptions were evaluated by normality tests (Kolomogorov-Smirnov and Shapiro Wilker)

and variance homoscedasticity through Levene (1960) and Brown-Forsythe (1974) tests. When necessary, data were transformed from square root and Box-Cox (1964) transformations. For comparing different factor levels, we used Tukey HSD post-hoc test.

Multivariate analyzes were performed to verify the variable importance on the sample variance. A similarity matrix with Gower (1971) distance index was calculated. To test differences among factors at the multivariate environment, a Permanova was performed. Graphical representation was made through a Redundancy Analysis (RDA) in Excel with XLSTAT (2014.5.03) for the reef microhabitats, and a Principal Coordinate Analysis for lab experiments (temperature x salinity and desiccation).

RESULTS

Microhabitats

Alga sampled at the exposed site showed the highest antioxidant activity (Figure 1), followed by alga sampled at plateau, differing only for Folin-Ciocalteu assay ($p > 0.05$). Except at DPPH and ABTS assays, the tide pool alga had the lowest activity. In general, the activity varied among the exposure gradient as following: exposed > plateau > sheltered > tide pool.

The influence of environmental parameters was assessed by a redundancy analysis (Figure 2), were the antioxidant activity presented by alga sampled at the exposed sites ($n = 3$), through DPPH and ABTS assays, were most influenced by the turbidity in this site. The activity measured by Chelators, Folin-Ciocalteu and FRAP assays had contrary responses to the abiotic parameters and related with only one algal sample from sheltered site. The low activities of alga from tide pool sites were influenced by most environmental parameters measured. The axes F1 and F2 explained 70.44 and 26.98% of the variation, respectively (Figure 2).

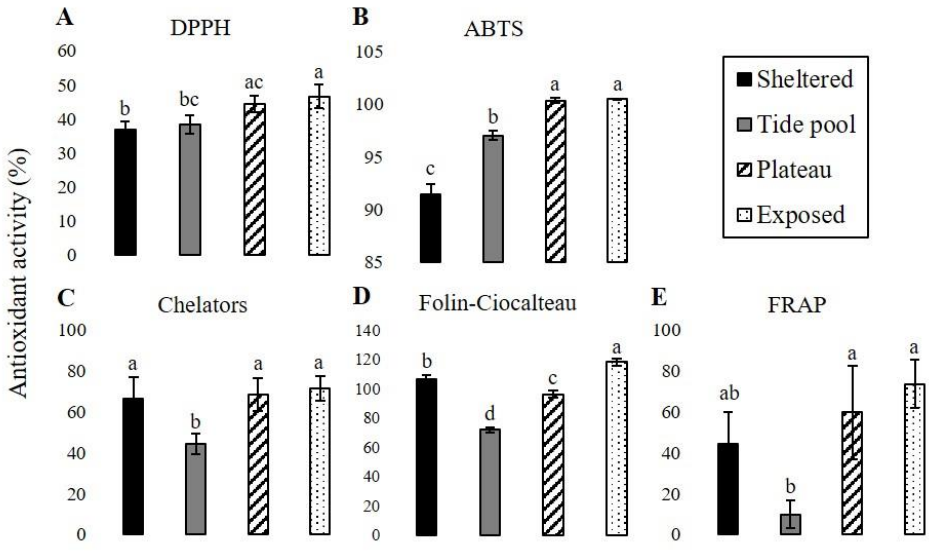

Figure 1 - Antioxidant activity (%) of *Palisada perforata* sampled in four reef microhabitats of Pernambuco, Brazil, measured through five methods, being, DPPH and ABTS radical scavenging, metal ion Chelators, Folin-Ciocalteu and FRAP reducing power. Different letters indicate significant difference among micro-habitats for each antioxidant assay (ANOVA, $p < 0.05$)

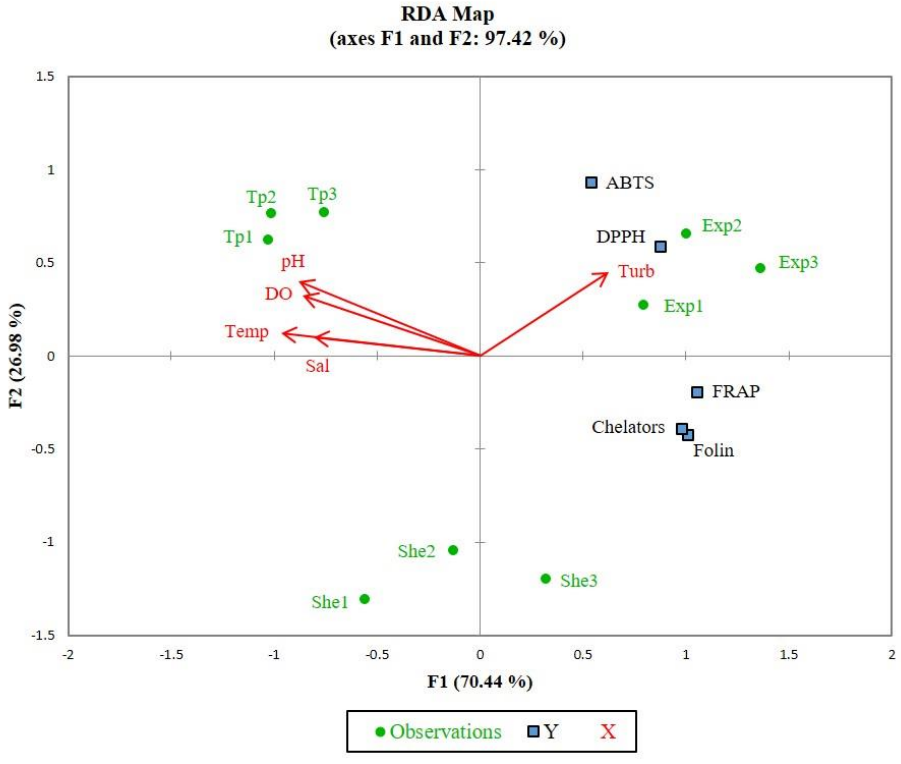

Figure 2 - Redundancy analysis for the influence of environmental data on the antioxidant activity (DPPH, ABTS, Chelators, Folin and FRAP assays) of *Palisada perforata* sampled at different reef microhabitats (exposed, sheltered and tide pool) in Pernambuco, Brazil. Observations: replicates of each microhabitat (n = 3); Y: antioxidant assays; X: environmental data - temperature (Temp), salinity (Sal), dissolved oxygen (DO), turbidity (Turb) and pH.

Laboratory experiments

At temperature x salinity experiment, we observed no great differences among treatments (Figure 3). At all assays, the control temperature (25°C) had the highest activities, with no differences between salinities. At DPPH, ABTS and Folin-Ciocalteu assays, the temperature of 25°C was different from the others, as it had higher activity. No differences were detected for Chelators and FRAP assays. We also tested the temperature of 40°C, however, as the specimens started to loose color, we terminated this treatment, as alga had perished.

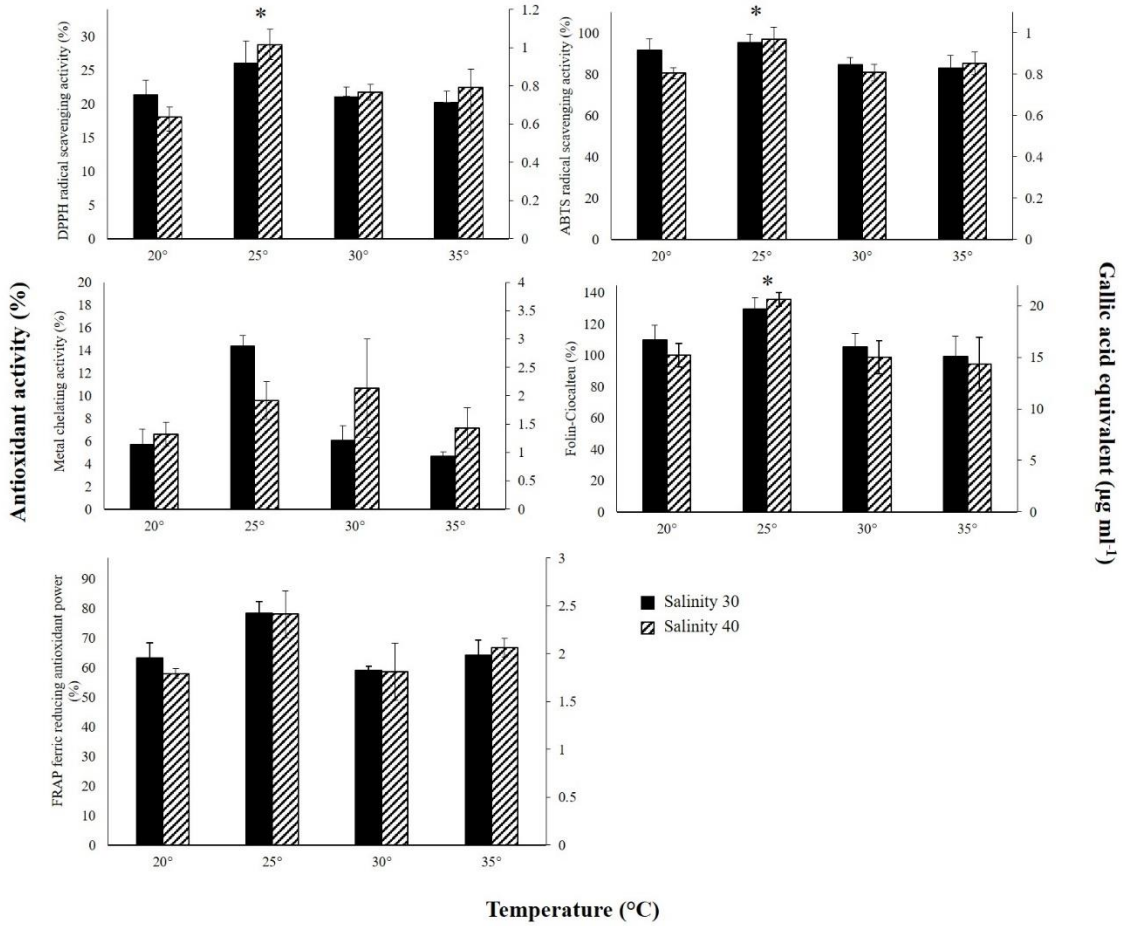

Figure 3 - Antioxidant activity of *Palisada perforata* through five methods under different temperature and salinity conditions. Results are expressed as percentage of activity and gallic acid equivalent (µg ml^{-1}). Asterisk indicate statistical differences among treatments for each assay.

Maximum quantum yield (Fv/Fm) varied little among different temperature and salinities during experiment. Effective quantum yield (Y(II)) decreased but not in significant levels, meaning that photosystem centers were not significantly affected by temperature and salinity variations (Figure 4), so those variances were not enough to cause oxidative stress to *P. perforata*.

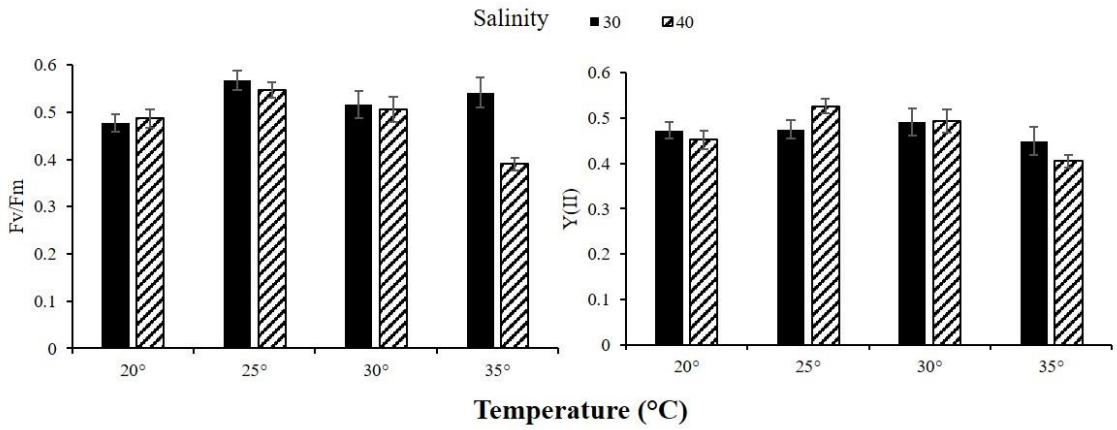

Figure 4 - Photosynthetic efficiency in *Palisada perforata* under temperature and salinity experiment measured through maximum quantum yield (Fv/Fm) and effective quantum yield (Y(II)).

Photosynthesis profiles estimated through electron transport rates in increasing irradiances are presented through ETRr x PAR curves, as shown in Figure 5 for the temperature x salinity experiment. All treatments showed similar curves, regardless the different treatments. At salinity of 30, the 30°C curve showed better fitness, followed by the 25°C, which had better antioxidant responses. Increasing salinity to 40, the 20°C curve was higher than all the others, evidenced by the area under the curve size. However, from those curves, differences on photosynthetic parameters were observed and are presented in Table 1.

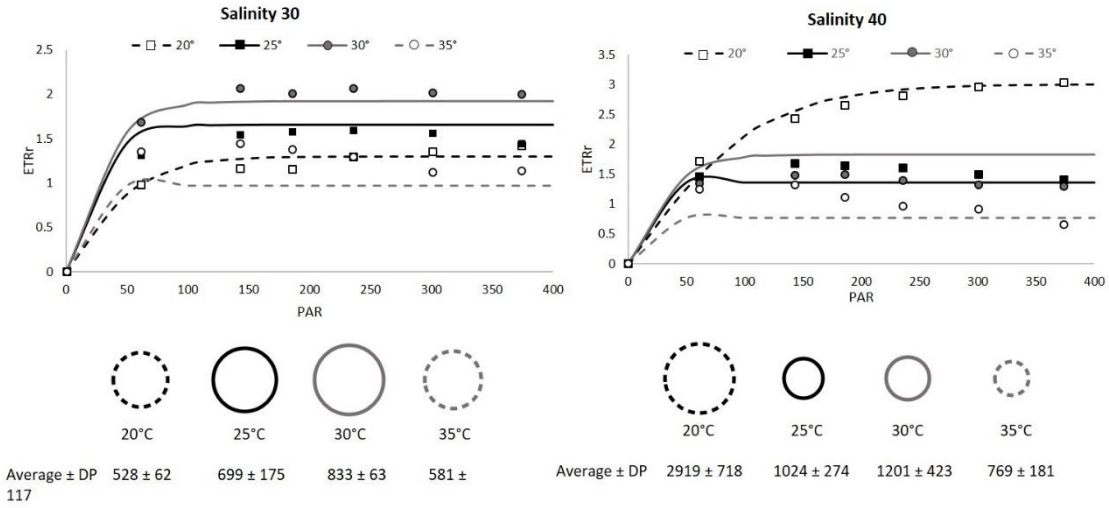

Figure 5 - ETR curves and area under the curve calculated for each treatment of *Palisada perforata* photosynthetic performance under temperature x salinity experiment.

The second experiment simulated the desiccation and rehydration effects of tidal cycles on *P. perforata* and results are shown in Figure 6. No great differences among treatments were observed, except for rehydrated treatment at Folin-Ciocalteu and FRAP assays., where the alga could not recover from damage.

Maximum quantum yield (Fv/Fm) varied little among desiccation experiment. Effective quantum yield (Y(II)) decreased in significant levels for both treatments, dehydrated and rehydrated, inferring that photosystem centers were significantly affected by desiccation stress (Figure 7). Indeed, to prevent from damage, the alga photosystem decreased its photosynthetic capacity even at maximum quantum yield, from control D to dehydrated treatment, which was maintained at effective quantum yield.

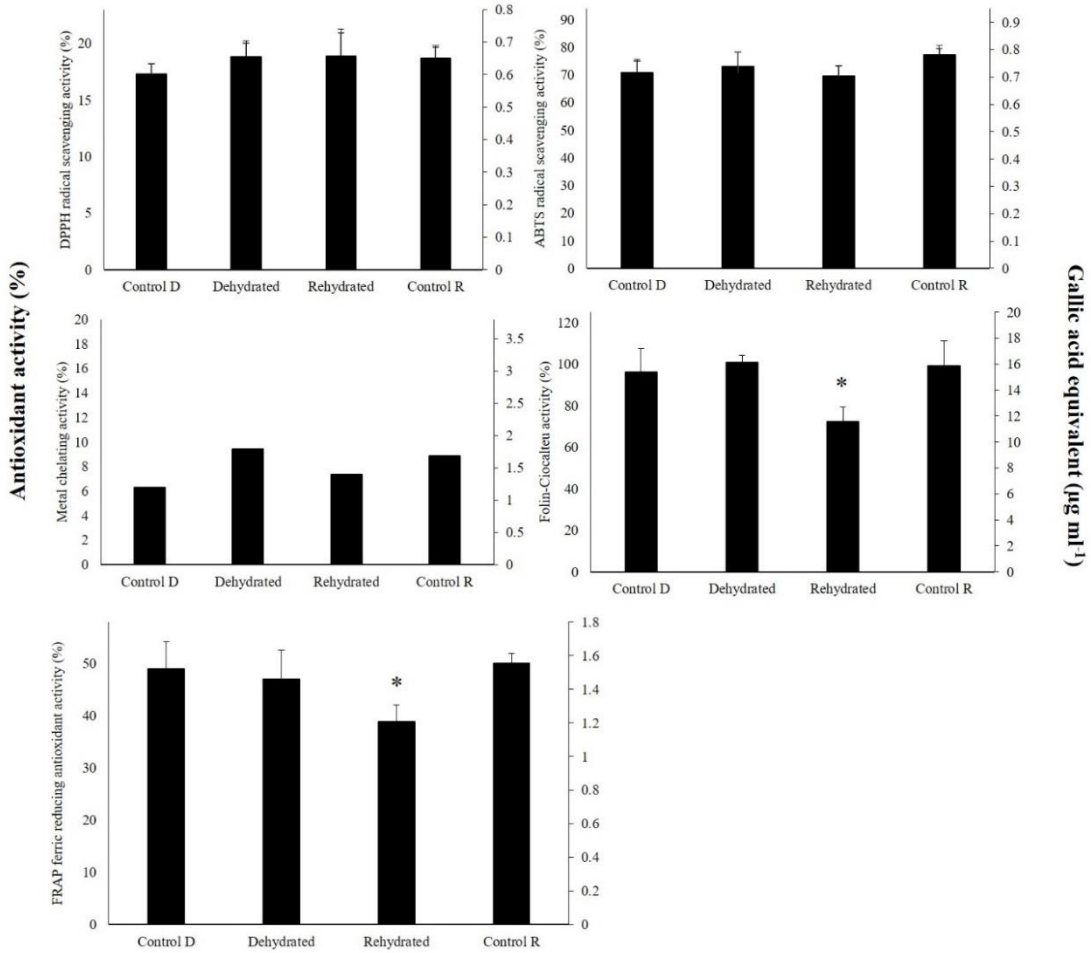

Figure 6 - Antioxidant activity of *Palisada perforata* through five methods under different desiccation levels. Results are expressed as percentage of activity and gallic acid equivalent (µg ml^{-1}). Asterisk indicate statistical differences among treatments for each assay.

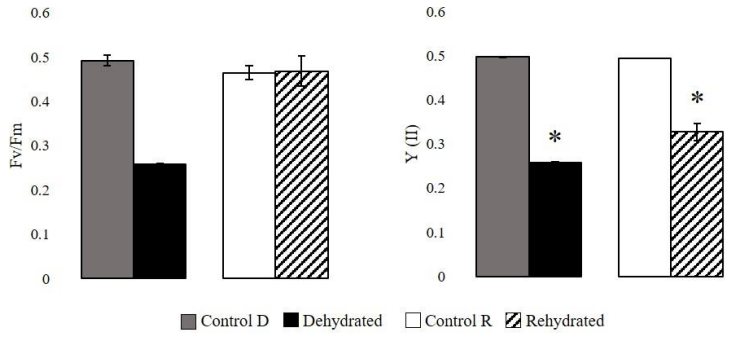

Figure 7 - Photosynthetic efficiency in *Palisada perforata* under desiccation experiment measured through maximum quantum yield (Fv/Fm) and effective quantum yield (Y(II)).

ETRr curves were also measured for *P. perforata* under desiccation experiment, as presented in Figure 6. We observe an overlap of the control D and rehydrated curves, followed by control R curve, and dehydrated presenting the lowest result, as evidenced by the area under

the curve. This result shows that, indeed, the alga under dehydration is suffering from it, decreasing its photosynthetic capacity to preserve the photosystems.

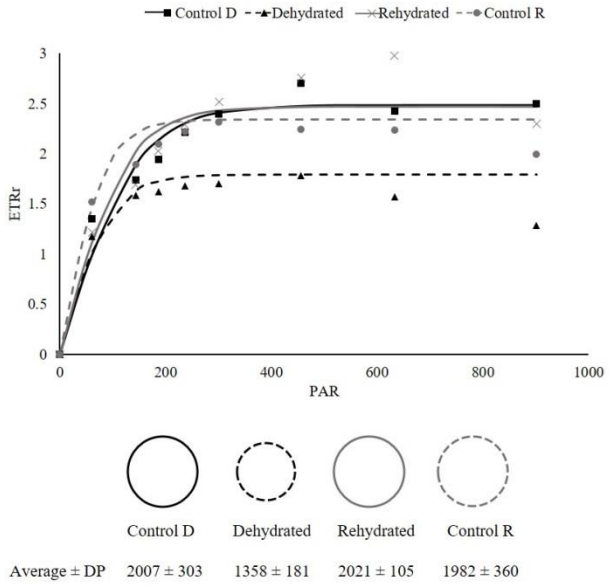

Figure 8 - ETR curves and area under the curve calculated for each treatment of *Palisada perforata* photosynthetic performance under desiccation experiment.

Coupled with ETRr curves, other parameters as ETR maximum, α and IK values, also calculated for *P. perforata* performance at both experiments (Table 1), also allows us to analyze it´s photosynthetic capacity and its possible damage due to oxidative stress.

Table 1 - Photosynthetic parameters calculated for *Palisada perforata* subjected to two experiments, temperature x salinity and desiccation. ETR max: maximum electron transport rate; α: photosynthetic efficiency; IK: photosynthesis saturation irradiance; obtained through ETRr x PAR curves.

	ETR max	**α**	**IK**
Temperature x Salinity experiment			
20°30	1.30 ± 0.25	0.02 ± 0.00	45.47 ± 6.08
20°40	3.01 ± 0.34	0.02 ± 0.00	113.51 ± 9.75
25°30	1.65 ± 0.47	0.04 ± 0.02	29.49 ± 10.59
25°40	1.35 ± 0.35	0.50 ± 0.22	1.80 ± 1.21
30°30	1.92 ± 0.14	0.04 ± 0.00	43.70 ± 3.96

30°40	1.82 ± 0.10	0.04 ± 0.01	47.01 ± 14.67
35°30	0.97 ± 0.21	0.42 ± 0.00	1.07 ± 1.35
35°40	0.76 ± 0.45	0.34 ± 0.00	1.15 ± 1.85
Desiccation experiment			
Control D	2.48 ± 0.28	0.01 ± 0.00	147.21 ± 0.00
Dehydrated	1.79 ± 0.23	0.01 ± 0.00	95.12 ± 0.00
Control R	2.33 ± 0.40	0.02 ± 0.00	76.39 ± 0.00
Rehydrated	2.46 ± 0.01	0.01 ± 0.00	143.96 ± 0.00

Multivariate analyzes were performed to obtain a general response of seaweeds to diverse tested variables at both experiments. Before analyzes, high correlation variables (α and IK) were removed to allow discernment of which variable is responding. Multivariate space was presented by a PCO and analyzes through Permanova.

Table 2 - Permanova results for temperature x salinity experiment with *Palisada perforata*.

PERMANOVA table of results

Source	**df**	**SS**	**MS**	**Pseudo-F**	**P(perm)**	**Unique perms**
Te	3	3757	1252.3	6.9907	0.001	998
Sa	1	48.344	48.344	0.26986	0.901	998
Te x Sa	3	740.85	246.95	1.3785	0.177	998
Res	24	4299.5	179.14			
Total	31	8845.7				

PERMANOVA Temperature Pairwise

Groups	t	P(perm)	Unique perms	P(MC)
20º, 25º	3.5586	0.001	999	0.001
20º, 30º	1.3475	0.092	999	0.151
20º, 35º	1.5212	0.02	999	0.064
25º, 30º	3.44	0.002	999	0.001

25º, 35º	3.3816	0.002	999	0.001
30º, 35º	1.1713	0.201	999	0.256

At temperature x salinity experiment, only temperature factor showed differences. Into the levels tested at Permanova pairwise differences were found only at 25°C level. The PCO (Figure 9) shows the separation and influence of variables DPPH, ABTS and Folin-Ciocalteu through a Person´s correlation (vectors).

Simper analysis shows each variable participation at the groups dissimilarity (Euclidian distance) (Table 3). After Simper analysis, it´s clear that the groups separation is due to the increase of the antioxidant activity in seaweeds at 25°C compared to the other temperature treatments.

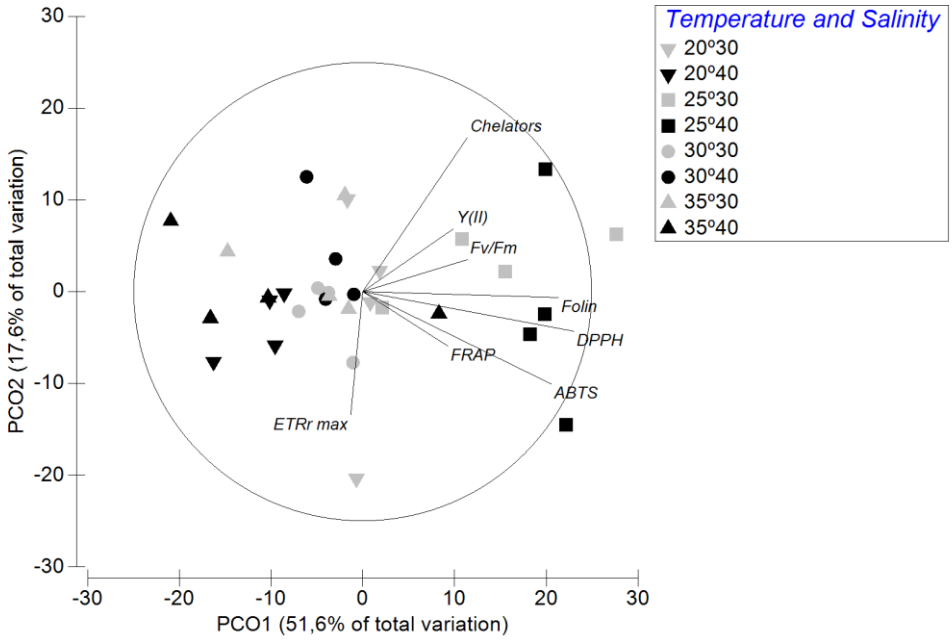

Figure 9 - Principal component analysis (PCO) to show influence of treatments on antioxidant and photosynthetic performances of *Palisada perforata* under temperature x salinity experiment. 2030: 20°C and salinity 30; 2040: 20°C and salinity 40; 2530: 25°C and salinity 30; 2540: 25°C and salinity 40; 3030: 30°C and salinity 30; 3040: 30°C and salinity 40; 3530: 35°C and salinity 30; 3540: 35°C and salinity 40.

Table 3 - Simper analysis results for temperature x salinity experiment with *Palisada perforata*.

SIMPER (% of dissimilarity)

Groups 20º & 25º

Average squared distance = 20.70

	Group 20º	Group 25º				
Variable	**v. Value**	**v. Value**	**Av. Sq. Dist**	**Sq. Dist/SD**	**Contrib%**	**Cum.%**
DPPH	-0.653	1.3	4.97	1.2	24.02	24.02
ABTS	-0.121	1.08	3.31	0.97	15.98	40
ETRr max	0.842	-8.76E-02	2.55	0.71	12.32	52.31
Chelators	-0.148	0.661	2.54	0.64	12.29	64.6
FRAP	-0.219	0.287	2.49	0.72	12.04	76.64

Groups 25º & 30º

Average squared distance = 16.57

	Group 25º	Group 30º				
Variable	**v. Value**	**v. Value**	**Av. Sq. Dist**	**Sq. Dist/SD**	**Contrib%**	**Cum.%**
ABTS	1.08	-0.573	3.75	1.08	22.63	22.63
FRAP	0.287	9.09E-02	2.9	0.97	17.5	40.13
DPPH	1.3	-0.19	2.72	1.3	16.44	56.57
Folin	1.23	-0.268	2.69	1.2	16.21	72.79

Groups 25º & 35º

Average squared distance = 27.35

	Group 25º	Group 35º				
Variable	**v. Value**	**v. Value**	**Av. Sq. Dist**	**Sq. Dist/SD**	**Contrib%**	**Cum.%**
Folin	1.23	-0.845	5.64	0.94	20.62	20.62
DPPH	1.3	-0.453	4.09	0.96	14.94	35.56
Y(II)	0.528	-0.613	3.92	0.43	14.34	49.9
Fv/Fm	0.663	-0.473	3.89	0.45	14.22	64.12
ABTS	1.08	-0.39	3.18	1.03	11.64	75.76

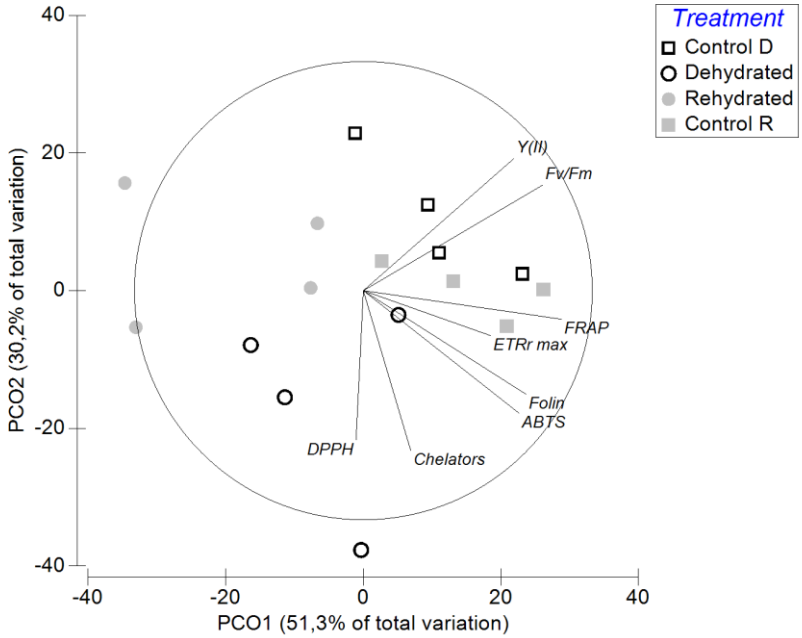

Figure 10 - Principal component analysis (PCO) to show influence of treatments on antioxidant and photosynthetic performances of *Palisada perforata* under temperature x salinity experiment. 2030: 20°C and salinity 30; 2040: 20°C and salinity 40; 2530: 25°C and salinity 30; 2540: 25°C and salinity 40; 3030: 30°C and salinity 30; 3040: 30°C and salinity 40; 3530: 35°C and salinity 30; 3540: 35°C and salinity 40.

For desiccation experiment, the same multivariate analyzes were performed. PCO graphically shows that control groups (controls D and R), are spatially identical, however, different from the treatments dehydration and rehydration, which is also confirmed by the Permanova (Table 4), where the difference among treatments and controls is evident.

Table 4 - Permanova results for temperature x salinity experiment with *Palisada perforata*.

PERMANOVA table of results						
Source	**df**	**SS**	**MS**	**Pseudo-F**	**P(perm)**	Unique **perms**
Tr	3	5123,8	1707,9	4,6979	0,001	998
Res	12	4362,7	363,56			
Total	15	9486,5				

PERMANOVA Desiccation Pairwise			
Groups	t	P(perm)	Unique perms
Control D, Dehydrated	2,2944	0,024	35

Control D, Rehydrated	2,2337	0,03	35
Control D, Control R	0,92557	0,559	35
Dehydrated, Rehydrated	1,818	0,019	35
Dehydrated, Control R	2,3291	0,03	35
Rehydrated, Control R	2,7904	0,024	35

Table 5 shows Simper analysis evidencing a greater participation of variables Y(II), Fv/Fm and ETRr max, so the photosynthetic parameters, as responsible for the groups control D and dehydrated and control R and rehydrated dissimilarity. The antioxidant responses were the main variables responsible for dissimilarity among dehydrated and rehydrated groups.

Table 5 - Simper analysis results for temperature x salinity experiment with *Palisada perforata.*

SIMPER (% of dissimilarity)

Groups Control D and Dehydrated

Average squared distance = 17.65

	Group Control D	Group Dehydrated				
Variable	**Av. Value**	**Av. Value**	**Av. Sq. Dist**	**Sq. Dist/SD**	**Contrib%**	**Cum.%**
Y(II)	0.898	-1.19	4.42	4.01	25.01	25.01
Chelators	-0.137	0.563	3.58	0.62	20.26	45.27
Fv/Fm	0.729	-0.788	3.21	1.34	18.2	63.47
DPPH	-0.762	0.265	1.95	0.73	11.05	74.52

Groups Dehydrated and Rehydrated

Average squared distance = 20.24

	Group Dehydrated	Group Rehydrated				
Variable	**Av. Value**	**Av. Value**	**Av. Sq. Dist**	**Sq. Dist/SD**	**Contrib%**	**Cum.%**
Folin	0.608	-1.36	4.08	2.35	20.13	20.13
Chelators	0.563	-0.377	3.51	0.58	17.36	37.49
ETRr max	3.30E-03	-0.666	3.01	0.58	14.84	52.34
FRAP	0.132	-1.24	2.78	0.86	13.72	66.06
DPPH	0.265	0.314	2.29	0.88	11.32	77.38

Groups Rehydrated and Control R

Average squared distance = 21.53

	Group Rehydrated	Group Control R				
Variable	**Av. Value**	**Av. Value**	**Av. Sq. Dist**	**Sq. Dist/SD**	**Contrib%**	**Cum.%**
Folin	-1.36	0.478	4.08	1.24	18.97	18.97
FRAP	-1.24	0.644	3.86	1.83	17.91	36.88
ETRr max	-0.666	0.274	3.47	0.57	16.13	53.01
ABTS	-0.645	0.885	3.14	1.08	14.57	67.58
Y(II)	-0.578	0.87	2.48	1.16	11.5	79.07

DISCUSSION

Palisada perforata is an intertidal seaweed that occurs from low to high heights in the reef environment. Considering that the reefs have several features due to its geomorphology, this species is found in four different micro-habitats, and each with different environmental factors within the reef formation: exposed site, plateau, tide pool and sheltered site. In view of that, we hypothesized that *P. perforata* should possess an efficient antioxidant system to protect its photosynthetic apparatus, thus enabling its occurrence at all micro-habitats.

As shown by our results, the position in the reef features have little influence on the antioxidant activity of *P. perforata*. The higher activity showed by algae sampled at exposed and plateau sites is explained in parts by the influence of environmental parameters such as turbidity for the first micro-habitat and higher UV radiation and oxygen at the second. Because we could not sample abiotic data from the plateau, we can only suppose that explanation. The tide pool algae were influenced by pH, DO, temperature and salinity, which seems obvious, due to increased evaporation rates at tide pools during low tides, increasing seawater´s temperature and salinity. Due to higher amount of seaweeds photosynthesizing, DO levels also increased in tide pools. Those factors contributed to the lower antioxidant activity by algae sampled in tide

pools, suggesting that the stress was so high that it was not able to increase protection. In the other hand, algae living in protected sites did not suffer from enough stress to produce antioxidant compounds, because it was not exposed to wave action, direct UV radiation, O_2 levels or temperature increase.

The effect of temperature can be primarily associated with the capacity of algae to photochemically quench excess solar energy (e.g. dynamic photoinhibition). Apparently, photosystem centers were not affected by the variance in temperature and salinity, as Y(II) decreased from Fv/Fm only by an average of 2.5%. In all specimens, irrespective of the treatment, there was a tendency of fully recovery of photosynthesis after experiment stress conditions. ETR curves also suggests that there was not enough influence of treatments to modify the electron transport rates. Plants acclimated to high temperatures, as tropical seaweeds, normally exhibit higher capacity for dissipating excess excitation than plants acclimated to low temperatures (Falk et al. 1990). This could be the reason why they didn´t suffer from temperature increase, or the temperature was not high enough.

Overall, the marked antioxidant capacity exhibited by *P. perforata* could be responsible for the low decrease in photochemical reactions occurring at the thylakoids (*e.g.* Fv/Fm and electron transport). As the integrity of the thylakoid membranes is a prerequisite for the necessary balance between light absorption and damage repair during photoinhibition (Demmig-Adams et al. 2008), the ability to photosynthesize at considerable rates during the onset of light and temperature suggest that ROS scavenging is a relevant anti-stress mechanism in these algae. For example, in the intertidal red alga *Stictosiphonia arbuscula* the antioxidative metabolism, especially of populations occurring at upper littoral levels and subject to extreme temperature and desiccation, minimizes lipid peroxidation and membrane damage after rehydration via an increase in the antioxidant enzyme activity (Burrit et al. 2002).

The effects of osmotic stresses on the primary processes of photosynthesis have been studied by chlorophyll fluorescence kinetics. These investigations were aimed primarily at measuring the effects of desiccation on photosynthesis. The two stresses (increasing salinity and desiccation) are comparable since they result in a reduction of the cellular water potential (Kirst 1989).

In a salinity stress experiment with *Acanthophora spicifera*, Pereira et al. (2017) observed that the species exhibited high tolerance to 25 to 40 salinities, with little change in physiology. However, 15, 20, 45 and 50 were the most damaging salinities and led to loss of biomass, depigmentation of apices, and the highest concentrations of antioxidant metabolites.

Desiccation stress was tested through dehydration and rehydration effects on *P. perforata* as they experience during tidal cycles. Despite at Folin-Ciocalteu and FRAP assays, the lack of significant differences between controls and treatments suggests that this alga must have a very efficient antioxidant system, because even during stress, the activity remained high, even higher than the control sometimes.

Yanl et al. (2005) have studied the photosynthetic recovery of intertidal seaweeds after rehydration. Based on their results, it is safe to say that is the ability to withstand desiccation stress (fast recovery during rehydration), but not that to avoid desiccation (water retaining ability) that determines the distribution of intertidal seaweeds.

In the same way, Flores-Molina et al. (2014) concluded that the presence and adequacy of functional responses to desiccation in seaweeds defines their altitudinal position in the intertidal zone, independent of the taxonomic group it belongs to. High intertidal species are tolerant to desiccation and that tolerance seems to be based on the various mechanisms that efficiently attenuate ROS excesses caused by desiccation (high antioxidant activity, biomolecule oxidation attenuation and photoinhibition tolerance) (Flores-Molina et al. 2014).

The functional status of the photosynthetic system of various seaweeds and plant models has been widely used as an indicator of stress caused by desiccation (Bell, 1993; Skene, 2004; Varela et al., 2006; Gylle et al., 2009; Pandey et al., 2010; Schagerl and Möstl, 2011). For example, in the resurrection plant *Selaginella bryopteris*, the rapid and complete recovery of Fv/Fm after rehydration clearly indicates the absence of marked photoinhibitory or thermal injury to PSII during desiccation. This is the result of a reversible reduction of the photosynthetic quantum yield due to down-regulation of PSII, where energy is thermally dissipated, process that was also observed in our study. Even though carbon fixation could be inhibited during desiccation, electron flow might continue and thus form ROS (Dinakar et al., 2012). Given this, photoinhibition could prevent ROS formation and help to overcome desiccation (e.g. Figueroa et al., 1997; Korbee et al., 2005; Contreras-Porcia et al., 2011).

With the photosynthetic performance results we can infer that the algae were under stress. The Fv/Fm measures the maximum photosynthetic efficiency and from control D to dehydrated plants, it decreased 23% its capacity in order to prevent PSII from damage, remaining low the Y(II). Electron transport rates were affected mainly at dehydrated treatment were it reached the lowest values.

Apparently, 2 h of air exposure did not significantly influence the photosynthesis of *P. perforata*, as observed by Yu et al. (2012) for other seaweeds, indicating a short-term desiccation effect followed by a complete recovery. However, Yu et al. (2012) observed that the photosynthetic yield of emersed thalli decreased sharply with increasing duration of exposure, possibly because biochemical resistance increased due to water limitation during desiccation, as already suggested by Bell (1993). With the extended desiccation and more loss of water the electron system operating between photosystem II (PSII) and photosystem I (PSI) may get interrupted due to decreased photosynthetic pigments coupled with enhanced ROS.

Yu et al. (2012) also observed that *Sargassum thunbergii* exhibits significant changes in photosynthetic activity in response to combined desiccation and temperature stress, as well as changing tidal pattern during the simulated diurnal light cycle. Desiccation appears to constitute the most acute stress affecting its photosynthetic activity. We did not combine temperature with desiccation experiments because our objective was to verify the algae response to each variable, separately, but their combined effects must cause a more severe stress effect on *P. perforata*.

Multivariate analyzes allow us to verify the influence of several variables on the algae antioxidant and photosynthetic performances. At temperature x salinity experiment, results from Permanova indicate that only the temperature had influenced the distribution of data, with 25°C being the control temperature, as it reached the maximum activities. The PCO also shows a clear separation of the controls, both at 25°C, and treatments. As 25°C had the highest activities, it was related with the antioxidant assays and Fv/Fm parameter. At desiccation experiment, PCO and Permanova show a clear segregation of control groups from treatment groups, as control groups had higher antioxidant activities and also higher photosynthetic capacities.

Field experiments conducted on *Fucus* adults demonstrated a decline in the PSII, suggesting that photoinhibition experienced during natural emersion is a sub-lethal stress, temporarily impairing photosynthesis (Davison and Pearson, 1996).

Photosynthetic reactions, which have been shown to be sensitive to desiccation, are electron transport between PSII and PSI and water splitting (Seddon and Cheshire, 2001). In this study, desiccation appears to have more important effect on Fv/Fm and Y(II) than temperature and salinity variations.

The production of ROS can be enhanced by stress and if the accumulation of ROS exceeds the capacity of plants to remove them, it will lead to oxidative stress (Kreslavski et al.,

2007). As a result, photosystems could be damaged because of DNA mutation, protein denaturation, lipid peroxidation, and chlorophyll bleaching as well as the loss of membrane integrity (Cherry and Nielsen, 2004). Meanwhile seaweeds could exhibit broad ranges of physiological responses to stressful conditions, notably including the immediate changes in the optimal fluorescence quantum yield (Fv/ Fm) (Parkhill et al., 2001). It has been rather well established that changes of Fv/Fm can be taken as an indicative parameter to reflect the state of PS II efficiency under adverse stresses (Maxwell and Johnson, 2000; Adams and Demmig-Adams, 2004; Pang and Shan, 2008).

Based on our results, we can conclude that *P. perforata* is more affected by desiccation stress than temperature or salinity variations. This is in agreement with the observed at the reefs, were algae found growing at plateau, and thus, exposed to air and UV radiation during low tides, had higher antioxidant activity to protect themselves from oxidative damage. The capacity to produce antioxidants to protect its photosynthetic apparatus make *P. perforata* an adapted seaweed to inhabit harsh environments as the intertidal reefs of tropical regions.

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9 CONCLUSÕES GERAIS

Este é o primeiro trabalho a avaliar o potencial antioxidante de macroalgas tropicais dos recifes de Pernambuco. Os recifes tropicais são ambientes severos, e as algas estão expostas a condições ambientais extremas, como elevado hidrodinamismo, radiação UV, flutuações de temperatura e salinidade, dessecação e outros fatores originados dos ciclos de maré. Para sobreviver neste ambiente, as macroalgas desenvolveram diversas estratégias de defesa, produzindo compostos com ação antioxidante.

Avaliando as espécies mais comuns de macroalgas marinhas dos recifes de Pernambuco, na seção 6 observamos que todas as espécies, por estarem expostas a fatores ambientais severos, possuem elevada atividade antioxidante, mesmo em baixas concentrações de extrato. *Padina tetrastomatica* foi a espécie com maior potencial, a qual poderia ser empregada em indústrias cosméticas, nutracêuticas, farmacológicas, entre outras.

Na seção 7, a fim de eliminar o efeito dos fatores ambientais, os quais podem variar de acordo com a posição da alga no recife, escolhemos a região inferior de recifes intermareais de Pernambuco para testar a atividade antioxidante das três macroalgas dominantes deste hábitat. Dentre as espécies *Sargassum furcatum, Bryothamnion triquetrum* e *Osmundaria obtusiloba*, esta última foi a mais promissora, com atividade próxima ao padrão utilizado. Ressaltamos o elevado potencial destas espécies tropicais, principalmente *O. obtusiloba*, representando uma nova fonte de antioxidantes naturais.

Para relacionar a atividade antioxidante das macroalgas tropicais com os fatores ambientais, na seção 8 realizamos amostragens de *Palisada perforata* em quatro micro-hábitats recifais e a exposição ao batimento de ondas e dessecação parecem ser os fatores que mais influenciaram as algas vivendo no lado exposto e no platô recifal, respectivamente. Com esta

mesma espécie, realizamos dois experimentos, para verificar a veracidade dos dados coletados em campo. Aparentemente, a temperatura e salinidade não influenciam fortemente a atividade antioxidante nesta alga. A dessecação, por outro lado, mostrou-se um forte fator estressante, diminuindo a eficiência fotossintética e elevando a atividade antioxidante de *P. perforata*.

Concluímos então que as macroalgas tropicais apresentam elevada atividade antioxidante, a qual está aparentemente relacionada com compostos lipofílicos e que a dessecação é o principal fator influenciando a atividade antioxidante, e assim, a distribuição das algas nos recifes.

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ANEXO A - RESULTADOS NÃO COMPILADOS

Capítulo 1

ANOVA Results:

Assumptions ANOVA

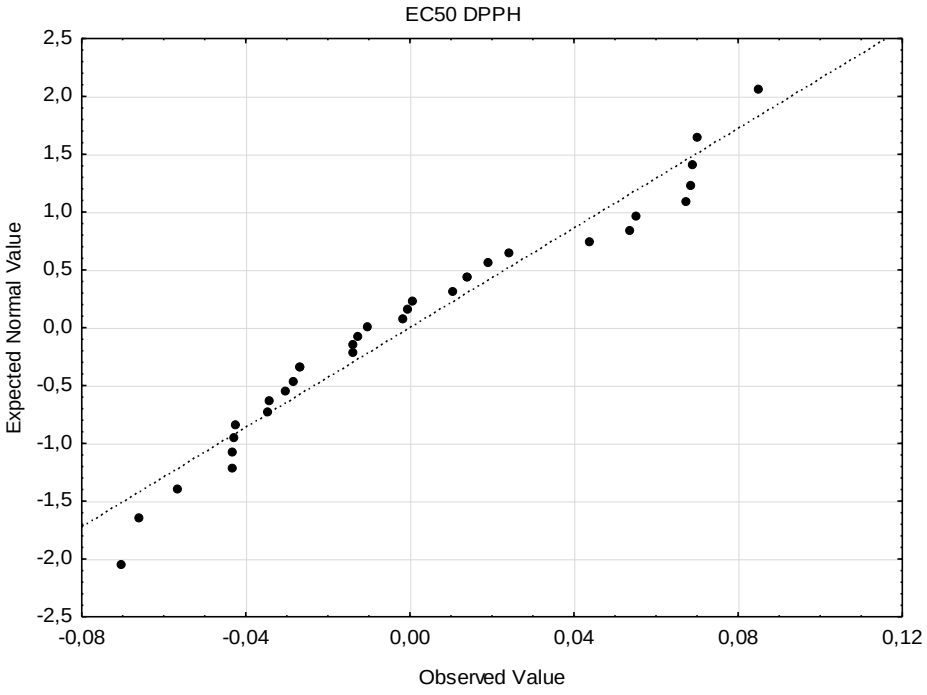

Figura 1. Residual normal values after Box-Cox transformation

Tabela 1. Levene's test for Homogeneity of variances

	MS effect	MS error	F	P
Levene	0,000620	0,000552	1,123188	0,389218

ANOVA one way

Tabela 2. Univariate results for ANOVA EC50 DPPH variable.

	Degree of freedom (BC)	Sums of squares (BC)	Means of squares (BC)	F (BC)	p (BC)
EC50 DPPH	10	27,96229	2,79623	1000,58	0,00
Error	22	0,06148	0,00279		
Total	32	28,02377			

*BC- Box-Cox transformation

Post-hoc ANOVA

Tabela 3. Post-Hoc Tukey HSD test for EC50 DPPH variable.

Species	Tukey HSD Test MS= 0,00279; df=22									
	G. caudata	*G. cearensis*	*P. perforata*	*C. acicularis*	*A. spicifera*	*B. pennata*	*C. cupressoides*	*C. racemosa*	*D. delicatulla*	*P. gymnospora*
G. caudata										
G. cearensis	0,999667									
P. perforata	0,371170	0,783385								
C. acicularis	0,000201	0,000201	0,000201							
A. spicifera	0,000201	0,000201	0,000201	0,000201						
B. pennata	0,000201	0,000201	0,000201	0,000201	0,000204					
C. cupressoides	0,000201	0,000201	0,000201	0,000201	0,000217	0,999869				
C. racemosa	0,000201	0,000201	0,000201	0,000201	0,000202	0,000201	0,000201			
D. delicatulla	0,000201	0,000201	0,000201	0,000204	0,000201	0,911261	0,583482	0,000201		
P. gymnospora	0,000201	0,000201	0,000201	0,000201	0,346826	0,000201	0,000201	0,000597	0,000201	
P. tetrastomatica	0,000201	0,000201	0,000201	0,000201	0,000201	0,000201	0,000201	0,000201	0,000201	0,000201

Redudance analysis (RDA) results:

Tabela 4. Eigenvalues and percentages of inertia (RDA).

	Enseada dos Corais		Boa viagem	
	F1	F2	F1	F2
Eigenvalue	3,0101	1,9899	3,6842	1,3158
Constrained inertia (%)	60,2021	39,7979	73,6841	26,3159
Cumulative %	60,2021	100	73,6841	100,0000

Total inertia	60,2021	39,7979	73,6841	26,3159
Cumulative % (%)	60,2021	100	73,6841	100,0000

Tabela 5. Redundance analysis results of explanatory and responses variables, and observations.

	Enseada dos Corais				**Boa viagem**			
	Standardized canonical coefficients		Scores (Explanatory var):		Standardized canonical coefficients		Scores (Explanatory var):	
	F1	F2	F1	F2	F1	F2	F1	F2
Temp	-0,2955	-1,232	0,0214	-0,9998	-0,2659	-0,6317	-0,4549	-0,8905
pH	0,4783	0,0635	0,9890	-0,1480	0,4692	-0,3865	0,8335	-0,5526
Turb	-0,4908	-0,1058	-0,9934	0,1148	0,5395	-0,2086	0,9534	-0,3017
DO	-0,5200	-0,2119	-0,9995	0,0303	0,5584	-0,1179	0,9848	-0,1736
Sal	0,3023	-0,4092	0,8634	-0,5045	0,4443	0,4527	0,7724	0,6351
	Scores (Obs):		Contributions (Obs):			Scores (Obs):		Contributions (Obs):
Local	F1	F2	F1	F2	Local	F1	F2	F1	F2
EC1	1,4272	0,2669	0,6441	0,0225	BV1	1,4222	-0,2926	0,6396	0,0271
EC2	-0,9448	1,1025	0,2823	0,3844	BV2	-0,4577	1,3779	0,0662	0,6004
EC3	-0,4824	-1,3695	0,0736	0,5931	BV3	-0,9645	-1,0853	0,2942	0,3725

	Enseada dos corais			Boa viagem	
	Scores (Response var):			Scores (Response var):	
	F1	F2		F1	F2
P. tetrastomatica	0,1444	0,7821	*P. gymnospora*	0,2915	0,7085
D. delicatulla	-0,1812	-0,7743	*A. spicifera*	0,8143	0,1857
G. caudata	0,7720	-0,1912	*B. pennata*	0,8901	0,1099
C. racemosa	0,7893	-0,0969	*G. cearensis*	0,7289	0,2711
C. cupressoides	0,7944	-0,0367	*C. acicularis*	0,9594	0,0406

Capítulo 2

Tabela 1. Results of ANOVA one-way.

		SS	Degr. of (Freedom)	MS	F	p
% DPPH	Intercept	211487.02	1	211487.02	65885.90	0
	Sp/Concentração	67807.84	14	4843.42	1508.90	0
	Error	96.30	30	3.21		
		SS	Degr. of (Freedom)	MS	F	p
GAE DPPH	Intercept	254.30	1	254.30	55006.94	0
	Sp/Concentração	132.79	14	9.49	2051.72	0
	Error	0.14	30	0.00		
		SS	Degr. of (Freedom)	MS	F	p
% ABTS	Intercept	125009.17	1	125009.17	43677.05	0
	Sp/Concentração	9672.70	8	1209.09	422.44	0
	Error	51.52	18	2.86		
		SS	Degr. of (Freedom)	MS	F	p
GAE ABTS	Intercept	53.49	1	53.49	32643.53	0
	Sp/Concentração	7.63	8	0.95	582.32	0
	Error	0.03	18	0.00		
		SS	Degr. of (Freedom)	MS	F	p
% MCA	Intercept	31743.69	1	31743.69	1322.93	0
	Sp/Concentração	3957.71	4	989.43	41.23	0
	Error	239.95	10	24.00		
		SS	Degr. of (Freedom)	MS	F	p
GAE MCA	Intercept	595.61	1	595.61	1273.54	0
	Sp/Concentração	77.14	4	19.28	41.23	0
	Error	4.68	10	0.47		
		SS	Degr. of (Freedom)	MS	F	p
% TPC	Intercept	61578.92	1	61578.92	2397.90	0
	Sp/Concentração	38184.86	9	4242.76	165.21	0
	Error	513.61	20	25.68		
		SS	Degr. of (Freedom)	MS	F	p
GAE TPC	Intercept	894.43	1	894.43	2106.41	0
	Sp/Concentração	609.95	9	67.77	159.61	0
	Error	8.49	20	0.42		
		SS	Degr. of (Freedom)	MS	F	p
% FRAP	Intercept	39703.05	1	39703.05	24400.88	0
	Sp/Concentração	16815.18	5	3363.04	2066.87	0
	Error	19.53	12	1.63		
		SS	Degr. of (Freedom)	MS	F	p
GAE FRAP	Intercept	216.36	1	216.36	12336.45	0
	Sp/Concentração	181.25	5	36.25	2066.87	0
	Error	0.21	12	0.02		

Capítulo 3

Habitats observation

ANOVA assumptions and results:

Tabela 6. Assumptions for ANOVA test, normal residual distribution and homogenity of variances

	Levene (Homogenity)				Kolmogorov-Smirnov (normality)	
Variable	MS effect	MS error	F	p	d	p
DPPH	0,66693	1,80966	0,36854	0,77793	0.11548	>0.2
ABTS*	0,18021*	0,25663*	0,70222*	0,57679*	0.26180	>0.2
Chelators	11,60278	7,83693	1,48053	0,29156	0.16759	>0.2
Folin	0,35386	0,97157	0,36421	0,78084	0.1686	>0.2
FRAP	81,45685	36,70695	2,21911	0,16344	0.15559	>0.2

*Results for Brown-Forsythe test of homogenety of variances.

Tabela 7. Results of ANOVA one-way (left side) and post-hoc Tukey HSD test (right side).

DPPH										
	SS	Degr. of Freedom	MS	F	p		Sheltered	Tide pool	Plateau	Expose
						Sheltered				
Intercept	20709,1	1,0	20709,1	2548,9	0,0	Tide pool	0,896			
Habitat	198,6	3,0	66,2	8,1	0,0	Plateau	0,046	0,123		
Error	65,0	8,0	8,1			Exposed	0,013	0,032	0,774	

ABTS										
	SS	Degr. of Freedom	MS	F	p		Sheltered	Tide pool	Plateau	Expose
						Sheltered				
Intercept	113642,7	1,0	113642,7	353320,9	0,0	Tide pool	0,000			
Habitat	161,8	3,0	53,9	167,6	0,0	Plateau	0,000	0,001		
Error	2,6	8,0	0,3			Exposed	0,000	0,001	0,991	

Chelators										
	SS	Degr. of Freedom	MS	F	p		Sheltered	Tide pool	Plateau	Expose
						Sheltered				
Intercept	47202,4	1,0	47202,4	801,4	0,0	Tide pool	0,034			
Habitat	1389,1	3,0	463,0	7,9	0,0	Plateau	0,981	0,020		
Error	471,2	8,0	58,9			Exposed	0,820	0,011	0,957	

Folin									
SS	Degr. of	MS	F	p		Sheltered	Tide pool	Plateau	Expose

		Freedom				Sheltered				
Intercept	115768,2	1,0	115768,2	20389,5	0,0	Tide pool	0,000			
Habitat	3464,8	3,0	1154,9	203,4	0,0	Plateau	0,004	0,000		
Error	45,4	8,0	5,7			Exposed	0,001	0,000	0,000	

FRAP

	SS	Degr. of Freedom	MS	F	p	Sheltered	Sheltered	Tide pool	Plateau	Expose
Intercept	26418,0	1,0	26418,0	109,2	0,0	Tide pool	0,100			
Habitat	6780,8	3,0	2260,3	9,3	0,0	Plateau	0,636	0,019		
Error	1934,6	8,0	241,8			Exposed	0,172	0,005	0,696	

Tabela 8. Eigenvalues and percentages of inertia (RDA).

	F1	F2	F3	F4	F5
Eigenvalue	3,2865	1,2586	0,0727	0,0476	0,0001
Constrained inertia (%)	70,4422	26,9757	1,5586	1,0203	0,0031
Cumulative %	70,4422	97,4179	98,9765	99,9969	100,0000
Total inertia	65,7306	25,1714	1,4544	0,9521	0,0029
Cumulative % (%)	65,7306	90,9020	92,3563	93,3084	93,3114

Tabela 9. Redundance analysis results of explanatory and responses variables, and observations.

	Standardized canonical coefficients:					Scores (Explanatory var):				
	F1	F2	F3	F4	F5	F1	F2	F3	F4	F5
Temp	-3,143	-3,218	0,907	-0,016	0,030	-0,965	0,122	-0,098	0,025	0,002
pH	0,682	2,284	-0,490	-0,540	0,003	-0,880	0,403	-0,107	-0,060	0,001
Turb	-0,244	0,279	0,410	0,061	0,004	0,627	0,452	0,478	0,035	0,013
DO	0,719	1,353	-0,344	0,579	-0,015	-0,863	0,325	-0,156	0,160	0,002
Sal	-0,147	0,128	0,136	0,037	-0,021	-0,810	0,099	0,047	-0,081	-0,038
	Scores (Obs):					Contributions (Obs):				
	F1	F2	F3	F4	F5	F1	F2	F3	F4	F5
Exp1	0,795	0,272	0,642	-2,524	12,957	0,096	0,011	0,043	0,324	0,135
Exp2	1,000	0,656	1,386	1,121	-1,405	0,152	0,066	0,200	0,064	0,002
Exp3	1,363	0,469	-1,123	0,412	-14,698	0,283	0,034	0,132	0,009	0,174
Tp1	-1,028	0,621	0,325	-2,320	-2,100	0,161	0,059	0,011	0,274	0,004
Tp2	-1,011	0,765	0,394	0,297	9,887	0,155	0,090	0,016	0,005	0,079
Tp3	-0,754	0,768	-1,247	1,689	-4,421	0,087	0,090	0,162	0,145	0,016
She1	-0,558	-1,309	0,728	0,628	10,761	0,047	0,263	0,055	0,020	0,093
She2	-0,129	-1,045	0,679	-0,855	-22,177	0,003	0,167	0,048	0,037	0,396
She3	0,322	-1,197	-1,783	1,551	11,195	0,016	0,220	0,332	0,122	0,101
	Scores (Response var):					Contributions (Response var):				
	F1	F2	F3	F4	F5	F1	F2	F3	F4	F5
DPPH	0,878	0,588	-0,002	0,168	-0,005	0,185	0,217	0,000	0,467	0,130
FRAP	1,056	-0,197	-0,070	-0,133	-0,008	0,268	0,024	0,053	0,292	0,363
ABTS	0,542	0,934	0,020	-0,111	0,006	0,071	0,548	0,004	0,204	0,173
Folin	1,009	-0,427	0,236	0,007	0,003	0,245	0,114	0,604	0,001	0,037
Quel	0,980	-0,392	-0,177	0,047	0,007	0,231	0,097	0,339	0,036	0,297

Salinity x Temperature experiments:

Tabela 10. Assumptions for ANOVA, homogenity of variances Levene and Brown-Forsythe (*) tests.

	MS Effect	MS Error	F	p
Folin	76,48267	41,87152	1,826603	0,128114
ABTS	2,955252	5,577369	0,529865	0,803209
DPPH	1,906526	1,988354	0,958846	0,482204
Chelators	0,558480	0,390433	1,430411	0,239291
FRAP*	211,0971	223,8891	0,942865	0,492929
Y(II)*	0,001926	0,002907	0,662652	0,700948
Fv/Fm*	0,004263	0,002337	1,823628	0,128721

Tabela 11. Results of ANOVA factorial (left side) and post-hoc Tukey HSD test (right side).

Folin										
	Degr. Of Freedom	SS	MS	F	p		20°C	25°C	30°C	35°C
Temperature	3	6286,845	2095,615	13,061	0,000	20°C				
Salinity	1	1,213	1,213	0,008	0,931	25°C	0,003			
Temp * Sal	3	335,178	111,726	0,696	0,563	30°C	0,974	0,001		
Error	24	3850,833	160,451			35°C	0,183	0,000	0,358	
Total	31	10474,069								
ABTS										
	Degr. Of Freedom	SS	MS	F	p		20°C	25°C	30°C	35°C
Temperature	1	227257,382	227257,382	11850,193	0,000	20°C				
Salinity	3	528,747	176,249	9,190	0,000	25°C	0,010			
Temp * Sal	1	24,525	24,525	1,279	0,269	30°C	0,572	0,001		
Error	3	213,353	71,118	3,708	0,025	35°C	0,866	0,002	0,952	
Total	24	460,261	19,178							
DPPH										
	Degr. Of Freedom	SS	MS	F	p		20°C	25°C	30°C	35°C
Temperature	3	257,329	85,776	15,506	0,000	20°C				
Salinity	1	0,078	0,078	0,014	0,907	25°C	0,000			
Temp * Sal	3	35,206	11,735	2,121	0,124	30°C	0,478	0,001		
Error	24	132,766	5,532			35°C	0,921	0,000	0,841	
Total	31	425,379								
ETRmax										
	Degr. Of Freedom	SS	MS	F	p		20°C	25°C	30°C	35°C
Temperature	3	2,058	0,686	6,968	0,002	20°C				
Salinity	1	0,036	0,036	0,367	0,551	25°C	0,231			
Temp * Sal	3	0,920	0,307	3,114	0,045	30°C	0,381	0,987		
Error	24	2,363	0,098			35°C	0,001	0,077	0,038	
Total	31	5,377								
Chelators										

	Degr. Of Freedom	SS	MS	F	p
Temperature	3	6,742	2,247	1,691	0,196
Salinity	1	0,445	0,445	0,335	0,568
Temp * Sal	3	2,106	0,702	0,528	0,667
Error	24	31,895	1,329		
Total	31	41,188			

FRAP

	Degr. Of Freedom	SS	MS	F	p
Temperature	3	0,516	0,172	0,097	0,961
Salinity	1	1,230	1,230	0,692	0,414
Temp * Sal	3	5,068	1,689	0,950	0,432
Error	24	42,678	1,778		
Total	31	49,491			

Fv/Fm

	Degr. Of Freedom	SS	MS	F	p
Temperature	3	0,024	0,008	2,143	0,121
Salinity	1	0,010	0,010	2,680	0,115
Temp * Sal	3	0,023	0,008	2,072	0,131
Error	24	0,088	0,004		
Total	31	0,144			

Y(II)

	Degr. Of Freedom	SS	MS	F	p
Temperature	3	0,019	0,006	2,361	0,097
Salinity	1	0,000	0,000	0,004	0,951
Temp * Sal	3	0,007	0,002	0,819	0,496
Error	24	0,065	0,003		
Total	31	0,090			

Expoisure experiment:

ANOVA assumptions and results:

Tabela 12. Assumptions for ANOVA, homogenity of variances Levene's test.

	MS Effect	MS Error	F	p
Folin	53,14414	14,72405	3,609343	0,04579
ABTS	2,049905	4,327163	0,473729	0,706305
DPPH	0,799026	0,379305	2,106554	0,152805
Chelators	0,55848	0,390433	1,430411	0,239291
FRAP	5,038042	4,333341	1,162623	0,364344
Y(II)	0,000382	0,000143	2,664413	0,095274
Fv/Fm	0,000574	0,000439	1,309091	0,316759

Tabela 13. Results of ANOVA one-way (left side) and post-hoc Tukey HSD test (right side).

Folin

	Degr. Of Freedom	SS	MS	F	p
Treatment	3	6286,845	2095,615	13,061	0
Error	24	3850,833	160,451		
Total	31	10474,07			

	CD	DH	RH	CR
CD				
DH	0,879262			
RH	0,016864	0,004765		
CR	0,967637	0,991502	0,007723	

ABTS

	Degr. Of Freedom	SS	MS	F	p
Treatment	3	116,23	38,74	2,262	0,133509
Error	12	205,51	17,13		
Total	15	321,74			

DPPH

	Degr. Of Freedom	SS	MS	F	p
Treatment	3	6,324	2,108	1,056	0,403647
Error	12	23,947	1,996		
Total	15	30,271			

ETRmax

	Degr. Of Freedom	SS	MS	F	p
Treatment	3	2,49E+08	8,29E+07	0,84847	0,493659
Error	12	1,17E+09	9,78E+07		
Total	15	1,42E+09			

Chelators

	Degr. Of Freedom	SS	MS	F	p
Treatment	3	0,70371	0,23457	0,34318	0,79466
Error	12	8,20228	0,68352		
Total	15	8,90599			

FRAP

	Degr. Of Freedom	SS	MS	F	p
Treatment	3	303,15	101,05	5,692	0,011644
Error	12	213,05	17,75		
Total	15	516,2			

	CD	DH	RH	CR
CD				
DH	0,908988			
RH	0,024792	0,077795		
CR	0,985110	0,747323	0,013570	

Fv/Fm

	Degr. Of Freedom	SS	MS	F	p
Treatment	3	0,019604	0,006535	2,905	0,078459
Error	12	0,026994	0,00225		

Total	15	0,046598

Y(II)

	Degr. Of Freedom	SS	MS	F	p
Treatment	3	0,05044	0,016813	39,866	0,000002
Error	12	0,005061	0,000422		
Total	15	0,055501			

	CD	DH	RH	CR
CD				
DH	0,000202			
RH	0,000335	0,142886		
CR	0,998924	0,000202	0,000369	