MARINHA DO BRASIL
INSTITUTO DE ESTUDOS DO MAR ALMIRANTE PAULO MOREIRA
UNIVERSIDADE FEDERAL FLUMINENSE
PROGRAMA ASSOCIADO DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA
MARINHA

SARAH DE JESUS CANTARINO

Quantificação e extração de caulerpina e seu potencial anti-incrustante

ARRAIAL DO CABO / RJ

2018

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QUANTIFICAÇÃO E EXTRAÇÃO DE CAULERPINA E SEU POTENCIAL ANTI-INCRUSTANTE

Trabalho de conclusão de curso, apresentado ao Instituto de Estudos do Mar Almirante Paulo Moreira e a Universidade Federal Fluminense, como requisito parcial para a obtenção do grau de Mestre em Biotecnologia Marinha.

Orientador: Prof. Dr. Ricardo Coutinho

Co-orientadora: Dra. Sabrina Teixeira Martinez

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Dissertação apresentada ao Instituto de Estudos do Mar Almirante Paulo Moreira e a Universidade Federal Fluminense, como requisito parcial para a obtenção do título de Mestre em Biotecnologia Marinha.

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Arraial do Cabo, 21 de setembro de 2018

FICHA CATALOGRÁFICA

Cantarino, Sarah de Jesus

Quantificação e extração de caulerpina e seu potencial anti-incrustante

50 Páginas, 10 Figuras, 2 Tabelas.

Orientador: Prof. Dr. Ricardo Coutinho

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Dissertação (Mestrado em Biotecnologia Marinha) –

1. Produtos Naturais; 2. *Caulerpa racemosa*; 3. Caulerpina; 4. Teste anti-incrustante; 5. Método de identificação; 6. Método de extração.

I. Coutinho, Ricardo. II. Universidade Federal Fluminense. III. Programa de Pós-Graduação em Biotecnologia Marinha, IEAPM/UFF.

AGRADECIMENTOS

Agradeço ao IEAPM e ao CNPq pelo apoio e suporte ao desenvolvimento do meu mestrado em Biotecnologia Marinha.

Ao Dr. Ricardo Coutinho, por ter aceito ser meu orientador e proporcionado essa oportunidade de para quem almejava aprender e se desenvolver nos estudos do mar.

À Dra. Sabrina T. Martinez, pela co-orientação que me guiou da bancada à síntese deste trabalho e por todo apoio e ensinamentos nas maravilhas do mundo das moléculas e dos produtos naturais.

Ao Dr. Lohengrin, ao Departamento de Química, ao Msc. Wladmir, à Dra. Daniela Fernandes, à Dra. Fernanda Braz, à Dra. Angélica Soares e ao Dr. Heitor Duarte, aos alunos Juliana e André, À Dra. Maria Helena, à Dra. Lu altvater, à Msc. Ellen Oliveira, ao Dr. José Eduardo, ao Hector Fabian Messano e à Msc. Ana Polycarpa pelo apoio no trabalho metodológico e nos testes anti-incrustantes.

Aos colegas e amigos do Biotecmar, que foram essenciais ao longo desta temporada em Arraial do Cabo, desde apoio no laboratório, nas coletas às animadas celebrações cabistas.

Aos meus amigos, que constantemente me fortaleceram para viver o essencial da vida. No apoio ao longo do mestrado, agradeço especialmente a: Ana T. Melo, Fernanda Gomes, João Depoli, Jean Vencioneck, Julia Cantarino, Ana Polycarpa, Mirelly Prado, Gessica Lima, Vitor Paganini Mayer, Camila Reveles, Noreyni Ndiaye, Ellen Oliveira, Priscila Ribeiro, Camila Haldek, Wladmir, Lucas Paes, Julio Cesar Monteiro e gatinha Saphyra.

Às famílias “de Jesus”, por terem me ajudado com abrigo no primeiro ano de Msc, e aos “Cantarinos”, pelo suporte na minha vinda para Arraial do Cabo.

À América e aos gêmeos, Thomas e Sven, à Surya, ao Yuri, por serem meus grandes amigos e irmãos, sempre juntos apesar da distância.

Aos meus pais, alicerces do meu desenvolvimento e apoiadores de todas as jornadas e buscas na minha vida.

Agradeço à Deus, por me guiar para o caminho de instrumento de vossa paz.

Aos meus pais e irmãos, pelo apoio e amor incondicional.

Aos avós in memoriam António dos Santos de Jesus e Mário Ribeiro Cantarino Filho, por serem fortes referências de trabalho duro e da visão científica

RESUMO

Caulerpina é um alcalóide bisindólico descrito como a substância principal do gênero *Caulerpa.* Este produto natural tem sido bastante estudada e de grande interesse devido as suas atividades biológicas. Porém ainda há uma grande distância da identificação desta substância à viabilidade comercial. Assim, este trabalho teve por objetivo identificar a caulerpina através de uma análise de rotina prática e econômica e ainda verificar uma potencial atividade biológica anti-incrustante. O método de quantificação por espectrofotometria Ultravioleta-visível (UV-vis) foi utilizado para estimar a quantidade de caulerpina nos extratos brutos. A alga *Caulerpa racemosa* foi submetida à maceração, extração por Soxhlet, extração assistida por ultrassom e extração assistida por micro-ondas. O método mais eficiente de extração teve os parâmetros de solvente verde, temperatura e tempo otimizados. As melhores condições para extração de caulerpina neste estudo foram obtidas com a irradiação de micro-ondas em etanol durante 7 minutos a 90 ^{o}C. O potencial anti-incrustante foi analisado mediante bioensaios de discos de difusão com bactérias isoladas de biofilme marinho, *Vibrio aestuarians*, *Pseudoalteromonas elyakovii*, *Polaribacter irgensii, Pseudomonas fluorescens* e *Shewanella putrefaciens*,e ensaios de assentamento larval com macroincrustantes *Bugula neritina* e *Tubastraea coccinea*. Não foi possível averiguar ação anti-incrustante da caulerpina e do extrato de *Caulerpa racemosa* a partir destas análises, porém diferentes alternativas foram apresentadas.

Palavras-chave: Produtos naturais. *Caulerpa racemosa*. Caulerpina. Métodos verdes. Extrato de algas marinhas. Bioincrustação.

ABSTRACT

Caulerpin is a bisindolic alkaloid described as the major substance of the genus. This natural product has been of great interest due to its biological activities. However, there is still a major distance from the identification of this substance to commercial viability. Thus, this work aimed to identify caulerpin through a practical and economic routine analysis and also to verify a potential anti-fouling biological activity. The quantification method of caulerpin content by Ultraviolet-visible (UV-vis) spectrophotometry was evaluated to estimate the quantity of caulerpin in the crude extracts. The algae *Caulerpa racemosa* was subjected to maceration, Soxhlet extraction, ultrasound-assisted extraction and microwave-assisted extraction. The most efficient caulerpin extraction method had the parameters green solvent, temperature and time consumed optimised. The best conditions for caulerpin extraction in this study were achieve with microwave irradiation in ethanol during 7 minutes at 90 oC. The antifouling potential were conducted bioassays of diffusion discs with bacteria isolated from marine biofilm, *Vibrio aestuarians, Pseudoalteromonas elyakovii, Polaribacter irgensii, Pseudomonas fluorescens* and *Shewanella putrefaciens*, and larval settlement assays with macrofouling *Bugula neritina* and *Tubastraea coccinea*. It was not possible to investigate the anti-fouling action of caulerpin and *Caulerpa racemosa* extract from these analyses, but different alternatives were presented.

Keywords: Natural products. *Caulerpa racemosa.* Caulerpin. Green methods. Seaweed extract. Biofouling.

SUMÁRIO

INTRODUÇÃO GERAL

O oceano é uma fonte de diversos produtos naturais. Estas substâncias atuam na mediadores de interações ecológicas no ambiente aquático, sendo estudadas para na identificação química e sua caracterização estrutural, na investigação da atividade biológica como fármacos e anti-incrustantes, no papel ecológico e evolutivo e no uso como marcadores taxonômicos, filogenéticos e biogeográficos (TEIXEIRA, 2013).

Nos últimos cinquenta anos, um total de 21.855 compostos marinhos foram descobertos (HALDAR & MODI, 2015). No campo dos fármacos, apenas quatro destes compostos foram transformados em drogas aprovadas pela *Food and Drug Administration* (FDA) e pela *European Agency for the Evaluation of Medicinal Products* (EMEA), conforme apresentado na Figura 1. Além destas, existem 13 compostos em diferentes fases de testes clínicos e vários outros em testes pré-clínicos (MAYER et al., 2010).

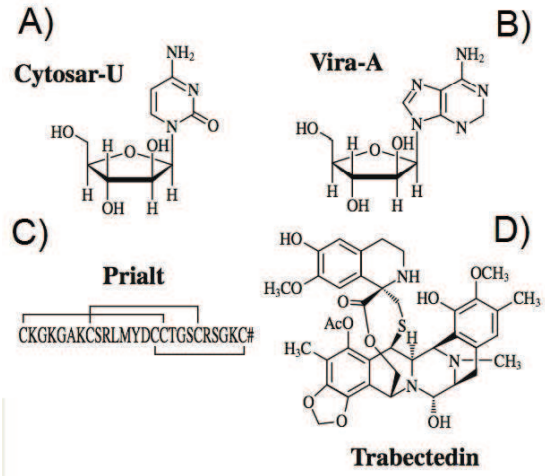

Figura 1: Drogas aprovadas pela FDA: A) cytarabine (Cytosar-U e Depocyt), B) vidarabine (Vira-A) e C) ziconotide (Prialt); Droga aprovada pela EMEA: D) Trabectedin (Yondelis). Adaptado de Haldar & Mody (2015).

Os passos para levar o produto natural identificado ao mercado passam por um longo processo de identificação, avaliação de potenciais usos e viabilidade comercial. Ainda não há um medicamento produzido a partir de produtos naturais de algas. Diante da necessidade de fortificar linhas de pesquisas como produtos naturais de algas no Brasil foi criada a Redealgas - Rede Nacional em Biotecnologia de Macroalgas Marinhas em 2005. Esta rede de pesquisa tem por intuito proporcionar a discussão de programas

de pesquisas e linhas de ações na biodiversidade brasileira de algas no tocante a Ciência, Tecnologia e Inovação (TEIXEIRA, 2013). O foco da maioria dos trabalhos relacionados a ecologia química de macroalgas no Brasil utilizou de extratos brutos ou substâncias majoritárias para verificar atividades como por exemplo antiherbivoria (PEREIRA et al., 2011).

Dentre as principais algas pesquisadas no Brasil quanto aos seus produtos naturais encontra-se a alga verde *Caulerpa racemosa*. Esta alga distribui-se em águas rasas tropicais e subtropicais, amplamente distribuída (YANG et al., 2014).

A substância majoritária encontrada em extratos de *C. racemosa* é a caulerpina (ROCHA et al., 2007). Esta foi isolada de outras espécies do gênero *Caulerpa* (AGUILAR-SANTOS, 1970, MCCONNELL et al., 1982, CAPON et al., 1983, MAO et al., 2006, SCHWEDE, et al. 1987, VEST et al., 1983), de algas verdes e vermelhas, *Codium decorticatum* (ANJANEYULU et al., 2010 apud GÜVEN et al., 2010), *Halimeda incrassate* (YAN et al., 1999 apud GÜVEN et al., 2010), *Laurencia majuscule, Hypnea concornis* e *Caloglossa leprieurii* (XU & SU, 1996 apud GÜVEN et al., 2010), *Chondria armata* (GOVENKAR & WAHIDULLA, 2000) e até mesmo de uma esponja desconhecida (ALTARABEEN et al., 2015).

A descoberta da substância foi obtida através de um estudo fitoquímico da alga comestível *Caulerpa racemosa* por Aguilar-Santos (1968; 1970) devido ao diferente sabor, registrado como picante. Esta substância causa uma suave sensação anestésica na língua e nos lábios (BHAKUNI & RAWAT, 2005). A toxicidade de alguns alcaloides pode ser captada por alguns mamíferos, como os humanos, em resposta a esse grande grupo de substâncias, por exemplo, através do sabor amargo no paladar (NETZ & OPATZ, 2015). Os alcaloides são "*componentes orgânicos cíclicos contendo nitrogênio em um estado oxidativo negativo que possui limitada distribuição nos seres vivos*" (PELLETIER, 1983, p.26). Ao nitrogênio é atribuída a atividade biológica para a maioria das substâncias deste grupo de metabólitos secundários que é um dos maiores e mais complexos (NETZ & OPATZ, 2015).

A caulerpina é um alcalóide bisindólico de nome ciclooctа(1,2-b:5,6-b')diindol-6,13-ácido dicarboxílico, 5,12-dihidro, ester dimetil (ChemIDplus). Sua estrutura foi elucidada pela primeira vez por Maiti et al. (1978) após ter sido incorretamente descrita como um

derivado de fenazina (Fig. 2) por Aguilar-Santos (1970). Até o presente momento é o único produto natural contendo um ciclooctatetraeno (BLOUIN et al., 2018).

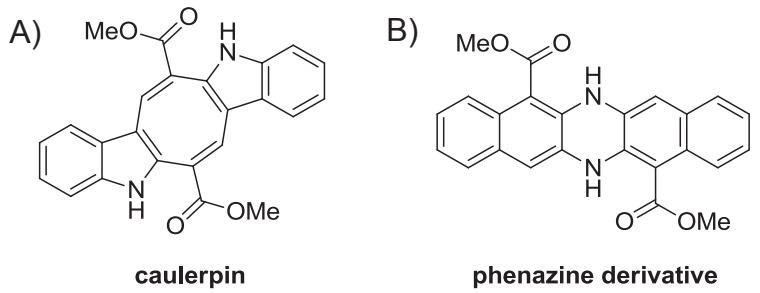

Figura 2: Estrutura química da (a) caulerpina e do (b) derivado de fenazina (C24H18N2O4).

A atividade biológica da caulerpina tem sido recentemente de grande interesse (MACEDO et al., 2012). Há pelo menos 8 patentes relacionadas que correspondem a: método de síntese, método de extração ou usos da substância (crescimento de plantas, anticancerígenos e afins) no PATENTSCOPE®. Dentre estas, destaca-se uma patente brasileira (n°. PI 1009162-9 A2) para a ação anti-inflamatória e contra dor (CAVALCANTE-SILVA et al., 2014; 2016). Ademais, várias atividades biológicas são descritas, como potenciais antitumoral, antibacteriana, antiviral, larvicida, regulador de crescimento vegetal e anticorrosivo (*eg.* ALARIF et al. 2010, CAVALCANTE-SILVA et al. 2013, 2014, 2016, CHAY et al. 2014, KAMAL AND SETHURAMAN 2012, LIU et al. 2009, LORENZO et al. 2015, MACEDO et al. 2012, MAO et al. 2006, RAUB et al. 1987, SENTHILRAJA et al. 2017).

Além da reconhecida aplicação farmacológica, destaca-se o seu potencial uso como inibidor de corrosão, verificado em testes com aço de baixo teor de carbono utilizado na extração de petróleo marinho (KAMAL & SETHURAMAN, 2012). Diante das demandas globais, as indústrias tem procurado inibidores seguros e ambientalmente adequados. Uma alternativa efetiva, com baixa ou nenhuma toxicidade natural, fácil disponibilidade e biodegradabilidade tem sido os extratos de plantas, em que suas atividades inibidoras de corrosão parecem estar atreladas a presença de substâncias heterocíclicas como alcaloides (RAJA & SETHURAMAN, 2008). Extensas pesquisas têm sido realizadas para avaliar o comportamento de materiais submersos em água do mar,

sendo a corrosão e a incrustação fatores de degradação. A bioincrustação é um dos principais desafios para a biotecnologia marinha (DA GAMA et al., 2002). Há uma busca crescente por alterativas ambientalmente adequadas, visto que tradicionalmente são empregados metais pesados e biocidas tóxicos em revestimentos de cascos de navios e afins (ABARZUA & JAKUBOWSKI, 1995; CLARE, 1996; PEREIRA et al., 2002). Várias algas foram reportadas com atividade anti-incrustante (eg. DE NYS et al., 1996; DA GAMA et al. 2008) e mais busca por novos compostos macroalgas tropicais são indicados a partir de uma abordagem multidisciplinar de química orgânica, microbiologia e ecologia para garantir aplicações biotecnológicas promissoras (DAHMS & DOBRETSOV, 2017).

A atividade anti-incrustante em extratos de *Caulerpa racemosa* foi verificada no estudo de Dobretsov et al. (2006). Estes autores indicam a necessidade de investigar quais compostos desta alga poderiam estar atuando contra os organismos incrustantes. A caulerpina, como substância majoritária da espécie, torna-se de interesse para esta investigação visto também a sua ação protetora à corrosão em meio marinho e baixa toxicidade (MOVAHHEDIN et al., 2014; ROCHA et al., 2007; VIDAL et al., 1984). Desta forma, avaliar sua ação anti-incrustante torna-se uma alterativa ambientalmente adequada em contraponto com o atual uso de metais pesados e biocidas tóxicos em revestimentos de cascos de navios e afins (PEREIRA et al., 2002; DA GAMA et al., 2003).

Apesar das várias aplicabilidades desta substância, o maior desafio está em sua obtenção. Uma das maiores dificuldades é como se obter a substância sem destruir os bancos naturais das algas (FERNANDES et al., 2014). O processo de síntese da caulerpina (*eg.* MAITI et al., 1978; TALAZ & SARACOGLU, 2010; CHAY et al., 2014), pode ser economicamente inviável em larga escala mas a obtenção de produtos naturais através do cultivo de algas é uma possibilidade devido as suas características de fácil e abundante produção (HONG, 2007).

Em todo modo, é fundamental que seja estabelecido um protocolo eficiente (*eg*. GROSSO et al., 2015) de extração da substância. Diante da investigação para possíveis aplicações da caulerpina, cabe questionar sua posterior viabilidade para uso comercial.

Uma forma de tornar viável é aumentar a praticidade e economia na identificação da substância. Os métodos fotométricos são amplamente empregados para análises de rotina de produtos comerciais (BRANQUINHO et al, 2012; SAMPAIO, et al. 2008;

SANGSTER & STUART, 1965; SREEVIDYA & MEHROTRA, 2003). Através do presente estudo busca-se identificar a substância a partir de seus picos de absorção em comprimentos de onda característicos na luz ultravioleta (222, 270, 292 e 317 nm - MAITI & THOMSON, 1977). Além disto, diferentes métodos de extração da caulerpina e seus rendimentos atrelados variam entre os estudos (*eg.* AGUILAR-SANTOS et al., 1970; MCCONNELL et al., 1982; CAPON et al., 1983; VEST et al., 1983; VIDAL et al., 1984; RAUB et al., 1987; SCHWEDE et al., 1987; GOVENKAR & WAHIDULLA, 2000; RANIELLO et al., 2006; MOVAHHEDIN et al., 2014; NAGAPPAN & VAIRAPPAN, 2014), assim, a otimização dos métodos para a quantificação e obtenção da caulerpina se torna essencial.

Diante do disposto, este trabalho apresenta no “Capítulo 1”, a quantificação de caulerpina em extratos de *C. racemosa* e a comparação técnicas de extração para obtenção de caulerpina e no Capítulo 2”, a avaliação do potencial anti-incrustante do extrato de *C. racemosa* e da caulerpina.

OBJETIVOS

OBJETIVO GERAL

Otimizar a metodologia de extração de caulerpina e investigar o potencial anti-incrustante do extrato de *Caulerpa racemosa* e da caulerpina.

OBJETIVOS ESPECÍFICOS

1. Avaliar método de identificação e quantificação do extrato de *Caulerpa racemosa* por espectrofotômetro;
2. Avaliar métodos de extração de caulerpina.
3. Testar a atividade da caulerpina e do extrato de *Caulerpa racemosa* em organismos incrustantes: bactérias formadoras de biofilme, briozoário *Bugula neritina e* coral *Tubastraea coccinea*;

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CAPÍTULO I

OPTIMISATION OF CAULERPIN EXTRACTION FROM *CAULERPA RACEMOSA* (FORSSKÅL) J.AGARDH 1873

Optimisation of caulerpin extraction from *Caulerpa racemosa* (Forsskål) J.Agardh 1873

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Abstract

Caulerpin is a bisindolic alkaloid described as the major substance of the genus *Caulerpa*. Caulerpin extraction techniques and its yields vary in the literature. The main objective of this paper is to quantify the caulerpin in the extracts and compare four extraction methods. The quantification method of caulerpin content by Ultraviolet-visible (UV-vis) spectrophotometry was evaluated and an analytical curve was elaborated to estimate the quantity of caulerpin in the crude extracts. The algae *Caulerpa racemosa* was subjected to maceration, Soxhlet extraction, ultrasound-assisted extraction and microwave-assisted extraction. Initially, all methods employed methanol as the solvent in the ratio of 10 mL/g (solvent/dried seaweed). The most efficient caulerpin extraction method had the parameters green solvent, temperature and time consumed optimised. The best conditions for caulerpin extraction in this study were achieve with microwave irradiation in ethanol during 7 minutes at 90 °C.

Keywords *Bisindole* alkaloid · Green methods · Extraction · Natural product

INTRODUCTION

Caulerpin (Fig. 1a) is a bisindolic alkaloid compound with an eight-membered cyclic ring with two esters and two indole rings, cycloocta(1,2-b:5,6-b')diindole-6,13-dicarboxylic acid, 5,12-dihydro-, dimethyl ester (ChemIDplus). This structure was elucidated for the first time by Maiti et al. (1978), after being incorrectly described as a phenazine derivative (Fig. 1b) (Aguilar-Santos 1970). Until now, is the only natural product described with a cyclooctatetraene (Blouin et al. 2018).

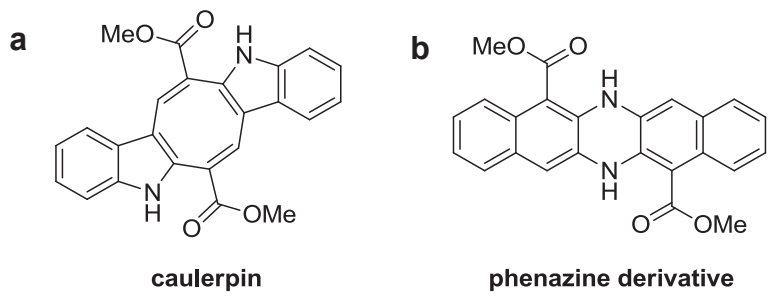

Fig. 1 Chemical structure of (a) caulerpin and (b) phenazine derivative ($C_{24}H_{18}N_2O_4$).

Initially, caulerpin was isolated from seaweeds of the genus *Caulerpa* (Aguilar-Santos 1970, McConnell et al. 1982, Capon et al. 1983, Mao et al. 2006, Schwede et al. 1987, Vest et al. 1983) and was then mentioned as a chemotaxonomic marker of *Caulerpa* (Schwede et al. 1987). But it was also isolated, in small quantity, from green and red algae, *Codium decorticatum* (Anjaneyulu et al. 2010 apud Güven et al. 2010), *Halimeda incrassate* (Yan et al., 1999 apud Güven et al. 2010), *Laurencia majuscule*, *Hypnea concornis* and *Caloglossa leprieurii* (Xu and Su 1996 apud Güven et al. 2010), *Chondria armata* (Govenkar and Wahidulla 2000) and even from an unknown sponge (Altarabeen et al. 2015).

The *genus Caulerpa* have a great amout of caulerpin (Vest et al. 1983) and occurs widely, notably in shallow-water tropical and subtropical areas (Yang et al. 2014). In the Indo-Pacific, the traditional harvesting practices and mariculture of many *Caulerpa* species are very common since they are edible seaweeds rich in protein, lipid, fatty acid, antioxidants, vitamins, minerals and secondary metabolites (Nagappan and Vairappan 2014, De Gaillande et al. 2017).

Currently, there is a great interest in this substance as a possible new anti-inflammatory and antinociceptive drug (Cavalcante-Silva et al. 2013, 2014, 2016, De Souza et al. 2009) and, also, because of its others biological activities such as anticancer (Liu et al. 2009, Senthilraja et al. 2017), antibacterial (Chay et al. 2014), antiviral (Macedo et al. 2012), larvicidal (Alarif et al. 2010), treatments of Alzheimer's disease (Lorenzo et al. 2015) and type-II diabetes and obesity (Mao et al; 2006), plant growth regulator (Raub et al. 1987) and anticorrosion (Kamal and Sethuraman 2012).

Secondary metabolites as natural products may be of interest to the pharmaceutical and technological industry, but still present a great barrier of industrial viability. The small amount of natural compounds from seaweeds and the difficulty isolating it in large quantities affects the continuity of pre-clinical applications (De Souza Barros et al. 2015). This can be one reason why there are no drugs from seaweeds yet, which is why the sustainable exploitation is needed (Fernandes et al. 2014).

One of the biggest challenges for commercial employment is how to obtain caulerpin. Many techniques are cited, achieving different yields (eg. Aguilar-Santos 1970, Mcconnell et al. 1982, Capon et al. 1983, Vest et al. 1983, Vidal et al. 1984, Raub et al. 1987, Schwede et al. 1987, Govenkar and Wahidulla 2000, Raniello et al. 2007, Movahhedin et al. 2014, Nagappan and Vairappan 2014).

Even though organic synthesis is one of the alternatives to obtain compounds whose natural source is in small amount, in the case of caulerpin, the developed methods present poor yields (Maiti et al. 1978, Chay et al. 2014). The caulerpin has been known for more than 50 years and cited in many articles, but an optimised protocol has not yet been proposed.

In this way, the Green Chemistry concept can be considerate. Some of these techniques are considered as ''green techniques'' as they comply with standards set by Environmental Protection Agency, USA. These include safe solvents auxiliaries, energy efficiency, use of renewable feedstock, reduce of derivatives, degradation prevention, atom economy, and time analysis for pollution prevention and inherently safer chemistry for the prevention of accident (Azmir et al. 2013, Anastas and Kirchhoff 2010). An efficient alternative is to analyse the target content in raw material with a rapid and reliable routine analysis (Sampaio et al. 2018). Ultraviolet-visible spectrophotometry (UV-Vis) as a quantification method of the caulerpin content in crude extract is explored in this article.

Extraction principle involves the hydration and swelling of the dried biological material followed by the transference of mass from the soluble compounds to the solvent by diffusion and osmotic processes. Stirring, temperature or vibration can enhance the mass transfer (Toma et al. 2001). Optimising the extraction method of a compound depends on decreasing time and amount of solvent consumed, expenses and labor, as well as exhausting and avoiding the degradation of the target compound, shortening operation techniques and enabling automation (Fulzele and Satdive 2005, Sticher 2008).

Now days, many extraction techniques are availed with significant improvement in extraction efficiency and selectivity with differences in the solvent volume, extraction time and extraction efficiency (Sticher 2008). The classic techniques of caulerpin extraction using maceration and Soxhlet apparatus are widely known (*eg.* Schwede et al. 1987, Nagappan and Vairappan 2014 for maceration and Movahhedin et al. 2014, Vidal et al. 1984 with soxhlet). Here these

methods were compared with green techniques (Sticher 2008) like ultrasound (Raniello et al. 2007) and microwave irradiation. Some reviews considered ultrasound-assisted extraction as a classic extraction procedures (Bucar et al. 2013), some not (Sticher 2008).

Thus, the aim of this work was to compare extraction techniques with crude extracts quantification of caulerpin content in order to increase the utilization of organic material, to reduce expenses and to choose the most efficient method.

MATERIALS AND METHODS

Samples and Extracts preparation

Samples of the seaweed *Caulerpa racemosa* were collected at the infralittoral zone (Orla Bardot, Búzios, Rio de Janeiro – Brazil, 22°45'05.7"S 41°53'03.0"W – Sisbio 57550-1) and transported in cooler box to the laboratory at the Almirante Paulo Moreira Institute of Marine Studies (IEAPM).

The material was kept in aquarium with aeration during the screening and washed in sea water and distilled water to remove sand and associated organisms. After that, the algae was stored in ultra-freezer (-80 ºC) and than, dried at room temperature for 5 days. The dried algae was submitted to four different methods of extraction (dynamic maceration, Soxhlet extraction, ultrasound-assisted extraction and microwave-assisted extraction). Methanol was used as the solvent in the proportion of 10 mL/g for the different extraction methods.

In dynamic maceration, the biological material was extracted with a magnetic stirrer IKA C-MAG HS 7. The extraction was performed for 72 hours, as described in the literature (Govenkar and Wahidulla 2000, Nagappan and Vairappan 2014). Follow the relation of time and temperature by Arhennius's law to accelerate the reaction (Arrhenius-based rate acceleration) (eg. Obermayer et al. 2016), the Soxhlet extraction for 23h at 65°C (methanol boiling point) and microwave-assisted extraction was performed for 21 min at 130 °C (Model Anton Paar Monowave 300). The ultrasound-assisted extraction (ultrasonic bath NOVA) was accomplished at room temperature in the same time of the microwave-assisted extraction (21 min), but the temperature with microwave was almost five times higher. All solutions were filtered and concentrated using a rotary evaporator. The yield of the extracts was calculated from the weight of the extract in relation to the weight of the dried seaweed, as followed:

$$\text{Total extract yield \%} = FM/IM \times 100$$

Where FM = final mass of extract (g) ; IM= inicial mass of dried seaweed (g).

The crude extract obtained from each extraction method were submitted to caulerpin quantification with spectrophotometer. The method that presented the highest efficiency of caulerpin extraction was selected to evaluated the interference of the green solvents (methanol, ethanol and water), different temperature (90 °C, 130 °C and 170 °C) and different extraction time (7min, 21 min and 35 min). For this purpose, new samples of *C. racemosa* was collected (Praia dos Anjos, Arraial do Cabo – Brazil, 22°58'43.9"S 42°01'08.8"W- Sisbio 57550-1).

Methods of analysis

The fingerprint of a known compound can be quickly verified by TLC (Bucar et al. 2013). To verify the presence of caulerpin, the thin-layer chromatography (TLC) was conducted as described by Alarif et al. (2010) on silica gel 60 PF254 plates and revealed with ceric sulfate reagent and ultraviolet light (UV) of 254nm and 365nm. Pure caulerpin was used as a standard to verify the substance in the extracts.

Quantification of caulerpin was proceed by photometric method. The analysis was carried out by scanning wavelenghts with Cary 60 UV-vis spectrophotometer (Agilent Technologies Inc) with UV quartz cell. The extract

samples were weight in an analytical balance (0.01 mg accuracy, AUW-220D Model – Shimadzu) and solubilized with assistance of the ultrasound (> 1 min). The stock solution was prepared from solid samples in EtOH of analytical grade for UV absorption. Measures of each extract samples at 0.5 mg/mL (extract/ethanol) were performed in triplicate. The results were showed as equivalent of caulerpin (mg/mL) and the values are presented as means ± standard deviation. The EtOH of analytical grade was used as white solution for the calibration. The major peak of caulerpin (λ_{max} 317 nm - Maiti and Thomson 1977) at the UV-vis absorption spectrophotometry was used with a serial dilution of this compound (n=10) to construct a calibration curve. A recovery rate of caulerpin was calculated (Sreevidya and Mehrotra 2003):

Recovery % = Observed concn (mg/mL) / actual concn (mg/mL) x 100

Where, observed concentration was estimated by the absorbance of each sample calculated with linear regression on the calibration curve and actual concentration was obtained from pure caulerpin.

Statistical analysis

The homogeneity of variances and normality of the observed concentration data was examined using the Shapiro-Wilk tests. To calculate the significance of the differences between the concentration of each extract a one-way analysis of variance (ANOVA) *post hoc* Tukey test was performed. The resulting p-values were considered to be statistically significant if $p < 0.05$.

RESULTS AND DISCUSSION

All extracts were analysed on a TLC plate at 254 nm and show a caulerpin band (Rf 0,61). The quantification of caulerpin in the crude extract was achieved through UV-vis spectroscopy.

The spectrophotometric method is a widely employed for routine analysis of commercial samples and natural products (Branquinho et al. 2012, Sampaio, et al. 2008, Sangster and Stuart 1965, Sreevidya and Mehrotra 2003).

The peak of 317 nm was used to estimate the caulerpin concentration because of the higher absorbance and linearity of its equation ($R^2 = 0,9943$):

$$y = 27,479x + 0,0709$$

Only the wavelengths of maximum absorbance (λ_{max}) is used to acquire the calibration curve, were concentrations are estimated from the absorbance in the linear relationship as described by the Lambert-Beer law. The method was selective to quantify caulerpin at a specific wavelength. The determination of caulerpin recovery concentrations were calculated with calibration curve points (n=6). The recovery rate of 101,67 % (± 8,59) indicating the accuracy and it is possible to quantify major significant differences between extracts. Sreevidya and Mehrotra (2003) recommend it use industrial routine analysis of plant materials containing alkaloids because of the practical and accurate results found with ajamalicine, papaverine, cinchonine, piperine and berberine.

Caulerpin has a maximum wavelength absorbance (λ_{max}) at 317 nm and absorption in the wavelength of 222, 270 and 292 nm (Maiti and Thomson 1977). Others substances present in *Caulerpa racemosa* extracts absorb UV-vis light. Although caulerpin is the major alkaloid found in *Caulerpa racemosa* extracts, there are other alkaloids recognized in this seaweed. They are monomethyl caulerpinate, caulerpin acid (Anjaneyulu et al. 1991), caulerchlorin (Liu et al. 2012), caulersin (Su et al. 1997), racemosin A, racemosin B (Liu et al. 2013) and racemosin C (Yang et al. 2014), showed in the fig. 2.

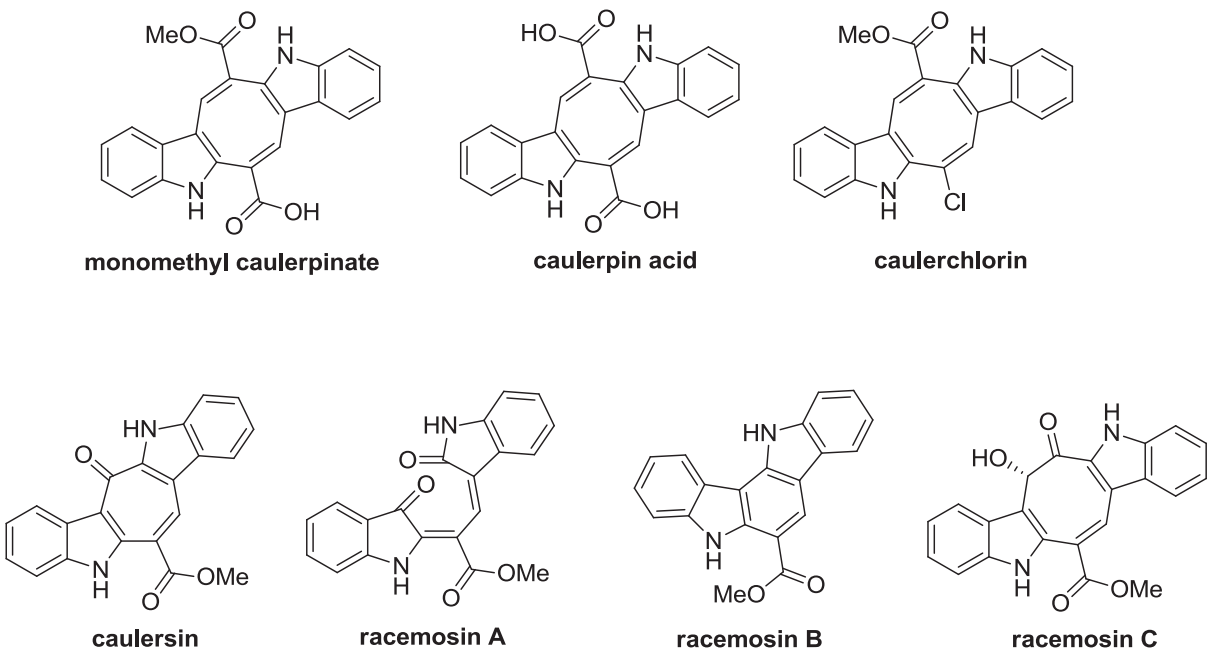

Fig. 2 Chemical structures of alkaloids described in literature for *Caulerpa racemosa* extracts.

However, the absorbance at 222 nm is the only wavelength that is similar for caulerpin, monomethyl caulerpinate, caulerpin acid and caulersin (table 1). This can interfere in the crude extract spectrum as a matrix effect. Since the absorption peaks at 220 to 225 nm are usually found in alkaloids and the free acids due to the unsaturated ester or acid (Sangster and Stuart 1965), they were not used to estimate the caulerpin content in the extracts.

Table 1 Maximum absorbance wavelengths from *Caulerpa racemosa* alkaloids.

Compound	ʎ max (nm)	Solvent	Reference
Caulerpin	**222**, 270, 292 and 317	Ethanol	Maiti and Thomson 1977, Aguilar-Santos 1970
Monomethylcaulerpinate	219, **221** and 306	-	Anjaneyulu et al. 1991
Caulerpin acid	**221** and 309	-	Anjaneyulu et al. 1991
Caulerchlorin	232, 265 and 299	Methanol	Liu et al. 2012
Caulersin	206, **220**, 265, 273 and 311	Ethanol	Su et al. 1997
Racemosin A	225, 273 and 342	Methanol	Liu et al. 2013
Racemosin B	217, 238, 284 and 355	Methanol	Liu et al. 2013

Racemosin C	210, 223 and 300	Methanol	Yang et al. 2014

Extraction method comparison

The first phase of this work consisted in obtaining *C. racemosa* extract by four different methods, classic methods (dynamic maceration and Soxhlet extraction) and non-conventional methods (ultrasound-assisted extraction and microwave assisted extraction). The highest extract yield was obtain with Soxhlet, 32.062 % (IM= 5g; FM = 1.6031 g). Followed by dynamic maceration with 19.502 % (IM= 5g; FM = 0.9751 g) and microwave-assisted extraction with 17.7 % yield (IM= 1.2g ; FM = 0.2124 g). The ultrasound-assisted extraction achieved only 11.84 % yield (IM= 1.2 g; FM =0.1421).

The extracts and the isolated compound have similar spectra curves (Fig. 3). The caulerpin at 0.125 mg/mL was used as pattern and the highest absorbance value in the wavelengths measures is observed in the microwave-assisted extraction spectrum.

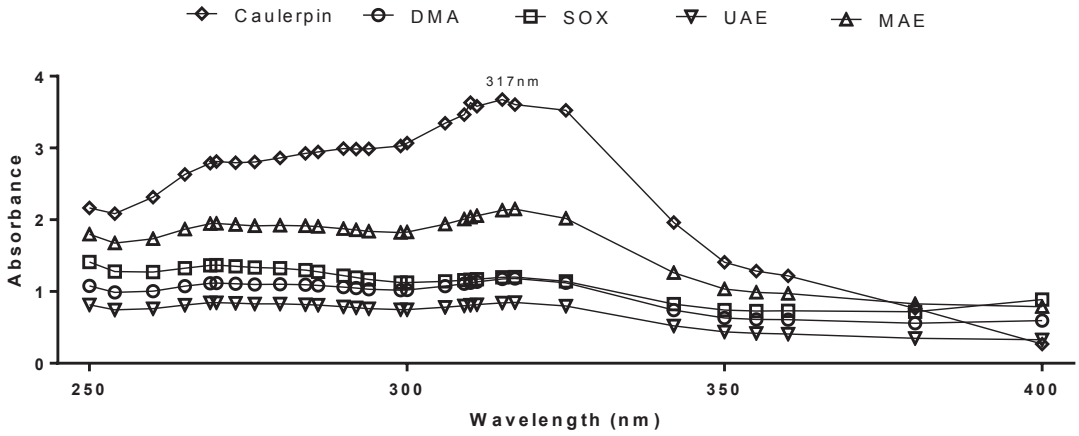

Fig. 3 UV absorption spectra of the extracts samples at 1 mg/mL and caulerpin at 0.125 mg/mL. DMA = Dynamic maceration, SOX = Soxhlet extraction, UAE = Ultrasound-assisted extraction, MAE= Microwave-assisted extraction.

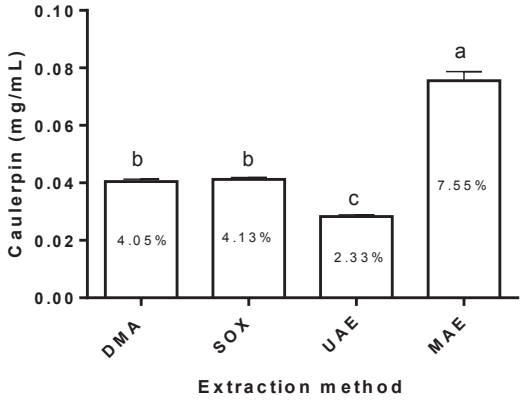

Fig. 4 Caulerpin contant (mg/mL) in each extract of *C. racemosa* obtained with different extraction methods. DMA = Dynamic maceration, SOX = Soxhlet extraction, UAE = Ultrasound-assisted extraction, MAE= Microwave-assisted extraction. Bars represents the mean ± standard error (n = 3) and percentages the concentration of caulerpin in each extract. Values identified by the same letters do not differ significantly (Tukey test, $p > 0.05$).

The microwave-assisted extraction presented the best results regarding the contain of caulerpin in the extract yield than other methods (Fig. 4). It yielded more than three times as much caulerpin as ultrasound-assisted extraction. Maceration and Soxhlet extraction showed no significant variation in caulerpin extraction. The first is known to consume high quantities of solvent, long extraction times and low extraction yields while Soxhlet can reduced solvent consumption but whit a possible degradation of thermo-labile substances (Bucar et al. 2013). For the isolation of natural products, Sticher (2008) indicates that the microwave-assisted extraction shows advantages in the extraction time, solvent consumption and yield of pure compounds in comparison with Soxhlet extraction. In this work, the microwave-assisted extraction was 60 times faster than the Soxhlet extraction. This work corroborates the use of microwave as a better methods for the extraction of natural compounds (Fulzele and Satdive 2005, Bagherian et al. 2011).

Microwave extraction optimisation

In order to increase the efficiency of caulerpin extraction, we evaluated changes in the parameters solvent, temperature and extraction time of this method. Figure 5 shows the quantified caulerpin of each extraction.

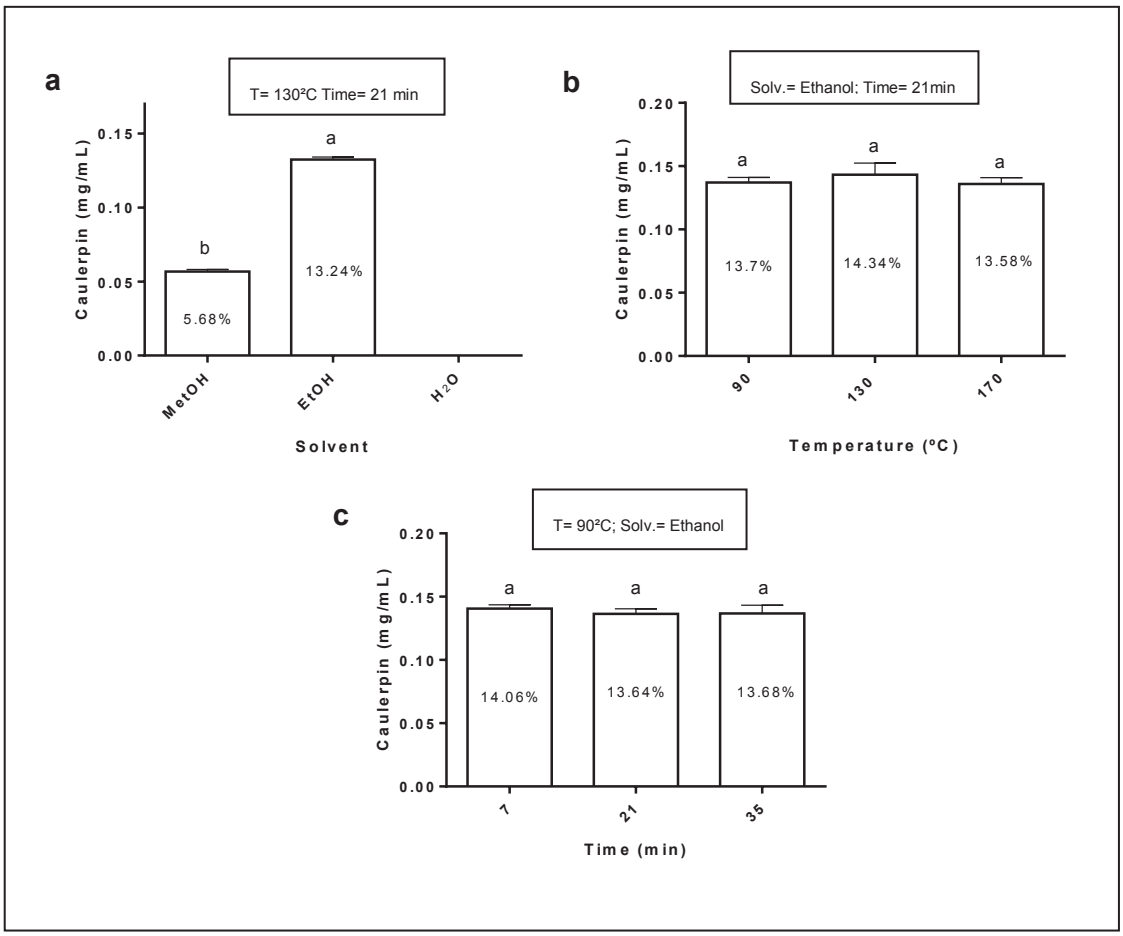

Fig. 5 Caulerpin contant (mg/mL) in microwave assistant extracts of *C. racemosa* comparing solvent (**a**), temperature (**b**) and time (**c**). Bars represents the mean ± standard error (n = 3) and percentages the concentration of caulerpin in each extract. Values identified by the same letters do not differ significantly (Tukey test, $p > 0.05$).

For the solvent parameter, methanol, ethanol and water were used at same time and temperature (21 min at 130 ° C). Ethanol present the higher percentage of recovery and caulerpin was not significantly detect with the uv-spectrophotometry. There solvents are suitable for microwave-irradiation, due to its high dielectric constant. There is a direct proportion between the dielectric constant and the absorbed energy by the molecules to reach the extraction temperature faster. Because of it, is expect that higher the dielectric constant of the solvent, better extractant is for microwave-assisted extraction (Romanik et al. 2007). However, this result was not found in this study. Although, water has the higher dieletric constant ($\varepsilon' = 78.3$), was not possible to determine the caulerpin content of the extraction with the water solvent for the tested conditions. This indicated that this extractor solvent is not suitable for the target compound. Methanol ($\varepsilon' = 32.6$) is used as an extractive solvent for caulerpin (Anjaneyulu et al. 1991) but was less efficiency in caulerpin extraction than ethanol ($p < 0.0001$). This solvent has the lowest dielectric constant ($\varepsilon' = 24.3$) in comparison with the others used and is the more environment friendly. Ethanol is known as bio-solvent due to its high solvent power, complete biodegradability and low toxicity. The selection of green extraction can ensure a safe and high quality product process and reduce the generation of hazardous substances (Chemat et al. 2012). Regarding, Bucar et al. (2013) appoint the necessity to considerate the polarity and stability of the compound extract, the toxicity, volatility, viscosity and purity of the extraction solvent, the possible artefacts formatted and the amount to be extract.

From the choice of the most suitable solvent, optimisation of the temperature, varying in a range of 40 °C was conducted. A wider variation in the temperature could fail due to the high pressure reached in the microwave. Thus, two new extractions were performed, 90 °C and 170 °C, during 21 minutes with ethanol as solvent. The comparison between the different temperatures did not differ, as showed in the

fig. 5b.These results suggest that the increase of temperature did not alter or degrade the caulerpin content, since caulerpin have a melting point of 317 °C (Aguilar-Santos 1970). Thermal degradation is one of the limitations of the microwave extraction method (Azwanida 2015). In spite of the fact that there was no significant yield variation, spending less energy power is better to reduce expenses. Therefore, the more suitable extraction temperature for forward optimisation was obtained at 90 ºC.

Changes in the extraction time was performed in 7 minutes, 21 minutes and 35 minutes with no significant variation (Fig. 5c). Thus, the choice of the shortest extraction time is more favorable to decrease the energy consumption. The improvements on the extraction process are required to allow an industrial development to be viable (Chemat et al. 2012).

The microwave-assisted extraction present the advantage of solvent reduction, level of simplicity and automation of the operating procedures. Martinez et al. (2014) indicate it use in natural products protocols for alkaloids. Nowadays extraction employing either diffused microwaves in closed systems or focused microwaves in open systems are established methods for obtaining natural products (Bucar et al. 2013, Sticher 2008). We opted for the first one, commonly named pressurised microwave extraction, where the solvent can be heated above its boiling point at atmospheric pressure by simply applying suitable pressure, thus enhancing both extraction speed and efficiency. The material in the vessel is affected by the dipole rotation, the ionic conductance and the interaction with the water in the solid matrix, leading to a rapid heating and increase of temperature. The microwave radiation generate a fast extraction with no degradation of the compounds, if the temperature is not too high (Sticher 2008). The solvent polarity can influence the extraction power, so choosing a suitable solvent could be crucial for the success of the extraction. To isolate a pure compound from organic matter in one step is still a distant possibility, but the application of more selective methods from extraction can accelerate the steps to obtain a final purified compound (Bucar et al 2013).

CONCLUSION

The search of the most suitable extract method for caulerpin from dried *C. racemosa* seaweed was proceed comparing classical extractions and non-convention extraction methods. The microwave-assisted extraction was the most effective in relation to dynamic maceration, Soxhlet extraction and ultrasound-assisted extraction. The changes of the method and the solvent were significant while temperature and extraction time for the tested conditions did not interfere in the extractive power.

The concept of green chemistry was applied in the quantification of caulerpin and to optimise the extraction. We employed a very simple and cheap method to quantify the caulerpin yield, the UV-vis spectroscopy. The extract prepared with ethanol by the microwave-assisted extraction was the most efficient. The lowest temperature and extraction time tested, at 90 ºC in 7 minutes, could improve the labor without lost in the caulerpin yield. Therefore, this work developed an extraction protocol to improve routine analysis by the green chemistry concept. The search for sustainable, practical, shorter time, low cost and environment friendly technologies are the new approach for the enable the industrial use of natural products.

ACKNOWLEDGMENTS

The authors thank the support of the National Council for Scientific and Technological Development (*CNPq*), the Foundation for Research Support of Rio de Janeiro State (FAPERJ) and the Brazilian navy's Almirante Paulo Moreira Institute of Marine Studies (IEAPM). We are also grateful to Dr. Lohengrin Fernandes and the department of chemistry at IEAPM for the laboratory facilities.

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CAPÍTULO II

INVESTIGATION OF THE ANTIFOULING POTENTIAL OF THE EXTRACT OF *CAULERPA RACEMOSA* (FORSSKÅL) J.AGARDH 1873 AND THE COMPOUND CAULERPIN

Investigation of the antifouling potential of the extract of *Caulerpa racemosa* (Forsskål) J.Agardh 1873 and the compound caulerpin

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ABSTRACT

Caulerpin is a bisindolic alkaloid described as the major substance of the genus *Caulerpa*. The main objective of this paper is to analyse the antifouling activity of caulerpin with fouling organisms such as bacterias of marine biofilm and macrofouling. Therefore, the algae *Caulerpa racemosa* was subjected to dynamic maceration with methanol to obtain a crude extract and to isolate caulerpin. The laboratory bioassays were conducted with marin bacteria, *Vibrio aestuarians*, *Pseudoalteromonas elyakovii*, *Polaribacter irgensii, Pseudomonas fluorescens* and *Shewanella putrefaciens* and larvae of *Bugula neritina* and *Tubastraea coccinea*. Anti-fouling activity was not detected with the tests performed but other approaches are raised.

KEYWORDS

Seaweed extract; biofilm; macrofouling; biofouling.

INTRODUCTION

One of the major problems of marine biotechnology is the biofouling (Abarzua and Jakubowski, 1995). Due to the economic and environmental impacts of fouling, the use of heavy metals and toxic biocides has been employed in ships and

naval structures. However, there is a search for environmental friendly alternatives through marine natural products (Abarzua and Jakubowski 1995, Pereira et al., 2003). Chemical compounds, such as secondary metabolites produced by seaweeds, have been extensively studied, ranging from intracellular action to ecosystem function (Watson and Cruz-Rivera 2003). Thus, several algae are reported with antifouling activity (De Nys et al. 1996, Da Gama et al. 2008), as well as various other biological activities such as antibacterial and antifungal.

The sustainability exploitation of seaweeds for screening studies must be considerate (Fernandes et al. 2014). For example, prospecting natural products could be sustainable with commercially cultivated macroalgae. As one cultivate, easy growing and widely consumed seaweed in Asian is the genus *Caulerpa* (Gaillande et al. 2016, Nagappan and Vairappan 2014). This alga have as major secondary metabolite the substance caulerpin (Schwede et al. 1987), which belongs to the group of alkaloids. These substances present complex structures, rare in terrestrial organisms, and activity of interest in areas of chemistry, pharmacology, physiology and medicine (Gul and Hamann 2016).

The highlights of caulerpin are the low toxicity (Movahhedin et al. 2014, Rocha et al. 2007, Vidal et al. 1984) and the broad biological activity (*eg*. Raub et al. 1987, Ayyad et al. 1994, Vairappan et al. 2004, De Souza et al. 2009, Liu et al. 2009, Alarif et al. 2010, Kamal and Sethuraman 2012, Macedo et al. 2012, Chay et al. 2014, Lorenzo et al. 2015, Cavalcante-Silva et al 2013, 2014, 2016, Raja et al. 2017). Among these activities, potential as corrosion inhibitor for mild steel used in the marine petroleum extraction was verified as a green inhibitor (Kamal and Sethuraman 2012). In this way, evaluating its antifouling action becomes an interesting alternative for coatings of steel in the marine environment.

When a substrate is submerged, physical, chemical and biological events act on the adsorption, colonization and development processes that micro and macro-organisms binds to the surface of the material. The establishment of the fouling organisms can occur in the first hours, through the biofilm formation, to a week later, by the establishment of micro and macro organisms (Abarzua and Jakubowski 1995).

For bacteria, a mechanism of signal communication named as *quorum sensing* was identify (Dobretsov et al. 2009). This communication acts in the formation of the biofilm and in the production of secondary metabolites. For the establishment of macro-organisms, studies shows that chemical signals influence in the substrate selection by the larvae (Dabrosov and Qian 2006, Dobretsov et al. 2007, Dobretsov et al. 2009). One of the main fouling species in tropical waters is the *Bugula neritina* Linnaeus, 1758 (Dobretsov et al. 2007) and in Brazil, one of the fouling invasive species with high dispersion rates is the sun coral *Tubastraea coccinea* Lesson, 1829 (Da Silva et al. 2014). Thus, an inhibitory action to bacteria growth and for the larval selection of fouling organisms is desired to inhibit the incrustation. In order to investiged the potencial of *Caulerpa racemosa* and one of it major secondary metabolite, caulerpin, against incrustation, bioassays in laboratory assessed the bacteria growth and larval development and settlement of the *Bugula neritina* and *Tubastraea coccinea* were develop in the present study.

METHODS

SAMPLES AND EXTRACTS PREPARATION

Samples of the seaweed *Caulerpa racemosa* were collected at the infralittoral zone (Orla Bardot, Búzios, Rio de Janeiro – Brazil) and transported in cooler box to the laboratory at the Almirante Paulo Moreira Institute of Marine Studies (IEAPM). The material was kept in aquarium with aeration, sorted and washed in salted water and distilled water to remove sand and associated organisms. After that, the algae was stored

in ultra-freezer (-80 ºC) and than, dried at room temperature for 5 days. The dried algae was submitted to a dynamic maceration with a magnetic stirrer IKA C-MAG HS 7 in methanol. The extraction was performed for 72 hours (Govenkar and Wahidulla 2000, Nagappan and Vairappan 2014). The solution was filtered and concentrated using a rotary evaporator FISATOM and the extract samples were weight in an analytical balance (0.01 mg accuracy, AUW-220D Model – Shimadzu).

ISOLATION AND IDENTIFICATION OF CAULERPIN

The caulerpin was isolated using column-chromatographic silica gel with hexane and hexane/ethyl acetate mixture (19:1, 9:1, 3:1), verified on thin-layer chromatography (TLC), and identified based on spectroscopic technique. The caulerpin content was visualized on TLC plates of silica gel 60 PF254 with hexane-ethyl acetate (3:2) mixture as mobile phase. The plates were revealed with ceric sulfate reagent and ultraviolet light (UV) of 254nm and 365nm. The samples were then analyzed in a Gas chromatography mass spectrometry (CG-MS) GCMS-QP2010 Plus Model – Shimadzu. The stock solution was prepared from solid samples in EtOAc of analytical grade for GC-MS analysis.

BIOLOGICAL ASSAYS

The antifouling potential of *Caulerpa racemosa* extract and caulerpin were conducted with organisms of the biofilm layer, corresponding to the fouling bacteria and of the secondary consumers, such as bryozoans and corals. Samples were weighed in analytical balance (accuracy of 0.01 mg). Stock solutions were prepared in volumetric flasks separately for each bioassay with ranges of concentration at scale of 10.

DISC DIFFUSION BIOASSAY

Antibacterial activity of *Caulerpa racemosa* extract and caulerpin follow disc diffusion assay as described by Devi et al. (2011) and by Carvalho et al. (2016).

The marine bacteria were components of biofilm: *Vibrio aestuarians*, *Pseudoalteromonas elyakovii*, *Polaribacter irgensii, Pseudomonas fluorescens* and *Shewanella putrefaciens.* The strains were obtained from the Collection of the University of Portsmouth (UK) and stored at IEAPM (Brazil). Bacteria were grown in Agar Marine Difco™ and replicate until the optical density of 1.5 – 2.0x10^8 cell/mL at 630 nm in a spectrophotometer.

Treatments of extract were diluted in methanol at concentrations of 0.01 μg/mL, 0.1 μg/mL, 1 μg/mL, 10 μg/mL and 100 μg/mL. The neutral control was methanol solvent and the positive, standard antibiotic Rifampicin (Sigma-Aldrich). For Caulerpin treatments solubilized was proceed in ethyl acetate at concentrations of 0.001 μg/mL, 0.01 μg/mL, 0.1 μg/mL, 1 μg/mL and 10 μg/mL. It neutral control was ethyl acetate solvent and Rifampicin as a positive control. All treatments and controls were performed on replicates (n = 5).

The extract and caulerpin were deposited at 10 μL in sterile paper discs of 6 mm diameter. Thereafter, the prepared discs were maintained for at least 20 min in ultraviolet light for sterilization and evaporation of the solvent. Measurer of the inhibition zone in the discs were determined after 24 h of incubation at 30°C.

LARVAL SETTLEMENT ASSAY

Bioassay of *Bugula neritina* and *Tubastraea coccinea* were carried out at with the same methodology. The bryozoans were collected in Guanabara Bay (Niterói, Rio de Janeiro), acclimated in a thermal box and transported to the laboratory. They were maintained in a tank with aeration. For the release of larvae, adults were kept for 24 hours in the dark and then exposed to light (Dobretsov and Qian 2004). The experiment with *C. racemosa* extract was conducted with replication (n=5) in 24-sterile polystyrene dishes (Falcon, Brookings, SD) containing 6.47 ± 3.63 larvae/mL larvae of *B. neritina*. The experiment with caulerpin was performed in 10 mL glass bequer with 6.20 ± 2.42

larvae/mL (n=5). Treatments for both experiments follow concentrations of 0.01 μg/mL, 0.1 μg/ml, 1 μg/ml, 10 μg/ml and 100 μg/ml. The negative control was only water and the white control was solvent, were methanol was used in the extract experiment and ethyl acetate for caulerpin. Experiments were run at a temperature of 21ºC and the larvae were counted in the optical microscope in the first hour (H1), in the sixth hour (H6) and after twenty-four hours (H24) (Dahms et al. 2004, 2007). The larvae were identified as free, settled or dead.

In the *Tubastraea coccinea* bioassay, adult specimens were collected by SCUBA diving on the rocky shore of Porco's island (Arraial do Cabo, Rio de Janeiro) and maintained in an aerated aquarium. The colonies were submitted to thermal stress (up to 30º C) to release larvae. After two months in the laboratory, larvae were released. These were separated in a bequer with larvae released in up to three days for the assembly of the experiment. Experiments were carried out in the same way as with *Bugula neritina.* There was deposited three larvae per replicate treatment (n = 5) at the same concentrations and same controls. The experiments were kept at room temperature and evaluation of fouling activity was conduct after 5, 10, 15 and 20 days under an optical microscope as proceed in previous studies at the Management and Control of Information on Biofouling and Bioinvasion project (GEBIO project) of IEAPM and PETROBRAS.

STATISTICAL ANALYSIS

The means and standard deviations were calculated. Mortality and settlement values of the larval assay were transformed into percentages. The homogeneity and normality of the data were analyzed with Shapiro-Wilk’s W test at a confidence level of 95%. The non-parametric test Kruskal-Wallis *post hoc* Dunn's test was used for statistical analysis with the GraphPad Prisma 6 Program to evaluate the difference in treatments of the *C. racemosa* extract and of caulerpin.

RESULTS

CHEMICAL IDENTIFICATION

The extract of *Caulerpa racemosa* and caulerpina were identified with CG-MS method, respectively represented in the Figures 1 and 2. The spectral data of caulerpin is similar to the reported in literature (Maiti et al. 1978). The mass spectra revealed the caulerpin as the major content of the *C. racemosa* extract (Fig. 2).

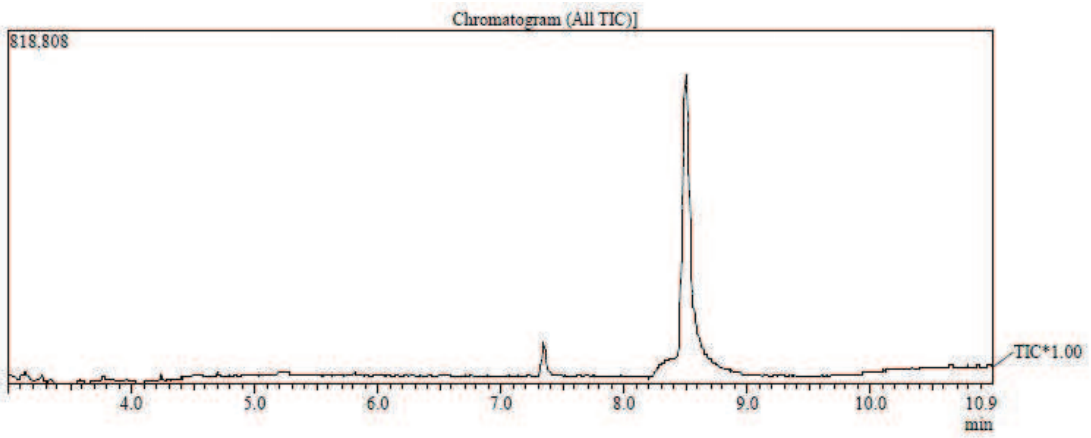

Figure 1. Chromatogram of *Caulerpa racemosa* extract (m/z 398.00). Retention time: 7.3 - 9.0. Mass peak at 40, 44, 57, 71, 85, 99, 113, 127, 207, 281 and 398.

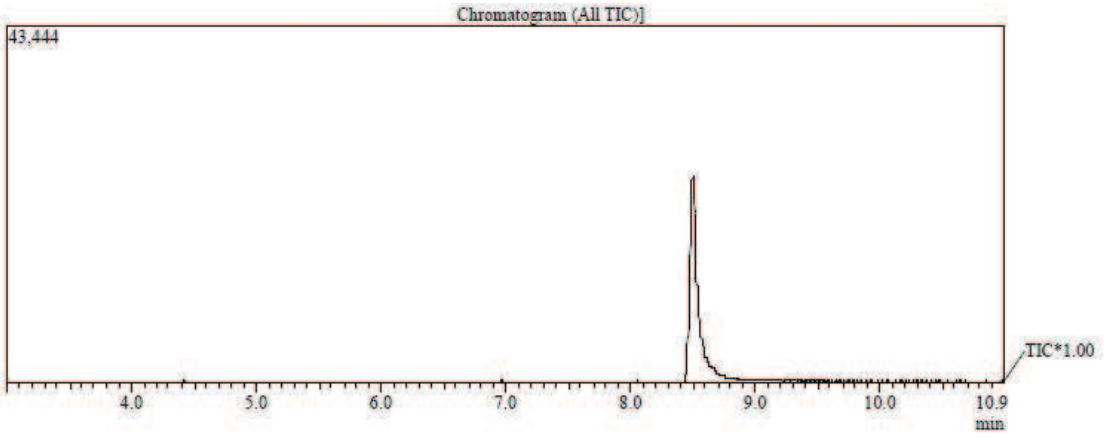

Figure 2. Chromatograms of caulerpin (m/z 398.00). Retention time: 8.533. Mass peak at 279, 306, 366 and 398.

BIOASSAYS

At the disc diffusion bioassay, the results of *Caulerpa racemosa* extract and caulerpin did not show significant inhibit growth of the bacteria tested (Table 1). Inhibition zones occurs in the extract of *Caulerpa racemosa* experimente for *Vibrio aestuarians* at 10

µg/mL and 0.01 µg/mL and for *Polaribacter irgensii* at 100 µg/mL and 0.01 µg/mL, while in the caulerpin experiment, inhibition zone was registered only for *V. aestuarians* at 10 µg/mL, 1 µg/mL, 0.1 µg/mL and 0.01 µg/mL. The classification of inhibition zone by Devi et al. (2011) is show at the Table 1. Only the positive control, the Rifampicin antibiotic, had significant inhibition zones.

Table 1. Antibacterial activity of *Caulerpa racemosa* extract and caulerpin.

Bacteria	*C. racemosa extract (*µg/mL)					Solvent control	Positive control	Caulerpin *(*µg/mL)					Solvent control	Positive control
	100	10	1	0.1	0.01			10	1	0.1	0.01	0.001		
Vibrio aestuarians	-	1.4 ± 1.4 *b	-	-	2.8 ± 1.72 *b	-	8.6 ± 0.25 **a	1.2 ± 1.2 *b	1.4 ± 1.4 *b	1.4 ± 1.4 *b	1.4 ± 1.4 *b	-	-	8.4 ± 0.29 **a
Pseudoalteromonas elyakovii	-	-	-	-	-	-	18.2 ± 1.32 ****a	-	-	-	-	-	-	18.6 ± 0.75 ****a
Polaribacter irgensii	1.3 ± 1.3 *b	-	-	-	3 ± 1.86 *b	-	11.1 ± 0.64 ***a	-	-	-	-	-	-	11.6 ± 0.68 ***a
Pseudomonas fluorescens	-	-	-	-	-	-	9.8 ± 0.74 ***a	-	-	-	-	-	-	13.2 ± 1.16 ***a
Shewanella putrefaciens	-	-	-	-	-	-	25.8 ± 0.58 ****a	-	-	-	-	-	1.8 ± 1.8 *b	24.6 ± 0.68 ****a

Values (mm) are expressed as the mean ± standard error (n=5). Same superscript letters are not statistically different ($p > 0.05$). * Weak activity (1 – 4 mm), ** moderate activity (5 - 8 mm), *** good activity (9 - 15 mm), **** highly active (16 - 25 mm) (Devi et al., 2011).

In the bioassay of *Bugula neritina,* there was significant variation in the larval settlement submitted to *Caulerpa racemosa* extract at the concentration of at 1 µg/mL and 10 µg/mL (Fig. 3a) and caulerpin at 100 µg/mL (Fig. 3b) experiments. Samples with significant variation of extract had lower settlement in the first hour ($H = 13.97$, $DF= 6$, $p < 0.05$) but it did not affect the settlement at 24 hours ($H =16.03$, $DF= 6$, $p < 0.05$). The inverse occurs with the caulerpin at 100 µg/mL, a higher settlement was

observed in the first hour (H =13.97, DF= 6, $p < 0.05$) but it did not affect the settlement at 24 hours (H =9.485, DF= 6, $p > 0.05$). Mortality rate was not significant in relation to the water and solvent control ($p > 0.05$).

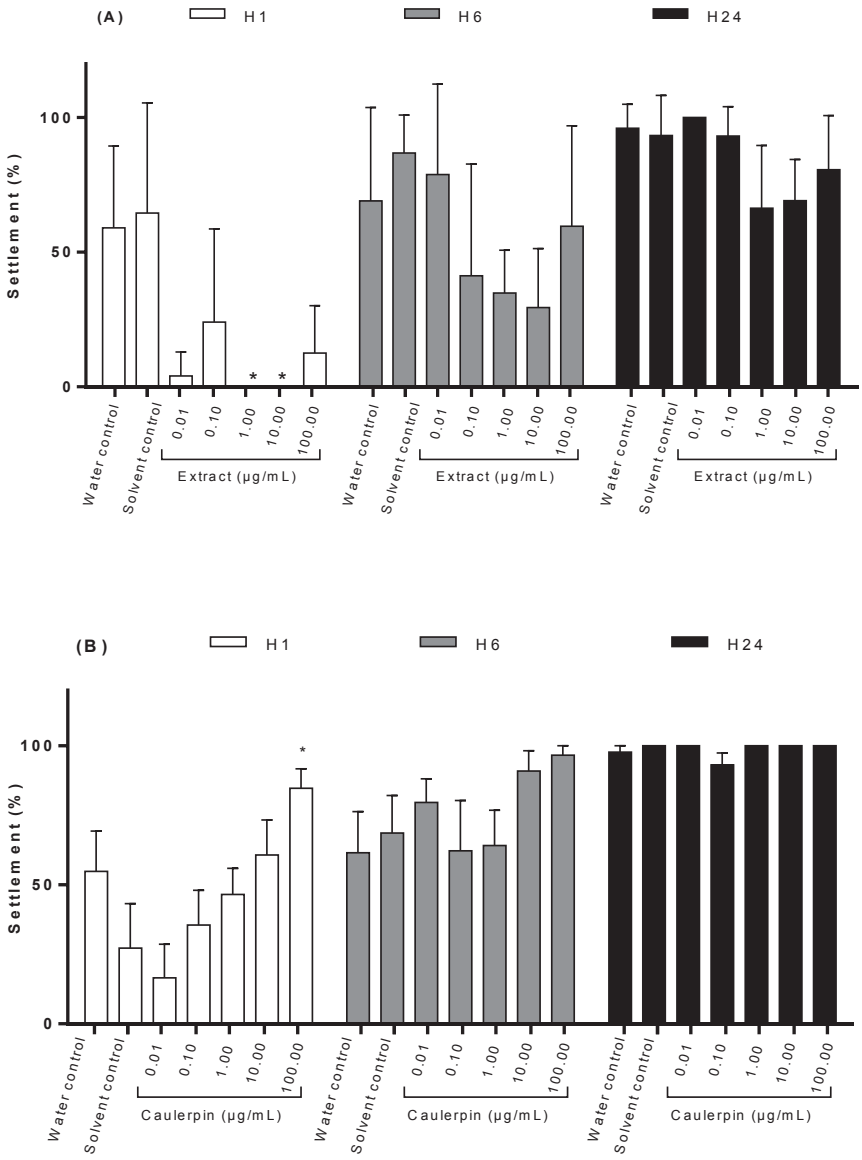

Figure 3. Larval settlement of *Bugula neritina* at: (A) extract experiment and (B) caulerpin experiment. Data are means ± SD (n=5) in percentage. Asterisks represent significant differences from solvent control ($p < 0.05$).

In the bioassay of *Tubastraea coccinea* (Fig. 4), there was no significant variations ($p > 0.05$) in the larval settlement percentage of *Caulerpa racemosa* extract

and caulerpin experiments, comparing with the water and solvent control because of the great variation in the replicates (of 0 to 100% of settlement in the treatment).

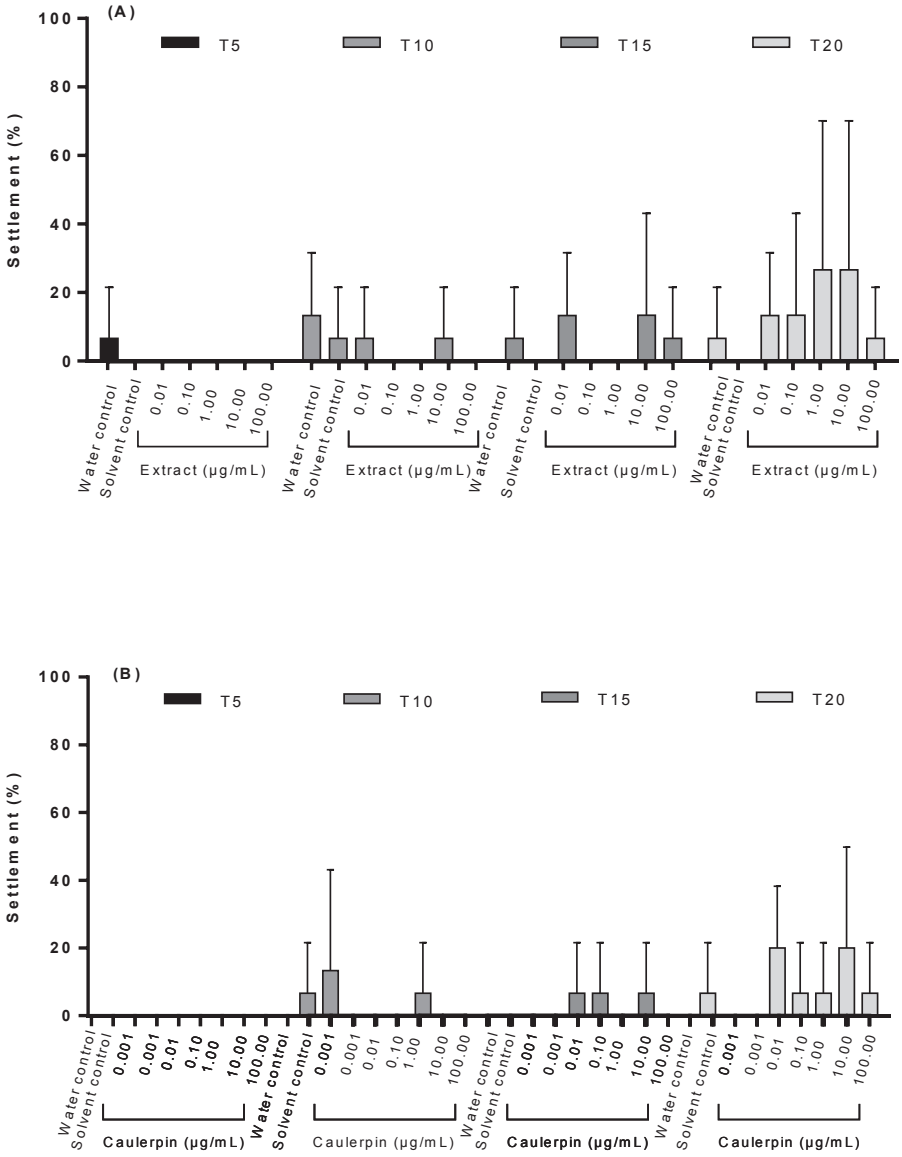

Figure 4. Larval settlement of *Tubastraea coccinea* at: (A) extract experiment and (B) caulerpin experiment. Data are means ± SD (n=5) in percentage. No significant differences were found ($p > 0.05$).

DISCUSSION

EFFECT AGAINST BACTERIA

In this study, non-significant growth inhibition response was verified for *Vibrio aestuarians*, *Pseudoalteromonas elyakovii*, *Polaribacter irgensii, Pseudomonas*

fluorescens and *Shewanella putrefaciens* at the disc diffusion bioassay with *Caulerpa racemosa* extract and caulerpin, but other authors found positive results in different bioassays with *C. racemosa* extract (Schroder et al. 1998, Skindersoe et al. 2008, Batista et al. 2014, Carvalho et al. 2017). Dobretsov et al. (2006) point that there are other bacterial responses to a chemical component beyond the inhibition of bacterial growth, such as repulsion, spreading and attachment. The authors investigated the relation of *C. racemosa* with microbial community by molecular fingerprinting techniques, investigation of the action of the metabolites on bacterial growth and bioassays of larval development and settlement fouling organisms. They proposed that the seaweed *C. racemosa* have a protection against micro and macro epibionts, as observed in field, and it could be due to: a mechanical defense, a not known concentration of a natural product or an active effect of this compound that is not bactericidal.

For a mechanical defense against epibionts, Da Gama et al. (2008) enforced the assumption that green macroalgae could have antifouling mechanisms of defense other than chemical. A mechanical action of caulerpin against steel corrosion is described by Kamal and Sethuraman (2012). Because of the low affinity with water, caulerpin do not dissolve in water and stick to the substrate. Kamal and Sethuraman (2012) investigated the action of HCl at the surface level but new researches with fouling organisms at surface level could asses a wider understanding of marine corrosion since it is influenced by living organisms (Baboian 2005) and elucidate if caulerpin affects directly the adsorption, colonization and development process of biofouling.

In order to prospect and identify valuable bioactive compounds, the concentration assessed for biological activities of natural extracts needs to be compatible with the sustainable exploitation of natural sources (Fernandes et al. 2014). For natural compound, an effective concentration 50% (EC50) lower than 25 µg/mL is

established for antifouling employment by the United States Navy (Qi et al., 2010) due to the difficulties to obtain natural products in a large amount (Rittschoff, 2001). However, Abarzua and Jakubowski (1995) highlights that algae can be used as "extraction organisms" due to the quick biomass farming and possible large production.

For an active effect that is not bactericidal, the understanding of the mechanism of action can be so important as its power. The antibiotic effect of caulerpin was not assessed in this study, but a molecular mechanism behind the antibacterial activity of caulerpin is described in the literature. Docking simulation of Aspartate β-semialdehyde dehydrogenase (ASADH) of *Vibrio anguillarum* with caulerpin found a high binding affinity for the site (Subramani et al. 2016). The ASADH is a target enzyme for the antibiotic effect due to the biosynthesis role in plants and microbes (Hadfield et al. 2001). This may be indicate a possible action against the formation of fouling biofilm. The development of biofilm bacteria on the algal can have negative effects on algae such as increased trawling, nutrient competition, sun blocking and gas exchange blocking (Stirk et al., 2007). Thus, control mechanisms of biofilm formation are important for algae survival.

Batista et al. (2014) test different macroalgae extracts for inhibition of *Quorum sensing* through the molecular signal of *Chromobacterium violaceum* CV017 and fixation signal of *Pseudomonas aeruginosa* PA01. The authors verify significant activity of the methanolic extract of *C. racemosa and* attribute this effect to interference on the bacterial signals from *Quorum sensing* LuxR (*C. violaceum* QS) and LasR (*P. aeruginosa* QS). Skindersoe et al. (2008) also described an inhibitory action of *Quorum sensing* for *Caulerpa* sp. extracts. Both studies did not identified the bioactive compound of the extract tested.

In addition, at a multigene resistance test of *Geodia cydonium* marine sponge,

caulerpin at 10 µg/mL had a resistance inhibition action and also enhanced the apoptotic effect of Tributyltin (Schroder et al. 1998). This may be suggest the potential use of caulerpin concomitant with non-toxic alternatives as anti-fouling agents to prevent microbial growth and biofilm formation. Dobretsov and Qian (2006) suggests that the biofilm composition affect directly the induction of larval settlement, as verified by changes in the composition of the bacterial communities of biofilms interfering the substrata evaluation and its selection by the larvae of *B. neritina*.

EFFECT AGAINST MACROFOULING

At the screening of *C. racemosa* from the field to laboratory, we found few or no fouling organisms. The same result was observed by Dobretsov et al. (2006), who suggested an antifouling control of epibiotic in *C. racemosa*. As the attempt with the substance caulerpenyne by Dobretsov et al. (2006), this work could not identify the compound responsible for the fouling defense of *Caulerpa racemosa* with the crude extract and the substance caulerpin in the bioassays employed.

In both larval assays, there was no significant settlement inhibition at the end of the experiments, in comparison to water and solvent controls.

Bryozoans are used as one of the main models of larval settlement assays in antifouling research (Dahms et al. 2007). A possible reduction of settlement at 1 µg /mL and 10 µg /mL of extract and an enhancing of the settlement at 100 µg /mL of caulerpin were observed in the first hour ($p < 0.05$) of *Bugula neritina* bioassay, but this was not significant after 6 hours and in the final settlement (24 hours). Althought the analysis of the antifouling activity can be made in the first hour, the ratio of the larval settlement success are observed after 24 h of experiment. The larvae are observe to swam free until the first two hours, cumulative settlement after five hour (Dahms et al. 2004) and at final 24 hours (Dahms et al. 2007). The extact of *C. racemosa* and caulerpin did not show significant alteration throughout the experiment.

A new antifouling assay, larval settlement assay with *Tubastraea coccinea,* is present here. At previous studies proceed at the GEBIO project of IEAPM and PETROBRAS, larval settlement was analysed and a maximum of 20 days was necessary to evaluate settlement and mortality rates. This sun coral, mistreated as "pest coral" for some authors, have been submitted to high effort methods of control and eradication, such as manual removal (Moreira et al. 2014). Here we propose a search for an environment friendly and sustainable approach for this non-endemic coral focus on prospecting a natural compound that acts in the larval selection of substrate for avoiding settlement. Although the extract of *Caulerpa racemosa* and caulerpin did not show activity for the *Tubastraea coccinea* larvae, other substances can be tested using this protocol.

FINAL CONSIDERATIONS

The antifouling potential of *Caulerpa racemosa* extract and its major compound, caulerpin, were investigated. Bioassays conducted with marine bacteria found in fouling biofilm and two important macrofouling components in Brazilian cost, *Bugula neritina* and *Tubastraea coccinea,* did not show a significant effect on the tested concentrations of extract and caulerpin. The bioassay with *T. coccinea* was first described and many relevant considerations were highlighted to elucidate the epibiotic defense of algae *Caulerpa racemosa.*

ACKNOWLEDGMENTS

The authors thank the support of the National Council for Scientific and Technological Development (*CNPq*), the Foundation for Research Support of Rio de Janeiro State (FAPERJ) and the Brazilian navy's Almirante Paulo Moreira Institute of Marine Studies (IEAPM).

DISCLOSURE STATEMENT

The authors report no conflict of interest.

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CONSIDERAÇÕES FINAIS

O método de quantificação por espectrofotometria UV-vis foi avaliado e empregado para identificação e quantificação de caulerpina no extrato bruto. Foi possível utiliza um método simples e econômico e desenvolver um protocolo de extração para a caulerpina para análises de rotina através do conceito de química verde. A extração assistida por micro-ondas foi a mais eficiente em relação à maceração dinâmica, extração de Soxhlet e extração assistida por ultrassom de acordo com a metodologia empregada. Este método mais eficiente foi otimizado nos parâmetros de solvente, temperatura e tempo de extração. Apenas o solvente apresentou alteração significativa nas condições testadas. A melhor condição foi estimada com extração etanólica assistida por micro-ondas por 7 minutos à 90 ^{o}C.

A ação anti-incrustante da caulerpina e do extrato de *Caulerpa racemosa* não foi significativa nos testes de difusão de disco com bactérias isoladas de biofilme marinho e testes de assentamento larval de dois macro-organismos incrustantes presentes na costa brasileira, *Bugula neritina* e *Tubastraea coccinea*. Possibilidade para investigar a função contra epibiontes foram levantadas. O ensaio biológico com *Tubastraea coccinea* foi descrito pela primeira vez como ferramenta para investigar possíveis substâncias que atuem na inibição ou atenuação no assentamento larval. Testes anti-incrustantes são maneiras simples para identificar potenciais substâncias de interesse para o controle da incrustação marinha. Esta é uma alternativa mais sustentável e menos agressiva para o controle da dispersão do coral sol.

Assim, este trabalho investigou os processos extrativos e de um potencial uso da caulerpina em busca de prospectar uma possibilidade de uso deste recurso natural marinho como um possível produto.