UNIVERSIDADE FEDERAL DE PELOTAS
Centro de Ciências Químicas, Farmacêuticas e de Alimentos
Programa de Pós-Graduação em Bioquímica e Bioprospecção

Tese de Doutorado

Bioprospecção em algas subantárticas no estudo do tratamento do câncer de bexiga.

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Pelotas, 2019

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Tese apresentada ao Programa de Pós-Graduação em Bioquímica e Bioprospecção (PPGBBio) da Universidade Federal de Pelotas, como requisito parcial à obtenção do título de Doutor em Ciências - Bioquímica e Bioprospecção.

Orientador: Prof. Dr. Tiago Veiras Collares

Co-Orientador: Prof. Dr. Claudio Martin Pereira de Pereira

Pelotas, 2019.

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Universidade Federal de Pelotas / Sistema de Bibliotecas
Catalogação na Publicação

A111b Amaral, Caril Constante Ferreira do

Bioprospecção em algas subantárticas no estudo do tratamento do câncer de bexiga / Caril Constante Ferreira do Amaral ; Tiago Veiras Collares, orientador ; Claudio Martin Pereira de Pereira, coorientador. — Pelotas, 2019.

128 f. : il.

Tese (Doutorado) — Programa de Pós-Graduação Bioquímica e Bioprospecção, Centro de Ciências Químicas Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, 2019.

1. Macroalgas chilenas. 2. Células T24. 3. Polissacarídeos. 4. Ácidos graxos poliinsaturados. 5. Integridade da membrana. I. Collares, Tiago Veiras, orient. II. Pereira, Claudio Martin Pereira de, coorient. III. Título.

CDD : 574.192

Elaborada por Gabriela Machado Lopes CRB: 10/1842

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Tese aprovada, como requisito parcial para obtenção do grau de Doutor em Ciências (Bioquímica e Bioprospecção), Programa de Pós-Graduação em Bioquímica e Bioprospecção, Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas.

Data da defesa: 25/10/2019

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Dedico este trabalho a todos que me desejaram êxito,
que compreenderam minhas falhas e limitações,
que estiveram junto nos desafios e tristezas,
e pelos quais divido a satisfação por esta realização.

Agradecimentos

À Universidade Federal de Pelotas, na pessoa de seu Magnífico reitor, Professor Pedro Curi Hallal, pela acolhida institucional.

Ao Programa de Pós-Graduação em Bioquímica e Bioprospecção e seus respectivos orientadores e colaboradores, pelo acompanhamento de minha formação e oportunidade de convívio com áreas do conhecimento multidisciplinares.

À CAPES, pela concessão e outorga da Bolsa de Doutorado Sanduíche (processo 88887.137286/2017-00) e suporte ao projeto (PGCI 88887.125421/2016-00).

Aos colegas de pós-graduação, pelo empenho que tratam seus projetos de pesquisa, mesmo abdicando de suas atuações profissionais pela dedicação a carreira acadêmica, ainda que em tempos pouco esperançosos.

Aos servidores técnico-administrativos vinculados ao PPGBBio e aos laboratórios de pesquisa, pela eficiência e colaboração imprescindíveis nas ocasiões em que foram solicitados.

Aos professores orientadores Tiago, Fabiana e Claudio, por todas as oportunidades que me foram oferecidas e por todos os ensinamentos que me foram partilhados.

Aos colegas de laboratório, pela compreensão com minhas limitações e correspondência com minhas expectativas. Graças a cada um de vocês essa trajetória pôde ser mais bem aproveitada.

Ao meu saudoso pai Martim, minha mãe Gasparina, minha irmã Tanyra, e minha namorada e companheira de vida Natália, por terem minha a dedicação de jamais decepcioná-los ou esquecê-los. Por tudo.

À Universidad de Magallanes, na pessoa do professor Andrés Mansilla e de todos de seu laboratório, minha gratidão pela acolhida e por terem tornado a vida em outro país ao longo de um ano uma experiência muito mais agradável.

Ao meu valioso amigo chileno Ricardo Cáceres Velastin, por ter me acolhido como um verdadeiro filho e por termos vivido incontáveis experiências cômicas, turísticas e gastronômicas ao longo de um ano, as quais certamente fizeram minha jornada ter sido mais bem aproveitada.

Aos meus amigos Eduardo Morais, Rodolfo Nogueira e Mateus Pinheiro, que puderam vivenciar uma viagem à Patagônia Chilena para me visitar, e de lá termos a forte convicção que as risadas são o amálgama de uma verdadeira amizade.

Aos meus compadres e amigos Igor, Rosane, Bruno, Tati, Murilo, Ariane, Junior, Fernanda, Jean e Juliana, por toda preocupação que já demonstraram pelo meu bem-estar e, mais que isso, por me proporcionarem isso na forma de suas amizades.

Aos novos colegas da Residência em Atenção à Saúde Oncológica do Hospital Escola da UFPel, preceptores e residentes das diferentes áreas, meu muito obrigado por me proporcionarem um olhar mais humanizado aos pacientes com câncer, e com estes poder aprender o comportamento de sua enfermidade, angústias e esperanças.

A todos aqueles que, ainda que não nominados, fizeram e/ou fazem parte da minha trajetória de modo singular, tenham de mim a gratidão por ajudarem a forjar quem sou.

Notas Preliminares

A presente Tese de Doutorado foi redigida segundo o Manual de normas UFPel para trabalhos acadêmicos de 2019, adotando o Nível de Descrição em Artigos, descrito no referido manual. Disponível em: https://wp.ufpel.edu.br/sisbi/normas-da-ufpel-para-trabalhos-academicos/. Acesso em: 20 de setembro de 2019.

Resumo

DO-AMARAL, Caril Constante Ferreira. **Bioprospecção em algas subantárticas no estudo do tratamento do câncer de bexiga.** Orientador: Tiago Veiras Collares. 2019. 128 f. Tese (Doutorado em Ciências) – Programa de Pós-Graduação em Bioquímica e Bioprospecção. Universidade Federal de Pelotas, Pelotas, 2019.

As macroalgas são reservas de biomoléculas importantes de interesse biotecnológico e farmacêutico, cujo conteúdo pode variar conforme estes organismos estabelecem respostas metabólicas frente aos desafios bióticos e abióticos nos quais estão envolvidos. Nos últimos anos tem crescido o número de investigações que buscam elucidar o comportamento de polissacarídeos e lipídeos de macroalgas como terapêutica antitumoral. A ecorregião de Magalhães, localizada na porção subantártica do Chile, é marcada como uma porção continental de alta latitude que apresenta baixo fotoperíodo no inverno e alto no verão. Variações entre espécies e sazonais podem influenciar seu estado reprodutivo e biomassa e, portanto, sua prospecção em alimentos, uso cosmetológico ou farmacológico. Este trabalho teve como objetivo investigar na literatura a estrutura e atividade antitumoral de fucoidan, um polissacarídeo sulfatado, e sua ação no câncer de bexiga (BC). Também foi objetivo deste trabalho determinar o perfil de ácidos graxos (FAs) da macroalga subantártica *Mazzaella laminarioides* nas fases vegetativa, tetrasporofítica e cistocárpica no inverno e no verão e avaliar a atividade antitumoral dessas FAs contra células tumorais de bexiga da linhagem T24. Os achados apontam para uma resposta favorável do uso de polissacarídeos e lipídeos derivados de macroalgas no BC. Dados da literatura apontam as fucoidanas como moléculas estratégicas na terapia antineoplásica no BC, na medida em que possuem atividade antiproliferativa, antiangiogênica e antimetastática, além de reduzir a toxicidade e produzir menos efeitos colaterais quando combinada com quimioterápicos covencionais. Sobre os lipídeos da macroalga subantártica *M. laminarioides*, foram encontrados dezenove FAs, e a composição variou de acordo com o estado reprodutivo e a estação em que as algas foram coletadas. A atividade citotóxica desses FAs foi avaliada em células de BC da linhagem T24, cuja viabilidade celular diminuiu de maneira dependente da concentração e do tempo. A atividade citotóxica mais significativa dos FAs, bem como a maior condensação da cromatina observada pela coloração com DAPI, foi observada na concentração de 200 µg/mL em 48 h. Tomados em conjunto, os resultados indicam que os polissacarídeos e lipídeos derivados de macroalgas têm potencial para reduzir a proliferação em células de BC, assim como prospectam a região subantártica chilena como favorável na obtenção de macroalgas com elevado valor biotecnológico.

Palavras-chave: Macroalgas chilenas; células T24; polissacarídeos; ácidos graxos poliinsaturados; integridade da membrana.

Abstract

DO-AMARAL, Caril Constante Ferreira. **Bioprospecting of Sub-Antarctic seaweed in the study of bladder cancer treatment.** Advisor: Tiago Veiras Collares. 2019. 128 p. Thesis. PhD in Sciences. Graduate Program in Biochemistry and Bioprospecting. Federal University of Pelotas, Pelotas, 2019.

Macroalgae are important reservoir of biomolecules with biotechnological and pharmaceutical interest, and the contents may vary as these organisms establish metabolic responses face to the biotic and abiotic conditions. In recent years, the number of studies that seek to investigate the behavior of macroalgae polysaccharides and lipids as antitumor therapy has increased. The Magellan ecoregion, located in the sub-Antarctic portion of Chile, is marked as a high latitude continental portion with low photoperiod in winter and high in summer. Variations between species and season may influence their reproductive status and biomass and, therefore, their prospecting in food, cosmetological or pharmacological use. This study aimed to investigate in the literature the antitumor structure and activity of fucoidan, a sulphated polysaccharide, and its action on bladder cancer (BC). The second objective of this study was to determine the fatty acid profile (FAs) of the *Mazzaella laminarioides* subanthartic macroalgae in the vegetative, tetrasporophytic and cystocarpic developmental phases in winter and summer and to evaluate the antitumor activity of these FAs against T24 bladder tumor cells. The findings point to a favorable response to the use of polysaccharides and lipids derived from macroalgae in BC. Literature data point to fucoidans as strategic molecules in antineoplastic therapy in BC, as they have antiproliferative, antiangiogenic and antimetastatic activity, as well as reducing toxicity and producing fewer side effects when combined with conventional chemotherapy. Nineteen FAs were found on sub-Antarctic macroalgae *M. laminarioides*, and the composition varied according to the reproductive status and the season in which the algae were collected. The cytotoxic activity of these FAs was evaluated in T24 BC cells, whose cell viability decreased in a concentration- and time-dependent manner. The most significant cytotoxic activity of FAs, as well as the highest chromatin condensation observed by DAPI staining, was observed at a concentration of 200 µg/mL within 48 h. Taken together, the results indicate that macroalgae-derived polysaccharides and lipids have the potential to reduce proliferation in BC cells, as well as prospecting the Chilean sub-Antarctic region as favorable in obtaining high-biotechnological macroalgae.

Keywords: Chilean seaweed; T24 cells; polysaccharides; polyunsaturated fatty acids; membrane integrity.

Sumário

1. INTRODUÇÃO E REVISÃO DA LITERATURA

Uma das doenças mais significativas que tem sido amplamente estudada nos recentes anos, o câncer é caracterizado por uma proliferação desordenada de células que, em casos mais severos, pode apresentar metástases. Não obstante os desafios de lidar com essa condição muitas vezes progressiva e debilitante, muitos são os dispêndios financeiros e sofrimentos causados pela doença. Um dos tipos de cancer mais significativos é o cancer de bexiga (BC), que está entre os dez tipos mais comuns, tendo atingido no ano de 2018 o registro de 549.393 novos casos e tendo causado aproximadamente 199.922 mortes no mesmo período (BRAY et al., 2018).

Os fatores de risco para o BC incluem fatores intrínsecos como aumento da expressão de protooncogenes e redução da expressão de genes supressores de tumor, além de fatores extrínsecos como o contato com poluentes atmosféricos, como o hábito tabágico e exposição ocupacional (CUMBERBATCH et al., 2018; WONG et al., 2018). Os tratamentos atualmente disponíveis para a maioria dos tumores de bexiga incluem ressecção cirúrgica total ou de parte do órgão, e em muitos casos o advento de quimioterapia neoadjuvante ou adjuvante (CHOU et al., 2016). Os medicamentos de ação antineoplásica possuem indicação para casos onde as abordagens cirúrgicas são contraindicadas ou em tumores que já apresentam metástases (SIO et al., 2018).

Recentemente, tem-se investigado novas estratégias terapêuticas antitumorais que sejam seletivas ao BC e que produzam menos efeitos colaterais, comuns com as quimioterapias convencionais. Produtos naturais, derivados do metabolismo de organismos como plantas, têm sido utilizados na prevenção tumoral ou supressão da malignidade, ao mesmo tempo que são percebidos menos efeitos colaterais e redução da resistência ao medicamento (CHANVORACHOTE et al., 2016; YUAN et al., 2017; MANN et al., 2005). Devido à complexidade de interações ambientais com de incidência de luz variável ao longo do ano e a resistência à dissecção pela posição intermareal, macroalgas da região subantártica chilena tem emergido como importantes organismos com biomoléculas ativas constituintes moleculares de intesse biotecnológico na terapia antitumoral (MANSILLA & ÁVILA, 2012; RUAN et al., 2018).

BIODISTRIBUIÇÃO

A Ecorregião de Magalhães é uma província biogeográfica localizada no sudeste do continente sul-americano (48º36 'a 56ºS; 66º25' para 75º40'O). Contendo uma área de 132.033 m², é o maior representante do ambiente subantártico, com um total de 391 espécies de macroalgas, das quais 75 são do filo Chlorophyta, 86 são Ochrophyta e 230 são Rhodophyta (ROZZI et al., 2008; MANSILLA & ÁVILA, 2012). As macroalgas são a base da cadeia alimentar de organismos marinhos, cujos metabólitos têm grande valor econômico na indústria farmacêutica (anticoagulantes, antitrombóticos e antioxidantes), alimentos (fibras dietéticas e funcionais), bioenergia (biocombustíveis) e biotecnologia (cosméticos, imunologia e bioprospectivas) (GRESSLER et al., 2011; GUARATINI et al., 2012; MATSUHIRO et al., 2014; LIRA et al., 2016; MCCAULEY et al., 2015; MILLEDGE; HARVEY, 2016). Além disso, as macroalgas são fontes de vários tipos de biomoléculas, como proteínas, vitaminas, aminoácidos, minerais, polissacarídeos e lipídios (DI MASCIO et al., 1995; HOLLNAGEL et al., 1996; CARDOZO et al., 2006). Apesar dos consideráveis progressos realizados nos últimos anos, o conhecimento atual sobre as propriedades farmacológicas e nutricionais de macroalgas da região subantártica do Chile ainda é limitado, uma vez que estudos sobre a composição química de macroalgas Sub-Antárticas são escassos.

MOLÉCULAS FUNCIONAIS

POLISSACARÍDEOS

Macroalgas pardas têm sido descritas como organismos que produzem uma importante quantidade de polissacarídeos de interesse biotecnológico e farmacêutico (ZUBIA et al., 2009; SHALABY, 2011). No grupo de polissacarídeos sulfatados derivados de macroalgas pardas, as fucoidanas são moléculas hidrossolúveis que possuem um grupo L-fucose e um grupo ester. A variabilidade da composição química entre fucoidanas de diferentes espécies de macroalgas se refere, em parte, a fatores como extração, parâmetros ecofisiológicos, estação do ano ou mesmo entre as diferentes partes das macroalgas (FLETCHER et al., 2017).

No que se refere à atividade antitumoral, as fucoidanas exercem efeitos terapêuticos altamente desejáveis em quimioterápicos antineoplásicos, que incluem efeitos antiproliferativo, proapoptótico, antiangiogênico e inibidor da migração celular

(LOWE and LIN, 2000). Importante mencionar, ao mesmo tempo que se mostram altamente promissoras pelos múltiplos efeitos metabólicos em células tumorais, as fucoidanas parecem não desempenhar interferência no metabolismo de células normais.

A atuação das fucoidanas no ciclo celular de uma célula tumoral pode ocorrer em uma ou mais fases distintas: G0, G1, S, G2 e M (SHERR, 1996). Há relatos de efeitos inibitórios na fase sub-G1 nas linhagens celulares tumorais de carcinoma gástrico humano, carcinoma pulmonar não-pequenas células, linfoma difuso de grandes células B, carcinoma de cólon, carcinoma de cabeça e pescoço e leucemia mielocítica aguda (SHAKERI et al., 2017; YANG et al., 2015; KIM et al., 2010; CHO et al., 2010; WEI et al., 2019) Além disso, a interrupção do ciclo cellular pode vir associada a outros eventos regulatórios, como dano genético e apoptose (ARUMUGAM et al., 2019). Em células tumorais da linhagem 4T1, as fucoidanas promovem infrarregulação da via de sinalização WNT/β-catenina, relacionada ao ciclo celular e apoptose (XUE et al., 2012). Em células de hepatocarcinoma (HepG2), fucoidanas derivadas da macroalga *Fucus vesiculosus* foram capazes de inibir a viabilidade celular e induzir apoptose (MARUDHUPANDI et al., 2015).

A apoptose é um fenômeno composto por distintos eventos do repertório bioquímico dependentes de energia que levam a uma morte programada das células (ELMORE et al., 2007). São duas as principais vias de apoptose: a via extrínseca e a via intrínseca. A primeira, também conhecida como via do receptor de morte celular, inclui a ativação de rotas metabólicas da família NF-ĸB, PI3K/Akt e MAPK/ERK. A segunda é conhecida como via mediada pela mitocôndria, uma vez que é característica a liberação citosólica do citocromo C da parede interna da mitocôndria e liberação de caspases do tipo 3 e 9 (LOWE e LIN, 2000). Estudos com fucoidanas em células tumorais apontam para uma indução de apoptose por ativação das duas vias, extrínseca e intrínseca (KIM et al., 2010; ATASHRAZM et al., 2015).

O crescimento volumétrico de um tumor depende essencialmente de uma rede de neovascularização capaz de levar nutrientes às camadas mais internas da massa tumoral. O processo de angiogênese tumoral inclui um ajuste dependente de sinais químicos que orientam a proliferação celular, diferenciação, sinalização celular e produção de moléculas como fator de crescimento de endotélio vascular (VEGF) ADAMS e ALITALO et al., 2007). A atividade antitumoral das fucoidanas também

inclui um efeito antiangiogênico, uma vez que são capazes de inibir o receptor tipo 2 do VEGF (KOYANAGI et al., 2003; CHEN et al., 2016).

A metástase é uma das características mais significativas na determinação da malignidade e potencial morbidade durante o tratamento do câncer. Uma vez que as células tumorais perdem a adesão intercelular e tecidual, podem migrar para tecidos circunvizinhos ou à distância do sítio primário (CAO et al., 2013). Já há relatos na literatura sobre a atividade antimetastática de fucoidanas em diferentes linhagens de células tumorais (HSU et al., 2013), cujos mecanismos incluem a regulação de metaloproteinases de matriz (MMPs) moduladas por fatores de transcrição e vias de sinalização como NF-κB, MAPK, PI3K-Akt e ERK (WANG et al., 2014). Em contexto, estudos com fucoidanas em linhagens de células tumorais somam mecanismos desejáveis para uma resposta favorável no tratamento antineoplásico.

Os desafios mais significativos do uso dos quimioterápicos convencionais envolvem a resistência medicamentosa e a ocorrência de efeitos colaterais (HSU e HWANG, 2019). Em situações onde foram administradas doses de fucoidanas junto a quimioterápico convencional (ciclofosfamida), foi notada uma resposta mais aprimorada por meio da inibição de metástase em modelo experimental (ALEKSEYENKO et al., 2007). O uso de fucoidanas com outros antineoplásicos, como tamoxifeno, cisplatina e paclitaxel exibiu um aumento da atividade antitumoral no que se refere à inibição de crescimento e taxas de apoptose (ZHANG et al., 2013). Estas vantagens se somam a outras já evidenciadas como maior tolerância à quimioterapia e menor sinais de fadiga (IKEGUCHI et al., 2011).

Pelos relatos da literatura até o momento, o uso de fucoidanas na terapia antitumoral tem apresentado vantagens bastante significativas, sejam elas relacionadas aos efeitos diretos sobre reguladores bioquímicos da sobrevivência celular, seja pela redução dos danos causados pelas quimioterapias convencionais. Em se tratando de uma linha de investigação emergente na terapia antitumoral baseada em componentes de organismos como macroalgas, mostra-se bastante promissora no tratamento do câncer.

LIPÍDEOS

Estudos com algas do filo Rhodophyta têm demonstrado um grande potencial econômico na produção de ácidos graxos poli-insaturados de cadeia longa (PUFAs), tais como o ácido araquidônico (C20:4n-6) e ácido eicosapentaenoico (C20:5n-3)

(SÁNCHEZ-MACHADO et al., 2004; KHOTIMCHENKO et al., 2005; CIAN et al., 2014; ASTORGA-ESPAÑA, 2014). O interesse em macroalgas marinhas estão nos PUFAs essenciais da família n-3 (ômegas 3), os quais têm sido associados à manutenção da saúde humana, principalmente na redução dos riscos de doença cardíaca (CHEN et al., 2011; ROBERTSON et al., 2013; YATES et al., 2014), processos inflamatórios e câncer (GIROS et al., 2009; POTTEL et al., 2014). Os PUFAs n-3 essenciais são altamente concentrados em regiões do cérebro, importantes para as funções cognitivas e comportamentais (CUTULI et al., 2016; LAURITZEN et al., 2016).

Macroalgas marinhas são organismos que vivem nos mais diversos ambientes e condições abióticas. Estas adversidades, muitas vezes extremas, como o ambiente Antártico e Subantártico favorecem estes organismos a desenvolver inúmeras rotas metabólicas, muitas de defesa para sua sobrevivência. Embora os fatores que podem influenciar os perfis de FAs nas macroalgas vermelhas ainda não estejam completamente elucidados, é importante mencionar que a região entremarés está sujeita a amplas flutuações ambientais, como radiação solar ou dessecação, que podem alterar o metabolismo das macroalgas (DUDGEON et al., 1990; KÜBLER & DAVISON, 1993; WILLIAMS & DETHIER, 2005). Portanto, a plasticidade bioquímica está intimamente ligada à quantidade de luz necessária para a fotossíntese, e esse fator é determinante para a sobrevivência e reprodução das macroalgas (FLORES-MOLINA et al., 2014). Sob condições naturais, a produção de lipídios por esses organismos é extremamente importante, pois os lipídios polares integram os componentes estruturais da membrana na forma de fosfolipídios e glicolipídios (GURR et al., 2002). Nesse contexto, lipídios neutros, como triacilgliceróis (TAGs), estão presentes e têm o papel de reservar fontes de energia (GUSCHINA & HARWOOD, 2009) que se mostram úteis para a sobrevivência em condições ambientais desafiadoras, como o inverno em regiões de altas latitudes (MANSILLA & ÁVILA, 2011). Além disso, é importante mencionar que tais algas toleram longos períodos de escuridão, com uma fotossíntese eficiente em níveis extrmamente baixos de luz (GÓMEZ et al., 2009).

Essencialmente, lipídios são um grupo de moléculas cujas principais funções têm sido relacionadas ao armazenamento de energia para reações metabólicas e como componente estrutural na constituição de membranas celulares, sendo um dos principais formadores da bicamada lipídica (SPOSITO et al., 2007). Hoje, porém, a

visão geral sobre o estudo de lipídios tem sido ampliada, principalmente pela descoberta de novas moléculas associadas a eles e suas ações na saúde humana (DAS, 2011), bem como, sua utilização como biomassa para energias renováveis (SIMON-PLAS et al., 2011).

Do ponto de vista estrutural, podemos dividir os lipídios em dois grandes grupos: os saponificáveis e insaponificáveis. Os lipídios saponificáveis, isto é, aqueles que contém ácidos graxos em sua constituição, podem ser também classificados como simples, onde estão os glicerolipidios (óleos e gorduras), os cerídeos (proteção de frutos e folhas) e o grupo formados por fosfolipídios e glicolipidios, chamados de lipídios complexos, onde encontramos os glicerofosfolipidios, esfingolipídios e esfingomielinas (CAMPBELL & FARRELL, 2007). Moléculas como terpenóides e carotenoides, formados por uma estrutura isoprenóide básica e fitoesteróis que possuem um núcleo ciclo-pentano-per-hidro-fenantreno como unidade básica, estão presentes na fração insaponificável dos lipídios, isto é, não contém ácidos graxos ligados a molécula principal (BERG et al., 2002).

Para uma adequada manutenção da saúde do organismo humano e realização das complexas funções que ocorrem em seu interior, é necessária uma boa alimentação. Uma dieta balanceada fornece de 200 a 400 mg de fitoesteróis, sendo necessário a ingestão de 2 g/dia de fitoesteróis para uma redução média de 10-15% dos índices de lipoproteína de baixa densidade (LDL). O mecanismo benéfico de ação dos fitoesteróis em nosso organismo está diretamente relacionado a não absorção do colesterol pelo organismo. Quando os fitoesteróis estão presentes na dieta, são quebrados em esteróis livres e ácidos graxos, sendo assim introduzidos nas micelas, impedindo a entrada ou o deslocamento do colesterol para elas. Devido à baixa absorção intestinal, os fitoesteróis deslocam o colesterol para fora da micela na luz intestinal, bloqueando parcialmente sua absorção, ou seja, reduzem a capacidade de transporte de colesterol pela micela, sendo então eliminado nas fezes (DAS, 2011).

Na classe dos lipídios polares e de grande importância estão os fosfolipídios, mais especificamente os esfingolipídios, que associados ao colesterol formam microdomínios chamados rafts, os quais são responsáveis por inúmeros direcionamento de processos celulares, como fixação de proteínas, intervenção na expressão gênica, biogênese da membrana e divisão celular. Estas novas

descobertas têm levado a novas drogas antibacterianas, antifúngicas e anti-inflamatórias que utilizam bases análogas aos esfingolipídios (SIMON-PLAS et al., 2011).

Neste contexto o conteúdo lipídico existente em matrizes vegetais tem se mostrado um estudo importante para áreas bioquímicas, farmacêuticas, nutricionais, entre outras. Os métodos convencionais se baseiam na extração dos lipídios utilizando grandes quantidades de solventes associado a tempos prolongados e calor excessivo, onde ocasionam principalmente a oxidação, peroxidação e hidrólise dos analitos com influências negativas ao resultado da análise (GUAN et al., 2007).

A bioatividade dos extratos lipídicos de algas marinhas tem sido estudada em células de melanoma, para avaliar e demonstrar a atividade citotóxica (ROCHA et al., 2007). Em um estudo sobre os efeitos citotóxicos de extratos de microalgas na linha de células de câncer gástrico humano, foram encontrados valores de IC50 de 6170 µg/mL para extrato rico em ácidos graxos saturados (SFAs) e 1260 µg/mL para extrato rico em PUFAs (SHAKERI et al., 2017). Alguns estudos em células de câncer de mama mostraram que a ingestão de PUFAs n-3 poderia reduzir o crescimento celular, o volume do tumor e prevenir a metástase por diferentes mecanismos, como modulação da expressão gênica e transdução de sinal, alterações no metabolismo do estrogênio, alterações na produção de espécies reativas de oxigênio (ROS), supressão da transformação neoplásica, inibição do crescimento celular e aumento da apoptose (LARSSON et al., 2004; WANNOUS et al., 2013). Essas observações reforçam o aspecto biológico do uso de macroalgas como fonte nutricional ou farmacológica, uma vez que as algas de ambientes frios têm sido prospectadas para esse fim (PEREIRA et al., 2012; VAN GINNEKEN et al., 2011).

O câncer de bexiga (BC) é um câncer urológico maligno comum em todo o mundo, geralmente ocorre no trato urinário e é a segunda principal causa de morte entre os tumores genito-urinários (SIEGEL et al., 2018). Com 76960 novos casos e 16390 mortes em 2016, aproximadamente 80% dos pacientes com BC apresentam tumores invasivos não musculares (Ta, T1 e Tis) (LEE et al., 2010; LI; DUYMICH et al., 2016). Estudos relatam que a ingestão de PUFA n-3 pode reduzir a incidência de vários tipos de câncer, como câncer colorretal (THEODORATOU et al., 2007; HALL et al., 2008), mama (THIÉBAUT et al., 2009) e próstata (GERBER, 2012; WILLIAMS et al., 2011). Todos os mecanismos subjacentes à forma como o PUFA n-3 podem modular o crescimento e desenvolvimento de células cancerígenas ainda não estão

claros. Evidencia-se que esses componentes podem interferir no ciclo celular ou na morte celular (CORSETTO et al., 2011) por um mecanismo apoptótico ou de autofagia (KIM et al., 2018). Um estudo de Corsetto e colaboradores (CORSETTO et al., 2012) apontou para interações que ocorrem entre PUFA n-3 e fosfolipídios da membrana em células cancerígenas, o que levaria a alterações na fisiologia da membrana.

JUSTIFICATIVA

Embora o considerável progresso feito nos últimos anos em relação à identificação da composição química das macroalgas, poucos relatos na literatura caracterizam a atividade antitumoral de matabólitos de macroalgas sobre BC. Em se tratando de polissacarídeos, com destaque para fucoidanas, os trabalhos na literatura têm apontado para uma atividade bastante favotável, porém até o momento não há revisões de literatura que abordem a atividade específica de fucoidanas sobre esse tipo de tumor. Adicionalmente, poucos relatos na literatura exploram as variações na quantidade de FA ou lipídios nas amostras das algas subantárticas chilenas. Até onde sabemos, não há relatos na literatura sobre a variação sazonal do conteúdo de FA na macroalga subantártica chilena *M. laminarioides*. Além disso, não há estudos sobre o efeito antitumoral de frações ricas em PUFAs obtidas desta macroalga contra células de BC. Considerando isso, o objetivo do presente trabalho foi e avaliar a atividade antitumoral da fração rica em PUFA n-3 contra células T24 de BC.

OBJETIVOS

GERAIS

- Investigar os trabalhos publicados que abordem a ação de fucoidanas sobre modelos de BC;
- Analisar os perfis de FAs em distintas fases de desenvolvimento das algas marinhas *M. laminarioides* no inverno e no verão e avaliar a atividade antitumoral da fração rica em PUFAs.

ESPECÍFICOS

- Apresentar a estrutura das fucoidanas derivadas de macroalgas;

- Abordar as fucoidanas como moléculas promissoras na terapia antitumoral;
- Discutir os trabalhos publicados na literatura que envolvam a ação de fucoidanas e modelos de BC.
- Analisar o papel das fucoidanas como terapia antitumoral adjuvante.
- Preparar e separar as amostras da macroalga *M. laminarioides*.
- Extrair os lipídeos das macroalgas e convertê-los em FAs.
- Determinar a citotoxicidade pelo método MTT para obter a fração rica em PUFAs e posteriormente testá-la em células T24 de BC.
- Relizar o teste de viabilidade celular LIVE/DEAD.
- Detectar a taxa de apoptose celular pela marcação para DAPI.

2. PROJETO DE PESQUISA

Caracterização e Avaliação do Potencial Antitumoral de Lipídeos de Macroalgas dos Filos Chlorophyta, Rhodophyta e Phaeophyta Originárias da Região Sub-Antártica do Chile

Participantes

Coordenador do Projeto: Claudio Martin Pereira de Pereira

Bolsista vinculado ao Projeto: Caril Constante Ferreira do Amaral

Rede de Colaboração:

- Universidade Federal de Pelotas, Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Laboratório de Heterociclos Bioativos e Bioprospecção, RS, Brasil.
- Universidad de Magallanes, Departamento de Ciências y Recursos Naturales, Punta Arenas, Chile.

Caracterização do Problema

Local - Universidade de Punta Arenas

A região de Magalhães, localizado no sudoeste do continente sul-americano (48º36 'a 56ºS; 66º25' para 75º40'W), é o maior representante do mundo do ambiente sub-antártico, com um total de 391 espécies de macroalgas, dos quais 75 são Chlorophyta, 86 são Ochrophyta e 230 são Rhodophyta. Muitas destas espécies são comercialmente importantes por seus alginatos e carragenanas, e várias espécies estão atualmente sendo comercializados para consumo direto por seres humanos. Apesar dos consideráveis progressos realizados nos últimos anos, nosso conhecimento atual sobre as propriedades nutricionais de macroalgas da região Sub-Antártica do Chile não é completamente elucidado, uma vez que estudos sobre a composição química de macroalgas Sub-Antárticas são escassos.

As algas são a base da cadeia alimentar de organismos marinhos que fornecem vários tipos de nutrientes, tais como proteínas, vitaminas, aminoácidos, minerais e polissacáridos e que servem como precursores de biomoléculas e lipídeos essenciais, tais como o ácido linoleico (18:2n-6) e ácido α-linolênico (18:3n-3), os quais não são sintetizados pelo corpo humano (DI MASCIO et al., 1995; HOLLNAGEL et al., 1996; CARDOZO et al., 2006). Além disso, os metabólitos de algas têm grande valor econômico em farmacêutica (anticoagulantes, atividades antitrombóticos e antioxidantes), alimentos (dietética e fibras funcionais), bioenergia (biocombustíveis) e indústrias de biotecnologia (imunológicos, cosméticas e antivirais) (GRESSLER et al., 2011; GUARATINI et al., 2012; PEREIRA et al., 2012; MATSUHIRO et al., 2014; MCCAULEY et al., 2015; LIRA et al., 2016; MILLEDGE & HARVEY, 2016).

Estudos sobre o filo Rhodophyta demonstraram um enorme potencial econômico na produção de ácidos graxos poli-insaturados de cadeia longa (PUFAs), tais como o ácido araquidônico (20:4n-6) e ácido eicosapentaenoico (20:5n-3) (SÁNCHEZ-MACHADO et al., 2004; KHOTIMCHENKO et al., 2005; ASTORGA-ESPAÑA, 2014; CIAN et al., 2014). No entanto, como as alterações climáticas, em particular a baixas temperaturas, fisiologicamente afetam estas algas causando um acúmulo de PUFAs, principalmente em sua estrutura de membrana.

O interesse particular em macroalgas marinhas são PUFAs essenciais da família n-3 (ômegas 3), os quais têm sido associados a manutenção da saúde humana, principalmente na redução dos riscos de doença cardíaca (CHEN et al., 2011; ROBERTSON et al., 2013; YATES et al., 2014), processos inflamatórios e câncer (GIROS et al., 2009; POTTEL; LYCKE et al., 2014). Os PUFAs n-3 essenciais são altamente concentradas nas regiões do cérebro, importantes para as

funções cognitivas e comportamentais (CUTULI et al., 2016; LAURITZEN et al., 2016).

Macroalgas marinhas são organismos que vivem nos mais diversos ambientes e condições abióticas. Estas adversidades, muitas vezes extremas, como o ambiente Antártico e Subantártico favorecem estes organismos a desenvolverem inúmeras vias metabólicas, muitas de defesa para sua sobrevivência.

Na interação com ambientes diferenciados, as algas se adaptam e criam rotas diferenciadas, onde podem produzir compostos de grande interesse biotecnológico ou simplesmente aumentar os níveis de compostos já existentes. Neste sentido, podemos citar o aumento dos níveis de ácidos graxos insaturados, mais precisamente os poli-insaturados, em ambientes gelados, utilizados pelas algas na integridade das membranas, sendo importante para manter as funções de todo seu sistema biológico. Estratégias metabólicas adaptativas ao frio intenso incluem a manutenção da fluidez das membranas biológicas pelo aumento de ácidos graxos insaturados que impedem a rigidificação das membranas compostas por lipídeos. Além disso, é importante mencionar que tais espécies toleram longos períodos de escuridão, com uma fotossíntese eficiente em extremos níveis baixos de luz (GÓMEZ et al., 2009). Sob condições de tempo frio, as algas mantêm sua fluidez na membrana, o qual é o principal responsável para o seu crescimento celular.

Com base nessas considerações, o objetivo do presente projeto é caracterizar o perfil lipídico, tais como, carotenoides, ácidos graxos e fitoesteróis de macroalgas da região subantártica do Chile, bem como, avaliar o potencial antitumoral e o grau de toxicidade celular em multilinhagens de células tumorais desses compostos isoladamente e em associação com fármacos comerciais, assim como os mecanismos de ação envolvidos.

Em consonância com os objetivos do projeto em conexão com as habilidades do discente de doutorado indicado para a bolsa, Caril Constante Ferreira do Amaral, doutorando do Programa de Pós-Graduação em Bioquímica e Bioprospecção, com experiência nas áreas de cultivo celular de células mesenquimais e tumorais, ensaios de toxicidade e expressão gênica necessários para a avaliação biológica dos compostos como aminoácidos, fitoesteróis e ácidos graxos entre outros obtidos das macroalgas marinhas.

JUSTIFICATIVA

Os lipídios são um grupo de moléculas formadas principalmente por ácidos graxos ligados a açúcares, álcoois, aminoácidos ou grupos fosfatos. As duas principais funções dos lipídios têm sido no armazenamento de energia para reações metabólicas e como componente estrutural na constituição de membranas celulares, sendo um dos principais formadores da bicamada lipídica (SPOSITO et al., 2007). Hoje, porém, a visão geral sobre o estudo de lipídios tem sido ampliada, principalmente pela descoberta de novas moléculas associadas a eles e suas ações na saúde humana (DAS, 2011), bem como, sua utilização como biomassa para energias renováveis (SIMON-PLAS et al., 2011).

Estruturalmente podemos dividir os lipídios em dois grandes grupos: os saponificáveis e insaponificáveis. Os lipídios saponificáveis, isto é, aqueles que contém ácidos graxos em sua constituição, podem ser também classificados como simples, onde estão os glicerolipidios (óleos e gorduras), os cerídeos (proteção de frutos e folhas) e o grupo formados por fosfolipídios e glicolipidios, chamados de lipídios complexos, onde encontramos os glicerofosfolipidios, esfingolipídios e esfingomielinas (CAMPBELL & FARRELL, 2007).

Moléculas como terpenóides e carotenoides, formados por uma estrutura isoprenóide básica e fitoesteróis que possuem um núcleo ciclo-pentano-per-hidro-fenantreno como unidade básica, estão presentes na fração insaponificável dos lipídios, isto é, não contém ácidos graxos ligados a molécula principal (BERG et al., 2002).

A constituição de nosso organismo e todas as funções que ocorrem em seu interior dependem principalmente de uma boa alimentação. Uma dieta balanceada fornece de 200 a 400mg de fitoesteróis, sendo necessária a ingestão de 2 g/dia de fitoesteróis para uma redução média de 10-15% do LDL-C. A ingestão de 3 a 4 g/dia de fitoesteróis pode ser utilizada como adjuvante ao tratamento hipolipemiante (SPOSITO et al., 2007).

O mecanismo benéfico de ação dos fitoesteróis em nosso organismo está diretamente relacionado a não absorção do colesterol pelo organismo. Quando os fitoesteróis estão presentes na dieta, são quebrados em esteróis livres e ácidos graxos, sendo assim introduzidos nas micelas, impedindo a entrada ou o deslocamento do colesterol para elas. Devido à baixa absorção intestinal, os fitoesteróis deslocam o colesterol para fora da micela na luz intestinal, bloqueando parcialmente sua absorção, ou seja, reduzem a capacidade de transporte de colesterol pela micela, sendo eliminado nas fezes (DAS, 2011).

Na classe dos lipídios polares e de grande importância estão os fosfolipídios, mais especificamente os esfingolipídios, que associados ao colesterol formam microdomínios chamados rafts, os quais são responsáveis por inúmeros direcionamentos de processos celulares, como fixação de proteínas, intervenção na expressão gênica, biogênese da membrana e divisão celular. Estas novas descobertas têm levado a novas drogas antibacterianas, antifúngicas e anti-

inflamatórias que utilizam bases análogas aos esfingolipídios (SIMON-PLAS et al., 2011).

Neste contexto o conteúdo lipídico existente em matrizes vegetais tem se mostrado um estudo importante para áreas bioquímicas, farmacêuticas, nutricionais entre outras. Os métodos convencionais se baseiam na extração dos lipídios utilizando grandes quantidades de solventes associado a tempos prolongados e calor excessivo, onde ocasionam principalmente a oxidação, peroxidação e hidrólise dos analitos com influências negativas ao resultado final da análise (GUAN et al., 2007).

A diversidade de novas matrizes a serem pesquisadas tem surgido ao longo dos últimos anos e tem obrigado aos pesquisadores procurarem não somente novos tipos de reagentes, mas também novos equipamentos e técnicas como por exemplo métodos extrativos em fase sólida, ultrassom e micro-ondas que separados ou associados tem ajudado a busca de moléculas de alto valor industrial, farmacêutico e alimentar como alcaloides, terpenóides, flavonoides entre outras (LI et al., 2013).

Paralelamente, pesquisas têm sido realizadas no sentido de procurar metodologias que utilizem quantidades reduzidas de reagentes e solventes, bem como que apresentem menor toxicidade e baixa geração de resíduos.

Neste sentido, métodos como Cromatografia Gasosa (GC-FID), cromatografia líquida de ultra eficiência (UHPLC), Cromatografia Gasosa acoplada a Espectrometria de Massas (GC-MS) e Cromatografia Líquida com Espectrometria de Massas (LC-MS) tem sido sugerida, pois se apresentam como métodos rápidos, com separação eficiente dos analitos, alta resolução e que utilizam volumes pequenos de amostras injetadas.

Aliado a estas técnicas, novas terapias para o câncer estão sendo desenvolvidas e isto envolve a busca por agentes antitumorais de origem natural e sintéticos. Essas pesquisas iniciaram por volta dos anos 1950, com o descobrimento e desenvolvimento de alcaloides da vinca, vimblastina e vincristina, isolados da planta Cantharanthus roseus (CRAGG & NEWMAN, 2005). Em meio àquela década também foi descoberta a podofilotoxina, constituinte principal das espécies da planta Podophyllum, popularmente usada no tratamento de carcinomas cutâneos. Posteriormente, foram desenvolvidos os derivados semissintéticos da podofilotoxina, o etoposídeo e teniposídeo, efetivos contra linfomas e cânceres testiculares (CRAGG & NEWMAN, 2005). Nesse caminho, ainda foram descobertos os taxanos, isolado do Taxus brevifolia e os compostos derivados da camptotecina, oriunda da planta Camptotheca acuminata (CRAGG & NEWMAN, 2005). Seguindo esse caminho, tanto nosso laboratório como outros tem dado especial atenção no desenvolvimento e pesquisa dos efeitos da administração da curcumina (derivado do açafrão) e seu potencial efeito antitumoral (ZANOTTO-FILHO et al., 2012). Os compostos derivados de macroalgas e seus potenciais efeitos antitumorais têm despertado especial atenção de nosso grupo. Estes compostos serão usados para ensaios comparativos de eficácia usando como base medicamentos já amplamente utilizados no tratamento do câncer. A incorporação de elementos e grupamentos químicos na síntese de novas moléculas e avaliação desse potencial efeito na regulação de oncogenes ou supressores tem sido o objetivo proposto e o caminho pelo qual buscaremos desenvolver novas abordagens terapêuticas.

OBJETIVOS

Levantamento das algas ambientalmente viáveis para coleta, conforme as leis ambientais do Chile;

Coleta de material de macroalgas em diferentes localizações da região Sub-Antártica;

Identificação do material coletado, conforme os Filos Chlorophyta, Rhodophyta e Phaeophyta;

Processo de secagem das algas e controle de umidade. Esse processo será executado respeitando as normas para extração de produtos naturais da Farmacopéia Brasileira;

Extração de carotenoides, ácidos graxos e fitoesteróis de macroalgas chilenas;

Bioprospecção inicial dos extratos de macroalgas quanto ao potencial de substâncias orgânicas, como, carotenóides, ácidos graxos totais e fitoesteróis;

Seleção do material algal apto para envio a Universidade Brasileira, Universidade Federal de Pelotas a fim de executar as análises de identificação química em Cromatografia Gasosa e Cromatografia Líquida;

Organização de dados iniciais de coleta, espécie/localização, potencial de bioprospecção para futura avaliação e posterior publicação e submissão de Depósitode Patente;

Ensaios biológicos preliminares dos compostos orgânicos derivados das macroalgas sobre células tumorais multilinhagens;

Identificação do perfil de resposta celular frente o contato com os compostos derivados das algas;

Ensaio de citotoxicidade para avaliação da viabilidade celular;

Bioprospecção dos derivados naturais de macroalgas na terapêutica docâncer, que inclui redação dos manuscritos para publicação e submissão de depósito de Patente baseado na atividade antitumoral dos compostos.

Organização de relatório para disponibilização da CAPES e aos Grupos de Trabalho Chile-Brasil.

METODOLOGIA

Extrações Lipídicas em Macroalgas Sub-Antártica

Ácidos Graxos (Brasil / Chile)

A extração de lipídios seguira o método Bligh & Dyer (BLIGH; DYER, 1959), o qual se baseia na extração a frio utilizando uma mistura de metanol / clorofórmio / água na proporção de 1:2:0,8. A fase orgânica (superior) que contém o n-hexano mais os ácidos graxos será removida e metilada segundo a metodologia de Hartman & Lago (HARTMAN; LAGO, 1973).

As extrações segundo método Bligh & Dyer (BLIGH; DYER, 1959) e Cheong (CHEONG; GUO; YANG; CHUA et al., 2011) serão testadas em micro-ondas e ultrassom. As derivatizações seguirão a metodologia segundo Hartman & Lago (HARTMAN; LAGO, 1973), o qual resumidamente se utiliza 5 mL de NaOH 0,5 mol L-1 em metanol, onde são misturados 0,2 – 0,25 g da amostra sob refluxo por um período de 5 min, sendo após, adicionado o agente esterificante , formado por 2 g de cloreto de amônio, 60 mL de metanol e 3 mL H2SO4 conc. Posteriormente a amostra e o agente esterificante são refluxados por 3 min, sendo após, transferido para um funil de separação contendo éter de petróleo e agua deionizada.

Fitoesteróis (Brasil / Chile)

Os fitoesteróis serão extraídos pelo método convencional segundo (LOPES; SOUSA; BERNARDO; ANDRADE et al., 2011), onde é feito a saponificação da amostra (0,5 g) usando 40 mL de solução etanólica de KOH 10 % a 70 ºC por 1 h e posteriormente uma extração liquido-liquido usando 3x10 mL de n- hexano para separação da fração insaponificável. As frações orgânicas serão evaporadas por evaporador rotativo a vácuo (MityVac) até a obtenção de um resíduo contendo os fitoesteróis. Na procura de extrações mais limpas será testada extrações de fitoesteróisassistidos por micro-ondas e ultrassom seguirão as metodologias de Xiao-Hua et al. (XIAO-HUA; ZHI-QUAN; GONG-KE, 2013) e Chemat et al. (CHEMAT; LAGHA; AITAMAR; BARTELS et al., 2004), respectivamente.

Carotenóides

As amostras de algas (0,5 g) a serem quantificadas serão pesadas em balão e saponificadas com 40 mL de KOH etanólico (10%) durante 1h, com agitação constante e temperatura de 70 ºC. Após resfriamento a amostra será centrifugada, sendo após colocada em funil de separação com 10 mL de n-hexano para separação da fração insaponificável. A fase orgânica após secagem com sulfato de sódio anidro será evaporada em rotaevaporador rotativo e o resíduo diluído em 1 mL de metanol-acetonitrila (30:70 v/v), sendo filtrado em membrana de 0,22 µm (LOPES; SOUSA; BERNARDO; ANDRADE et al., 2011).

Procedimentos Cromatográficos

2.4.2.1 Cromatografia Gasosa GC-FID (Brasil)

Análise de Ácidos graxos por GC-FID

As análises cromatográficas serão feitas utilizando padrão Mix F.A.M.E. C4C24 (Supelco). O padrão será preparados na concentração de 2000 mg.L-1 em hexano (grau HPLC) marca Malinckrodt. A identificação dos compostos será feita pela comparação do tempo de retenção do composto analisado e aquele obtido para o padrão cromatográfico. A concentração será calculada pelo programa GC Solution, utilizando a área normatizada.

Na identificação dos ácidos graxos será utilizado um cromatógrafo a gás GC/FID 2010 marca Shimadzucom uma coluna capilar Elite-Wax (Polietilenoglicol) 30 m x 0,25 mm i.d. x e 0,25 µm de espessura do filme; hidrogênio como gás carreador na vazão de 1,2 mL.min-1; sendo o programa de temperatura inicial da coluna de 100 ºC permanecendo nesta temperatura por 0,5 min. subindo na razão de 7 ºC.min-1 até 175 ºC após aumentando na razão de 5 ºC.min-1 até 190 ºC permanecendo 1 min. nesta temperatura, subindo a razão de 1,2 ºC.min-1 até 230 ºC permanecendo por 11,45 min; temperatura do injetor e detector em 230 ºC.

Análise de Fitoesteróis por GC-FID

As análises de fitoesteróis serão realizadas utilizando padrões de beta sitosterol (pureza ≥ 95 %, Sigma, S1270); campesterol (pureza aprox. de 65 %, Sigma, C5157); estigmasterol (pureza de aprox. 95 %, Sigma, S2424). A preparação dos padrões será na concentração de 1000 mg.L-1 em n-hexano (grau HPLC). A identificação dos compostos ocorrerá através da comparação do tempo de retenção do analito e o padrão cromatográfico. As concentrações serão calculadas por área normatizada através do programa GC solution do GC-2010. Na identificação dos fitoesteróis o aparelho utilizado será um cromatógrafo a gás GC/FID 2010 marca Shimadzucom uma coluna capilar DB-5 (5% fenil e 95% metilsiloxano) 30 m x 0,25

mm i.d. x e 0,25 µm de espessura do filme; como gás carreador hidrogênio na vazão de 1,2 mL.min-1; o programa de temperatura empregado será o seguinte: temperatura inicial da coluna de 150 ºC na razão de 20 ºC.min-1 até 300 ºC permanecendo nesta temperatura por 12,5 min; temperatura de injeção de 320 ºC e do detector de 320 ºC.

Análise de Carotenóides por UHPLC

As análises de carotenóides serão realizadas em umCromatógrafo Liquido de Ultra Eficiência UltiMate 3000 (Thermo Scientific Dionex) com detector ultravioleta (UHPLC – UV) acoplado a um detector ELSD (Agilent, Mod. 385), utilizando uma coluna C18 de fase reversa (5µm x 250 mm x 4,6 mm i.d). A eluição cromatográfica será realizada a partir de uma corrida isocrática com fase móvel constituída de eluente A (diclorometano: metanol: acetonitrilo: água, 5,0: 85,0: 5,5: 4,5, v / v) e eluente B (diclorometano: metanol: acetonitrilo: água, 25,0: 28,0: 42,5: 4,5, v / v). Separação dos carotenóides foi alcançado pelo procedimento seguinte gradiente: 0% de B durante 8 min; um gradiente linear de 0 a 100% de B em 6min; 100% de B durante 40 min, a um caudal de 1,0 mL / min. O espectro de absorção de carotenóides serão exibidos entre 250 e 700 nm.

Ensaios Biológicos

Cultivo Celular

As linhagens celulares utilizadas estão armazenadas no Banco de Células Tumorias dos Laboratórios de Oncologia Celular e Embriologia Molecular do CDTec/UFPel, adquiridas através do Banco de Células do Rio de Janeiro (PABCAM, Universidade do Rio de Janeiro, RJ, Brasil). As células tumorais serão cultivadas em meio apropriado para cada linhagem e mantidas em incubadora a 37˚C, com

atmosfera umidificada (95%) e saturação de CO2 (5%) controlado (Heal Force®, China) até atingirem o estágio de subconfluência (~90%). Neste estágio as células serão recolhidas com uma solução de tripsina com 0.25% e 0.04% de EDTA (v/v 1:1) (Vitrocell Embriolife) na proporção de 1:3. Todos os experimentos serão realizados com as células na sua fase logarítmica de crescimento.

Avaliação da Morfologia Celular

As células serão incubadas em placas (200 µL/poço) de cultura de 96 poços (TTP, Switzerland) a uma densidade de 2 x 104/mL e mantidas na incubadora por 24 horas. Então, o meio de cultura será aspirado e substituído por outro contendo diferentes concentrações das substâncias a serem testadas (a concentração testada depende do composto antitumoral proposto, variando de 5 a 100 µM). Após o período de 24, 48 e 72 h as células serão visualizadas e fotografadas (aumento original de 100x e 400x) com uma câmera acoplada a um microscópio invertido com fluorescência Olympus IX71 (Olympus Optical Co., Japan).

Ensaio de Viabilidade Celular (MTT)

Os meios de cultura serão removidos e substituídos por outro contendo 0,5 mg/mL de MTT (sal tetrazolium [3-(4,5-dimetltiazol-2-yl)-2,5-difeniltetrazolium brometo]). Após a remoção dos materiais experimentais, 180 µL/poço de meio de cultura e 20 µL de MTT serão adicionados em cada poço e mantidos na incubadora por 3 horas a 37ºC. Então o meio contendo MTT é aspirado e 200 µL/poço de DMSO serão adicionados a cada poço para solubilização dos cristais de formazan decorrentes da redução do MTT pela desidrogenase mitocondrial. Após incubação os valores de absorbância serão capturados com o auxílio de um espectrofotômetro com o comprimento de onda calibrado a 570 nm (Thermo Plate TP-Reader).

Análise Estatística

A normalidade e a homocedasticidade dos dados serão analisadas e os mesmos serão submetidos ao teste paramétrico ou não paramétrico mais adequado. Para todos os testes, será fixado em 5% ($p < 0,05$) o nível de rejeição da nulidade da hipótese.

3. RELATÓRIO DE TRABALHO DE CAMPO

Este estudo contou com o apoio financeiro e logístico do Programa Antártico Brasileiro (PROANTAR/MCT/CNPq-nº 23/2009; 64/2013) e da Marinha do Brasil. Também foram fundamentais o apoio financeiro e de bolsas de estudo das agências brasileiras de fomento à pesquisa Fundação para Apoio à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), Fundação para apoio à pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Pesquisa Científica Desenvolvimento Tecnológico (CNPq), Programa Geral de Cooperação Internacional - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (PGCI/CAPES), Programa Nacional de Pós-Doutorado - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (PNPD/CAPES), Proyecto Conicyt PIA, Instituto de Ecología y Biodiversidad (IEB) e o Programa FONDECYT.

O período correspondido ao Doutorado Sanduíche deu-se de maio/2017 a abril de 2018. A partir da chegada ao Chile foi iniciado o período de adaptação ao novo país e o aprendizado da rotina laboratorial. As rotinias iniciais incluíram o acompanhamento da coleta de material biológico junto aos colegas de laboratório em seus projetos de pesquisa, bem como o processamento das amostras. Durante os dias iniciais de ambientação e acompanhamento laboratorial ocorreram reuniões com os co-orientadores chilenos no intuito de ajustar os objetivos do projeto incialmente proposto, no intuito de refinar as análises e obter resultados mais robustos. Dessas conversas e reuniões houve a proposta e incentivo para que investigássemos em algas pardas subantárticas o conteúdo de polissacarídeos sulfatados, como fucoidanas, bem como a atividade antitumoral desses compostos. Ao conversar com os orientadores no Brasil, optou-se por prosseguir com o projeto inicialmente proposto e realizar uma busca direcionada à atividade de fucoidanas no câncer de bexiga, no intuito de fornecer subsídio teórico robusto para futuras investigações.

Uma vez integrado ao ambiente e nova equipe, foram iniciadas as etapas de bioprospecção das algas que seriam selecionadas para os estudos. As buscas na literatura eram direcionadas ao metabolismo das macroalgas subantárticas, impacto ecológico e potencial econômico. Após extensa análise, optou-se por avaliar a composição de lipídios e atividade antitumoral da macroalga vermelha *Mazzaella laminarioides* em diferentes estações do ano e em diferentes fases de

desenvolvimento, a saber: vegetativa, tetraesporofítica e cistocárpica. Ao mesmo tempo, também foram coletadas as macroalgas pardas *Lessonia flavicans* e *Lessonia searlesiana* no intuito de avaliar futuramente a influência da sazonalidade na composição química e atividade antitumoral de fucoidanas derivadas dessas algas.

Durante os meses de julho e agosto de 2017 foram feitas as coletas relativas ao inverno. Por aproximadamente 3 semanas as idas ao terreno tinham como objetivo buscar material ao laboratório vinculado a este e outros projetos em desenvolvimento pela equipe. Em muitos casos as idas foram dificultadas por conta do clima e maritmidade, circunstâncias que muitas vezes eram identificadas como inviáveis no momento em que se chegava aos pontos de coleta, aproximadamente 50 km ao sul da cidade. Tornou-se particularmente desafiador coletar a macroalga *Lessonia searlessiana*, uma vez que a mesma ocorre naturalmente em uma região subersa de aproximadamente 10 metros de profundidade. A formação de ondas com amplitude moderada atua como um efeito complicador, uma vez que as coletas em profundidade foram realizadas com o auxílio de equipamento de mergulho e as condições de visibilidade precisam estar bem ajustadas e sem interferências. Por isso, muitas vezes era necessário voltar em outro dia para conseguir efetuar a coleta. Ao obter quantidade de material suficiente, este era encaminhado ao labotarório para etapas subsequentes de processamento. As algas coletadas foram limpas em água corrente para remoção do sal. As macroalgas vermelhas foram separadas conforme a fase do desenvolvimento em que se encontravam. As macroalgas pardas foram fragmentadas em partes, a saber: frondas, estipes e disco. Todo o material coletado foi seco em laboratório com o auxílio de uma estufa ventilada a 20 °C por 5 dias. A biomassa seca, então, foi triturada com um moinho de facas e acondicionada em sacos plásticos do tipo “zip”, para melhor preservação do material até o envio ao Brasil.

Dados relativos a este projeto foram apresentados no IX Congreso Latinoamericano de Ciencia Antartica realizado em Punta Arenas/Chile entre os dias 04 e 06 de outubro de 2017. A apresentação constituiu o referencial teórico, a proposta de cooperação internacional, a representatividade do estudo tanto do ponto de vista bioquímico quanto ficológico e a bioprospecção dos recursos naturais disponíveis na região de Magalhães, a metodologia implementada, os dados já obtidos relativos a coleta e o cronograma de execução em andamento. Junto a este

trabalho foi apresentado outro estudo do grupo que buscou investigar a caracterização química dos ácidos graxos derivados de uma alga antártica (*Desmarestia anceps*) conforme a ocorrência em 5 pontos distintos de coleta, que mostrou diferenças significativas com consequente maior quantidade no ponto próximo a Estação Comandante Ferraz (EACF) e em Punta Ullmann com coleta profunda realizada com auxílio de robô (PU-R).

Entre o final de outubro e início de novembro de 2017 foram realizadas as coletas relativas à primavera. Entre janeiro e fevereiro de 2018 ocorreram as coletas de verão e no mês de abril de 2018 foi realizada a coleta de outono. Conforme mais coletas eram efetuadas, maior a segurança em trabalhar com o material. Ao mesmo tempo, a ajuda aos colegas de laboratório era constante, o que contribuiu para o aprendizado e empenho em fazer um bom trabalho assim que este material chegasse ao Brasil.

Evidentemente, muitos foram os desafios durante a realização desse trabalho. Algumas análises do projeto original (mencionado acima) foram substituídas e outras acrescentadas sem prejuízo ao escopo inicialmente previsto, com vistas a aprimorar os resultados e conter resultados mais robustos. Em termos gerais, a saída do país e a mudança dos rumos do projeto anteriormente estabelecido naturalmente alimentavam preocupações relacionadas a prazos. Do ponto de vista técnico, acadêmico e científico, esta experiência pode ser considerada bem-sucedida, na medida em que proporcionou engrandecimento em todas as áreas mencionadas, além de proporcionar o convívio com investigadores do mais alto gabarito nessa área.

4. ARTIGO 1

Antitumoral effects of fucoidan on bladder cancer

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§*Artigo redigido e submetido conforme as normas do periódico "Algal Research"*

Abstract

Fucoidans are natural compounds derived from brown algae with various known biological activities. The mechanisms underlying these activities are being widely studied and not yet fully understood. The results obtained so far have pointed fucoidans as promising molecules capable of serving as an adjunct to anticancer therapy, or even as dietary supplementation in patients with cancer. Many of the fucoidan-related effects such as antiproliferative, proapoptotic, antiangiogenic, and migration inhibitor may occur concurrently. Thus, knowledge of the cellular processes involved in metabolic pathways has fundamental importance for the identification of fucoidans-related antitumor potential. Although recent advances in understanding the biological activity of fucoidans have been made with different cancer cell lines, studies with experimental bladder cancer (BC) models are still scarce. Thus, this review addresses the molecular structure of brown algae-derived fucoidans, extraction, mechanisms that confer biological activity, and focuses on studies involving BC. This study points to a perspective of the use of fucoidans as a therapeutic possibility, supplementation of the diet of cancer patients and also with the possibility of reducing the side effects of conventional chemotherapy.

Keywords: Brown algae; sulfated fucan; antiproliferative; antioncogenic; chemotherapy; dietary supplementation;

Introduction

Cancer is a multifactorial disorder of various etiologies characterized by uncontrolled cell growth and eventually metastasis in the most severe cases, causing economic burdens and patient sufferings. Among various types of cancers, BC is the tenth most common cancer worldwide with 549,393 new cases in 2018, and 199,922 deaths were estimated in the same year (Bray *et al.*, 2018). BC is considered a cancer of industrialized countries, and its incidence rate is three-fold greater in high-resources versus low-resources nations (Greiman, Rosoff and Prasad, 2017). As part of a global effort to understand the magnitude of resources devoted to the diagnosis and treatment of BC patients, the estimated EU spending on BC in 2012 was € 4.9 billion, with public health care estimated at around 2.9 billion euros (Leal *et al.*, 2016).

The most significant risk factors for BC initiation and progression include intrinsic factors, which include higher proto-oncogenes expression and lower tumor suppressor genes; and extrinsic factors such as tobacco smoke habit and contact with risk factors such as air pollution and occupational exposure. (Hwang *et al.*, 2003; Li and Hemminki, 2004; Bailey-Wilson *et al.*, 2004; Thorgeirsson *et al.*, 2008; Hung *et al.*, 2008; Burger *et al.*, 2013; Cumberbatch *et al.*, 2018; Wong *et al.*, 2018). Although the literature provides inaccurate data regarding the surgical indication for bladder resection and lymph node dissection, other treatment modalities have been promising (Chou *et al.*, 2016). Chemotherapy has been shown to be beneficial in cases where surgical treatments are contraindicated, or in tumors with metastatic disease (Sio *et al.*, 2014). Despite advances in understanding tumor development in recent years, the most successful and preferred targets for new therapeutic agents include regulation of apoptotic signals. (Owens *et al.*, 2013; Morgensztern *et al.*, 2015; Bagrezaei *et al.*, 2018).

Innovations in bladder cancer therapies are needed to selectively eliminate cancer cells with as few side effects as possible. In context, natural agents are being used to prevent or suppress tumor progression and malignancy and, consequently, reduce the side effects of conventional therapies (Mann, Backlund and DuBois). Many chemotherapeutic agents have been discovered by evaluating the cytotoxic effects of compounds obtained directly from plant extracts (Kinghorn, 2008; Chanvorachote *et al.*, 2016). Additionally, it has been reported in the literature that natural products serve as effective substances against drug resistance during chemotherapy (Yuan *et al.*, 2017). As a consequence of the growing demand for new therapeutic strategies from natural resources, marine organisms such as seaweeds have emerged as an important source of new bioactive molecules on cancer therapy (Gammone *et al.*, 2016; Ruan *et al.*, 2018).

Seaweeds are a heterogeneous group of eukaryotic and photosynthetic organisms that play an important role in marine biodiversity and can be classified essentially into three main taxa: green algae (Phylum Chlorophyta), red algae (Phylum Rhodophyta) and brown algae. (Phylum Ochrophyta). Macroalgae have also been identified as an extremely important reservoir of unique bioactive secondary metabolites with pharmaceutical properties, especially sulfated polysaccharides (Kijjoa and Sawangwong, 2004; Shalaby, 2011; Zubia *et al.*, 2009). The production of these bioactive molecules by their secondary metabolism occurs through a response to ecological pressures such as space competition, predation, and successful reproduction (Haefner, 2003).

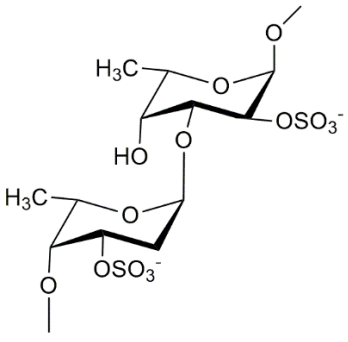
Within the group of sulfated polysaccharides derived from macroalgae, fucoidans have been extensively studied for their anticancer activity. This review summarizes the structure that attributes the functional characteristics of fucoidans. Moreover, this work highlights the experimental studies using fucoidans that have shown the antitumor activity against BC cells. This review provides a compilation of studies based on the mechanisms and functional

perspective of this important component in algal metabolism, and its promising antitumor properties in BC.

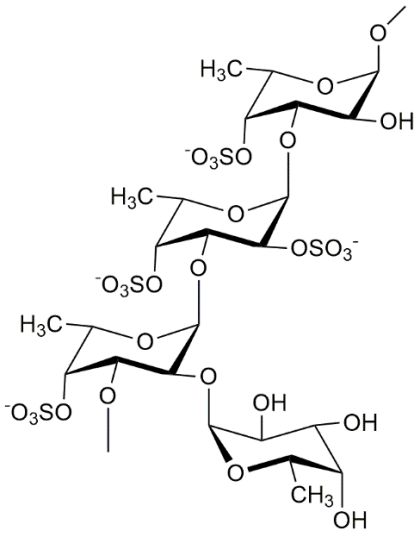
Structure of marine-derived fucoidan

Sulfated polysaccharides extracted from algae are a class of compounds found with a great variety of structures. In algae from Rhodophyta phylum, are the sulfated galactans (Barahona *et al.*, 2012) and in Chlorophyta phylum there are a variety of sulfated carbohydrates, as rhamnose and xylose (Li *et al.*, 2019). In this sense, brown algae (Ochrophyta) has fucoidan, one of the most explored class of sulfated polysaccharide.

Fucoidans are water-soluble polysaccharides essentially consisted of L-fucose and sulfate ester groups. The first isolation of fucoidan is dated from 1913, by Kylin in brown algae as *Fucus vesiculosus* and and *Laminaria saccharina* (Kylin, 1913). Fucoidan extracted from different algae results in variation of polymeric compositions, as exemplified in Figure 1. Moreover, its composition is extremely variable according to extraction procedure, seasonal conditions, eco-physiological parameters and even in different parts of the seaweed (Fletcher *et al.*, 2017).



Fucus vesiculosus



Laminaria saccharina

Figure 1. Repeating chemical structures of fucoidans from different brown algae (Ale and Meyer, 2013; Chollet *et al.*, 2016), redrawn by ChemBioDraw.

Many of the brown algae have fucoidans with simple structure, especially in the order of Fucales, as *F. vesiculosus*, which is composed by fucose and sulfate. On the other hand, some algae produce molecules that can be very complex, containing other monosaccharides (i.e. glucose, mannose, xylose) and proteins, among other compounds. Table 1 exemplifies the variety of fucoidans chemical composition obtained from different brown algae reported in literature.

Obtaining fucoidans from seaweed, in general, involves three steps: i) collection and preparation of algae; ii) pre-treatment of algae and; iii) extraction/purification of fucoidan. The preparation step involves the cleaning with sea water of algae after the collection, in order to remove dirt and epiphytes. The cleaned biomass is then dried and milled to obtain highest yields in extraction procedures (Fletcher *et al.*, 2017). The second step, pre-treatment, is on the subject of remove unwanted compounds during fucoidan extraction, as lipids (defatted), proteins (deproteinated), phenols (dephenolated) and chlorophyll (Hahn *et al.*, 2012). In order to minimize this coextraction of compounds, the most commonly extraction method is performed by using a mixture of organic solvents, as methanol/chloroform/water (Lim *et al.*, 2019), ethanol/water (Usoltseva *et al.*, 2017), acetone/ethanol (Dinesh *et al.*, 2016) or acetone alone (Marques *et al.*, 2016).

The extraction methods are characterized using organic solvents with a great variability of conditions (i.e. temperature and time). The extraction procedure used to obtain fucoidan from algae is a critical point. Different methods of extraction could result in distinct chemical composition of functional components and consequently, different/novel biological activities associated (Atashrazm *et al.*, 2015). The first fucoidan extraction was reported by Kylin in 1973, describing the use of acetic acid solution to the extraction of this polysaccharide. The most commonly extraction methods involves the use of hot water and/or acidic solutions (70-100 ºC) during several hours (Hahn *et al.*, 2012). Nowadays, modern techniques are used to obtain to reduce extraction time of fucoidans, as well as obtain it in major yields, as microwave and ultrasound assisted extraction (Zhao *et al.*, 2018).

Fucoidan as a promising molecule on cancer therapy

The most successful therapeutic strategies against cancer cells include antiproliferative, proapoptotic, anti-angiogenic and cell migration inhibitory properties (Lowe

and Lin, 2000). Some of these pathways may occur concurrently in cancer regulatory effects, such as cell cycle arrest, genetic damage, and apoptosis (Arumugam *et al.*, 2019). In 4T1 mouse breast cancer cells, for example, fucoidan promotes down-regulation of Wnt/β-catenin signaling, an important target of cell cycle and apoptosis (Xue *et al.*, 2012). In hepatocellular carcinoma HepG2 cells, fucoidan from the brown algae *F. vesiculosus* could inhibit cell viability and induced cell apoptosis (Marudhupandi *et al.*, 2015).

In general, the cell cycle is the event characterized by cell growth and differentiation (Collins, Jacks and Pavletich, 1997) and presents the following distinct phases: G0 (quiescence), G1, S (synthesis), G2 (interphase) and M (mitosis) phase (Sherr, 1996). Studies have shown that normal cell lines are resistant and have no cell cycle interference in the presence of fucoidans, while cancer cells are selectively affected in their cell metabolism. Several studies have shown that fucoidan inhibits dose-dependent cell viability and induces disruption of the sub-G1 cell cycle in cancer cell lines. This phenomenon has been observed in a wide range of cancer cells, such as human gastric carcinoma (Shakeri *et al.*), non-small cell bronchopulmonary carcinoma cells, diffuse large B-cell lymphoma (Yang *et al.*), human colon cancer cells (HT29), human colorectal carcinoma cells (Kim *et al.*), head and neck squamous cell carcinoma (Blaszczak *et al.*) and acute myelocytic leukemia (THP-1 cells) (Cho, Lee and You, 2010; Wei *et al.*, 2019; Yang *et al.*, 2015). Furthermore, in HepG2 cells, a fraction of oligo-fucoidan showed higher antitumor activity by stopping the G1-S cell cycle than high molecular weight fucoidan (Yan, Lin and Hwang, 2019).

Apoptosis is the process of programmed cell death characterized by distinct morphological characteristics and energy-dependent biochemical mechanisms (Elmore, 2007). Many human disorders are involved in unappropriated apoptosis and anti-proliferative control, including cancer. There are two types of apoptosis signaling pathways: a) the cell death receptor pathway (extrinsic), that includes activation of the NF-κB, PI3K/Akt and

MAPK/ERK family pathways; b) the mitochondria-mediated pathway (intrinsic), that includes the selective production of pro- and anti-apoptotic proteins of the Bcl-2 family, release of cytochrome c from the mitochondria and activation of caspases, such as -9 and -3 (Lowe and Lin, 2000). Fucoidans could induce apoptosis by activation of both extrinsic and intrinsic pathways. In the extrinsic pathway, fucoidan increased the level of apoptotic ligands FasL and TRAIL to their specific receptors (Fas and DR5) in human colon cell lines (Kim *et al.*, 2010), and downregulation of ERK pathway in human HS-Sultan cells (Aisa *et al.*, 2005). Similarly, studies have shown that fucoidan could mediate cell death via apoptosis by caspase-9 activation (Atashrazm *et al.*, 2015; Kwak, 2014).

The complex architecture of the angiogenesis process requires rigorous adjustment of cell proliferation, differentiation, migration, matrix adhesion, and cell-cell signaling during chemical signaling-mediated tissue remodeling and maturation (Adams and Alitalo, 2007). It has been reported that Fucoidans have anti-tumor and antiangiogenic functions that include inhibition of vascular endothelial growth factor (VEGF) receptor 2 (Chen *et al.*)/Erk/VEGF signaling pathway (Chen *et al.*, 2016a), down-regulation of MMP activity -2 and VEGF/hypoxia-induced factor 1 signaling (HIF-1) (Ou *et al.*, 2017) and reduction of angiogenesis by the AKT/MMP-2 signaling pathways by activating JNK and p38 (Kim *et al.*, 2014).

The different fucoidans derived from marine macroalgae could act in distint forms on angiogenesis-related reactions of different cancer cells (Hsu and Hwang, 2019), as follows:

- Fucoidan from *S. fusiforme* (FSF): inhibits angiogenesis of human microvascular endothelial cells and microvessel formation by lung cancer cell with inhibition of VEGFR2/ERK/VEGF signaling pathways in vitro and in vivo in nude mice (Chen *et al.*, 2016a). FSF: anti-angiogenic effects on the human hepatocellular carcinoma cell lines Bel7402, SMMC7721 and Huh7 (Cong *et al.*, 2014).

- *S. thunbergii*-derived fucoidan: inhibits angiogenesis by downregulating MMP-2 activity and VEGF/hypoxia-inducible factor-1α (HIF-1α) signaling in HUVEC cells and inhibits lung cancer cell A549 migration and proliferation (Ou *et al.*, 2017).
- Fucoidan from *S. integerrimum* (Liu *et al.*): reduces A549 cell viability and induces cell apoptosis, loss of mitochondrial membrane potential, generation of ROS and G2/M phase cell cycle arrest (Liu *et al.*, 2016).
- *F. vesiculosus*-derived fucoidan: interfering to the binding of basic fibroblast growth factor (Soeda *et al.*, 2000) and VEGF (Koyanagi *et al.*, 2003).

These findings reforce the potential pharmacological application of fucoidan as a antiangiogenic agent against cancer.

One of the most significant characteristics that gives cancer a morbidity and potential mortality status is the possibility of metastasis. The process of metastasis corresponds to the spread of tumor cells within the surrounding normal tissue. Cancer can also spread regionally to nearby lymph nodes, tissues, or organs at sites distant from the first affected tissue, involving complex and redundant signal transduction pathways (Cao *et al.*, 2013). Studies have investigated the influence of fucoidan on anti-metastasis activity in different cancers (Haneji *et al.*, 2005; Hsu *et al.*, 2013). Fucoidan anti-tumor signaling pathways relevant to metastasis migration and invasion envolve: the expression of matrix metalloproteinases (MMPs) modulated by transcription factors NF-κB, AP-1 and upstream MAPK and phosphoinositide 3-kinase (PI3K)-Akt and ERK signalling pathways (Wang *et al.*, 2014).

Importantly, fucoidans are capable of inhibiting P-selectins-mediated platelet binding of cells (Cumashi *et al.*, 2007). This is a well-established stage of metastasis onset and progression (Yue *et al.*, 2015). During tumor cell migration, most of these circulating cells do not survive attack from imune cell infiltration or the shear forces of the blood stream. Then, the tumor cells can adere to platelets, inducing platelet aggregation and surviving. With the

inhibition of P-selectin residing on the platelet surface by fucoidan, the number of attached tumor cells reduce. In this context, it´s is important to emphasize that fucoidan can also inhibit adhesion molecules such as integrins residing on the tumor cell surface and can modify distribution of their subunits (Atashrazm *et al.*, 2015).

Fucoidan and Bladder cancer

Actually, although scientific research has advanced significantly in understanding cancer biology and tumor microenvironment, treatment for many tumor types remains challenging. Some studies have shown that natural marine substances such as fucoidans have favorable preventive effects against cancer cells, especially BC cells (Fukahori *et al.*, 2008; Chen *et al.*, 2016b). Several cellular mechanisms have been affected due to the physicochemical properties that give fucoidans a specific action on BC cells (Table 2). According to the literature to date, fucoidans have shown promise as a therapeutic agent.

Studies involving fucoidans in bladder cancer are recent. The first, by Cho (Cho, Kim and Moon, 2014), presented a new understanding of the molecular mechanisms by which fucoidans are capable of inhibiting tumor cell proliferation and migration in 5637 and T24 BC cell lines. Previously, other investigators had described some mechanisms by which fucoidans affect cell growth by inhibiting ERK, JNK, and MAPK signaling pathways, as well as cell cycle regulation and apoptosis pathways (Aisa *et al.*, 2005; Xue *et al.*, 2012; Lee, Kim and Kim, 2012).

The findings of this study are quite significant and prominent because they were the first to present the molecular mechanisms involved in fucoidan-induced AKT activation. AKT has a well-recognized role in tumor aggressiveness and cell survival, as its signaling pathway is involved in the progression, migration and invasion of a wide range of tumors (Osaki, Oshimura and Ito, 2004; Song, Ouyang and Bao, 2005). Considering this, it was reported in this work that fucoidan treatment promoted AKT activation, which is linked to an inhibition

of MMP-9 expression by reducing the binding activity of NF-ƙB and AP-1 in BC cells, effects related to suppression of cell migration and invasion (Cho, Kim and Moon, 2014).

In the same year the work mentioned above was published, Park et al. (Park *et al.*) investigated the effects of fucoidan on cell proliferation, cell cycle arrest, and apoptotic cell death in the T24 cell line of human urinary BC. Overall, the study demonstrated that fucoidan inhibits T24 cell growth by inducing apoptosis in these cells. The apoptotic mechanism was by inhibition of caspases 8 and 9, acting on the extrinsic and intrinsic pathways. In the extrinsic pathway there is participation of the Faz/FasL system that follows the caspase-8 phosphorylation, thereby leading the cell to the apoptosis route (Jin and El-Deiry, 2005). In the intrinsic pathway, the release of cytochrome c from mitochondria and consequent mitochondrial electrochemical membrane failure leads to increased caspase-9 activity (Brenner and Mak, 2009). Additionally, fucoidan induced cell cycle disruption in G1 phase, with significant reduction of expression of cyclin D1, cyclin E and Cdks. Fucoidan also interfered with RB gene phosphorylation, affecting its role on E2Fs transcription factors.

A transition between cell cycle phases is dependent on cyclins that are subjected to pRB phosphorylation (Paternot *et al.*, 2010), and the presence of fucoidan favored the transition from G1 to S phase. In summary, this study could demonstrate the mechanisms that led to cell cycle disruption, thereby leading to inhibition of cell proliferation and consequently fucoidan-induced caspase-dependent apoptosis in T24 cells.

In 2015, a study by Chen et al. (Chen *et al.*) evidenced the antiangiogenic role of seaweed-derived fucoidans in T24 cells. The critical role of tumor angiogenesis plays in volumetric growth and, consequently, potential dissemination to surrounding tissues is well established (Carmeliet and Jain, 2000; Harper and Moses, 2006). Chen's results were quite robust in showed that fucoidan treatment promoted inhibition of HIF-1 and VEGF in T24 cells. HIF-1 is produced in hypoxia situations and has a mechanism that involves the

transcription of pro-angiogenic genes, such as VEGF (Semenza, 2000). Correspondingly, HIF-1 blockade is associated with a suppression of tumor growth, progression and, consequently, malignancy via VEGF blockade (Zhong *et al.*, 1999; Tang *et al.*, 2004). According to Chen, fucoidan treatment also inhibited the phosphorylation of the AKT/mTOR/p70S6K/4EBP-1 cascade in T24 cells. These results are important because the participation of this pathway and its relationship with VEGFR2 is known to promote angiogenesis and tumorigenesis (Jiang and Liu, 2009). In vivo assays showed a similar response, with tumor size reduction from inhibition of HIF-1 and VEGF. This feature makes the regulation of HIF-1 an important target in antitumor therapy.

Apoptosis is crucial for all multicellular organisms to control cell proliferation and maintain tissue homeostasis. *In vitro* studies allowed the investigation of apoptosis-induced fucoidan in bladder cancer cells. A study of Han (Han *et al.*) brought a new perspective by demonstrating that fucoidan treatment was able to induce apoptosis in 5637 BC cells by producing reactive oxygen species (ROS), and then promoted inhibition of telomerase activity by the PI3K/AKT signaling pathway. ROS are related to the electron transport chain in the mitochondrial membrane. Once released, they depolarize the mitochondrial membrane promoting increasing permeability with consequent release of pro-apoptotic proteins (Sinha *et al.*, 2013; Kardeh, Ashkani-Esfahani and Alizadeh, 2014; Bhat *et al.*, 2015). The apoptotic effect of fucoidan was accompanied by significant inactivation of telomerase, which was associated with diminished hTERT, Sp1, and c-Myc gene expression. It's important to highlight the potential to induce apoptosis has become an important target in the development of anti-tumor agents and the recent findings have suggested that fucoidans could play this role in future.

Cancer is a highly debilitating disease. Most deaths related to patients with cancer are due to muscle-related loss of muscle mass and systemic inflammation (Tuca, Jimenez-

Fonseca and Gascón, 2013). Literature reports consistently point to an anti-inflammatory and anti-tumor effects of fucoidans (Cumashi *et al.*, 2007; Senthilkumar *et al.*, 2013). Based on this, in a different approach the study by Chen (Chen *et al.*) aimed to evaluate the metabolic effects of fucoidan administration in mice with BC. The implementation of fucoidans in mice diet was able to reduce mortality, muscle atrophy, muscle loss-related genes, inhibited the production of proinflammatory cytokines and activated IGF-1-mediated cell signaling. These results reinforce the broad action of fucoidans in the treatment of BC, both in vitro and in vivo.

Taken together, these data and studies already performed *in vitro* and *in vivo* models of BC pointed fucoidan as a strategy with wide potential to antitumor therapy. Evidently, further studies are needed in this field to better understand the cellular mechanisms involved in tumor suppression, as well as to evaluate whether the use of fucoidans in combination with other drugs can bring better result in tumor inhibition with reduction of symptoms of cancer patients or side effects that are so common in conventional chemotherapy. Based on the results and evidences until now, we can consider optimistic the inclusion of fucoidans as extremely promising molecule from seaweed origin for the treatment of BC, alone or combined.

Fucoidan as adjuvant treatment

The chemotherapeutics agents used routinely in cancer treatments are frequently known by their side effects and drug resistance (Hsu and Hwang, 2019). Some studies reported that the fucoidans and combinations of fucoidan with clinical drugs in adjuvant settings may reduce the toxicity of certain anti-cancer drugs. In this context, fucoidan has been investigated as a dietary supplement or synergetic anti-tumor agent to reduce the toxicity and/or enhance the efficacy of chemotherapeutic drugs.

The association of therapies promoted the inhibition of metastases in LLC-transplanted C57BL/6 mice in vivo when fucoidan (from *F. evanescens*) was combined with cyclophosphamide (Alekseyenko *et al.*, 2007). Another effect is the decrease in oxidative stress and matrix metalloproteinases due to treatment with fucoidan and vitamin C complex, that can suppress tumor invasion (Saitoh, Nagai and Miwa, 2009). It has been already reported the co-treatment with fucoidan and tamoxifen, cisplatin and paclitaxel, showing enhance the anticancer activities such as inhibition of cell growth, apoptosis and cell cycle arrest in human MDA-MB-231 and MCF-7 breast cancer cells (Zhang *et al.*, 2013). Some advantages with these combinations were observed in treatment of advanced or recurrent colorectal cancer (Ikeguchi *et al.*, 2011). When fucoidan (from *C. okamuranus*) was combined with FOLFOX, the patients that received these therapeutic protocols could have the prolonged chemotherapy time without any signs of fatigue and showed favorable prognosis.

The effect of combining fucoidans with chemotherapeutic agents has also been tested to evaluate the potential inhibition of lung cancer in vivo and in vitro. It has been suggested that fucoidan up-regulates the intrinsic apoptosis pathway via TLR4/CHOP-mediated caspase-3 and PARP activation and thereby promotes increased cisplatin-induced cytotoxicity in human lung cancer cells with a positive synergistic effect (Hsu *et al.*, 2018). These results indicate that fucoidan exerts a greater antitumorigenic effect as an adjuvant, and, according to the author, more potent when administrated prior to cisplatin. Overall, cisplatin remains a first-line anticancer drug for chemotherapy in the clinical treatment of human lung cancer (Hsu and Hwang, 2019), but fucoidan supplementation has shown promise. It is important the development of *in vivo* and preclinical studies in the future to assess the safety and usefulness of combined fucoidan treatments, whether in patients with lung cancer or otherwise tumors.

Whether as an adjunct to therapeutic chemotherapy or dietary supplementation, fucoidans have been very promising for cancer patients. As shown in this review, the

structural components that confer fucoidan biological activity are related to specific effects on regulators of the metabolic repertoire of tumor cells and also to the reduction of normal cell toxicity (van Weelden *et al.*, 2019). This understanding provides support for considering the therapeutic strategy as plausible even in individuals with late cancer, as fucoidans may provide anti-inflammatory effects in these patients (Takahashi *et al.*, 2018).

Conclusions

Ideally, the desired cancer treatment should be able to eliminate tumor cells and preserve healthy cells, with reduced or without side effects. However, it is known that conventional chemotherapeutics have numerous side effects. Natural compounds derived from marine macroalgae have been shown to have an important antitumor activity. In this context, this review pointed to the importance of the study of fucoidans from a structural and functional point of view, focusing on the molecular mechanisms involved in the antitumor activity in bladder cancer. The results have shown that fucoidans produce adjuvant effect on the treatment of bladder cancer in experimental and clinical trials. The expansion of therapeutic possibilities from the bioprospecting of natural compounds allows the link between biomedical research and clinical application. In conclusion, the understanding of the mechanisms underlying the antitumor effects of fucoidans as well as their advantages for therapeutic application combined with the search for new approaches and investigations make the fucoidans a promising allied in the new combined therapies of natural products and thereby improve the quality of life of cancer patients with fewer side effects.

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Table 1. Chemical composition of fucoidan polymers from different algae species (data are presented as molar ratio%).

Algae	Composition (% mol)	Reference
Laminaria hyperborea	fucose (97.8), galactose (2.2), sulfate (53.8)*	Kopplin et al., 2018
Fucus vesiculosus	fucose (44.5), xylose (7.1), mannose (2.7), galactose (3.1), glucose (1.3), sulfate (28.4)*, uronic acid (4.9)*	Ho et al., 2015
Undaria pinnatifida	fucose (29.7), xylose (0.4), mannose (1.0), galactose (22.7), glucose (0.8), rhamnose (0.3) sulfate (29.1)*, acetyl (2.5)*, uronic acid (2.1)*	
Padina boryana	fucose (61), galactose (31), mannose (4), glucose (3), acetyl and sulfate (18.2)*, polyphenols (4.3)*	Shevchenko et al., 2017
Sargassum feldmannii	fucose (39), galactose (57), mannose (1), xylose (1) glucose (1), sulfate (21)*, polyphenols (2.4)*	
Sargassum henslowianum	fucose (1), mannose (0.05), galactose (0.05), xylose (0.38),	Cuong et al., 2015

	glucose (0.92), uronic acid (5.8)*, sulfate (25.20)*	
Cladosiphon okamuranus	fucose (70.13), arabinose (0.34), xylose (1.80), rhamnose (0.17), mannose (0.54), galactose (0.80), glucose (0.31), glucuronic acid (9.27), sulfate (15.16)*	Lim et al., 2019
Laminaria japonica	fucose (48.7), galactose (18.4), mannose (10.2), rhamnose (4.03), glucose (5.43), xylose (1.21), glucuronic acid (10.8), N-acetyl glucosamine (1.23), sulfate (21.43)*, uronic acid (6.78)*	Chen et al., 2017
Sargassum duplicatum	Fucose (51.3), galactose (48.6), sulfate (31.7)*	Usoltseva et al., 2017
Nemacystus decipiens	fucose (75), xylose (1.2), galactose (1.8), glucose (1.5), glucuronic acid (20.5), sulfate (20.3)*, protein (2.2)*	Cui et al., 2018

* % of sample weight (mass)

Table 2. Application of fucoidan against bladder cancer by *in vitro* and *in vivo* studies.

Author/ Year	Design	**Objectives**	**Principal findings**
Cho et al., 2014	*In vitro* Culture of human bladder carcinoma cell lines 5637 and T24.	To report the novel roles of the AKT activation in inhibiting cell growth and migration in fucoidan-treated bladder cancer cells.	- Fucoidan induced the inhibition of cell growth via p21WAF1-mediated G1-phase cell-cycle arrest by the activation of AKT signaling; - The activation of AKT appeared to be linked to an inhibition of MMP-9 expression through reduction of the binding activity of NF-ƙB and AP-1 in fucoidan-treated bladder cancer cells, resulting in a suppression of the migration and invasion of the cells.

Park et al., 2014	*In vitro* Culture of human bladder cancer cells (T24 cell line).	To evaluate the anti-proliferative effects of fucoidan in human bladder cancer T24 cells.	- Fucoidan decreased the viability of T24 cells through the induction of G1 arrest and apoptosis; - Fucoidan-induced G1 arrest is associated with the enhanced expression of the Cdk inhibitor p21WAF1/CIP1 and dephosphorylation of the pRB along with enhanced binding of p21 to Cdk4/6 as well as pRB to the transcription factor E2Fs.
Chen et al., 2015	*In vitro* *In vivo* Culture of human bladder cancer cells (T24 cell line).	To evaluate the anti-angiogenic activity of Low Molecular Weight Fucoidan (LMWF) in bladder cancer T24 cells.	- LMWF can inhibit hypoxia-stimulated HIF-1 accumulation and transcriptional activity VEGF secretion, and the migration and invasion in hypoxic human bladder cancer cells. - LMWF also downregulated hypoxia-activated phosphorylation of I3K/AKT/mTOR/p70S6K/4EBP-1 signaling pathway in T24 cells.

			- LMWF inhibited HIF-1 and VEGF *in vivo*, reducing tumor growth.
Han et al., 2017	*In vitro* Culture of human bladder cancer cells (5637).	To determine the pro-apoptotic effects of fucoidan in the human bladder cancer cell line 5637; To investigate the role of hTERT expression and telomerase activity during the fucoidan-induced 5637 cell apoptosis.	- Fucoidan treatment increased the generation of intracellular ROS, whereas the overelimination of ROS by N-acetylcysteine, an anti-oxidant, attenuated fucoidan-induced apoptosis, inhibition of hTERT, c-myc, and Sp1 expression, and reversed fucoidan-induced inactivation of the PI3K/Akt signaling pathway.

Chen et al., 2016	*In vivo* Treatment of mice with bladder cancer.	To investigate whether LMWF supplementation ameliorates chemotherapy. induced muscle atrophy in mice with bladder cancer.	- LMWF promoted a significant reduction of body weight loss, muscle atrophy, and intestinal injury and dysfunction. - LMWF promoted myostatin, activin A, and pro-inflammatory cytokine production, FoxO3 expression and activation, NF-κB activation, MuRF-1 and MAFbx/atrogin-1 expression. - LMWF leads to the IGF-1 expression and formation, and IGF-1-regulated mTOR/p70S6k/4EBP-1 protein synthesis signaling.

5. ARTIGO 2

Lipidic profile of sub-Antarctic seaweed *Mazzaella laminarioides* (Gigartinales, Rhodophyta) in distinct developmental phases and cell cytotoxicity in bladder cancer

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[§]*Artigo redigido e submetido conforme as normas do periódico "Food and Chemical Toxicology"*

Abstract

The Magellan ecoregion, located in the sub-Antarctic portion of Chile, is marked as a continental portion of high latitude that presents low periods of photoperiod and luminosity in the winter and high in the summer. Seasonal variations in the chemical composition of seaweeds may influence their reproductive state and biomass, and thus their prospection into food, cosmetological or pharmacological use. The objectives of the present study were to determine the fatty acids (FAs) profile of the red seaweed *Mazzaella laminarioides* in vegetative, tetrasporophitic, and cystocarpic phases in winter and summer, and asses the antitumoral activity of these FAs against T24 bladder cancer cells. The algal extracts consisted of nineteen FAs, and composition varied according to the reproductive state and the season the algae were collected. The cytotoxic activity of these FAs was evaluated in T24 bladder cancer (BC) cells. Cell viability decreased in a concentration- and time-dependent manner. Most significant cytotoxic activity of FAs, as well the highest chromatin condensation observed by DAPI staining was at a concentration of 200 μg/mL in 24 and 48 h. The results indicate that FAs derived from *M. laminarioides* have potential to reduce the proliferation in BC cells.

Keywords: Sub-Antarctic macroalgae; T24 blader cancer cells; Polyunsaturated fatty acids; seasonal variation; membrane integrity.

Introduction

The Magellan Ecoregion is a biogeographic province located in the southwest of the South American continent. Containing an area of 132.033 m², it is the largest representative of the sub-Antarctic environment, with a total of 391 macroalgae species, of which 75 are of the Chlorophyta, 86 are of the Ochrophyta and 230 are of the Rhodophyta (Rozzi *et al.*, 2008; Mansilla, Ávila and Yokoya, 2012). Macroalgae are the basis of the food chain of marine organisms, whose metabolites have great economic value in the pharmaceutical industry (anticoagulants, antithrombotics and antioxidants), food (dietetics and functional fibers), bioenergy (biofuels) and biotechnology (cosmetics, immunology and bioprospectives) (Gressler *et al.*, 2011; Guaratini *et al.*, 2012; Matsuhiro *et al.*, 2014; McCauley *et al.*, 2015; Lira *et al.*, 2016; Milledge and Harvey, 2016). In addition, macroalgae are sources of several types of biomolecules such as proteins, vitamins, amino acids, minerals, polysaccharides and lipids (Di Mascio *et al.*, 1995; Hollnagel *et al.*, 1996; Cardozo *et al.*, 2006).

Among macroalgae of the Rhodophyta from sub-Antarctic region, the *M. laminarioides* stands out for its high economic potential as a producer of carrageenans. This macroalgae is distributed along Chile coast strip (30ºS to 55ºS) and is characterized by an alternation of isomorphic generations (Buschmann et al., 2001). It is located in an intertidal zone and its different reproductive phases can coexist during the year (Luxoro and Santelices, 1989; Varela *et al.*, 2006). Some essential lipids, such as linoleic acid (C18:2*n*-6) and α-linolenic acid (C18:3*n*-3), are not synthesized by the human body, and then the macroalgae are an important source of these compounds. Moreover, omega-3 and -6 (*n*-3 and *n*-6) polyunsaturated fatty acids (PUFA) play an important role in the formation and integrity of the lipid bilayer of the cellular plasma membrane. Studies indicate that

Rhodophyta algae can produce significant amount of PUFAs, such as arachidonic acid (20:4*n*-6) and eicosapentaenoic acid (20:5*n*-3) (Khotimchenko, Vaskovsky and Titlyanova, 2005; Cian *et al.*, 2014). High amounts of essential *n*-3 PUFAs have been reported to be important in maintaining human health, especially in reducing the risk of heart disease (Chen *et al.*, 2011), inflammatory processes and cancer (Giros *et al.*, 2009; Pottel *et al.*, 2014).

Bladder cancer (BC) is a common malignant urologic cancer worldwide, often occurs in the urinary tract and it is the second leading cause of death among genitourinary tumors (Siegel, Miller and Jemal, 2018). With 76960 new cases and 16390 deaths in 2016, approximately 80% of BCs patients have non-muscle invasive tumors (Ta, T1, and Tis) (Li *et al.*, 2016; Lee *et al.*, 2010). Studies have reported that intake of *n*-3 PUFA may reduce the incidence of several types of cancer, such as colorectal (Hall *et al.*, 2008; Theodoratou *et al.*, 2007), breast (Thiébaut *et al.*, 2009) and prostate cancer (Williams *et al.*, 2011; Gerber, 2012). The whole mechanisms underlying how *n*-3 PUFA can modulate cancer cell growth and development remains still unclear. It is evidenced that these components can interfere in cell cycle or cell death (Corsetto *et al.*, 2011) by an apoptotic or autophagy mechanism (Kim *et al.*, 2018). Another study of Corsetto et al. (Corsetto *et al.*, 2012) pointed to interactions of *n*-3 PUFA in membrane phospholipids on cancer cells, which would lead to changes membrane physiology.

Even though the considerable progress made in recent years in the chemical composition of macroalgae, few reports in the literature explore the variations in FA or lipid amounts in samples from the Chilean Sub-Antarctic seaweeds (Ortiz *et al.*, 2009; Mansilla and Ávila, 2011). To our knowledge, there are no reports in the literature about the seasonal variation in the FA content in the Chilean Sub-Antarctic

macroalgae *M. laminarioides*. Additionally, there are no studies regarding the antitumoral effect of *n*-3 PUFA rich fractions obtained from this seaweed against BC cells. Considering this, the aim of the present investigation was to analyse the FAs profiles in distinct developmental phases of the seaweed *M. laminarioides* in winter and summer, and evaluate the antitumoral activity of *n*-3 PUFA rich fraction against T24 BC cells.

Material and Methods

1 Algae collection

Samples

Samples of *M. laminarioides* were collected at low tide in intertidal region, when they were exposed in the rocky habitat, during the winter in July 2017 and summer in January 2018. The collect geographical area is known as Santa Ana (53° 44' S, 70° 58' W) and is located 70 kilometers south of Punta Arenas, Strait of Magellan, Chile. The macroalgae were carefully collected and separated according to the morphological differences between the vegetative and reproductive phases of development (tetraesporophytic and cystocarpic), with no damages or evident epiphytes at the time of collection. The materials were taken to the laboratory in a plastic box with seawater from the region where they were obtained for the drying procedures. The general information of algae collection, developmental phases and subsequent morphological identification under specified characteristics were carried out in the Herbarium of the Antarctic and Sub-Antarctic Marine Ecosystems Laboratory (LEMAS).

Samples Preparation

After being washed with deionized water with the aims to remove salt and residual epiphyses, each sample was dried on absorbent paper at room temperature (20 °C) during 5 days. The dried algae biomass was crushed using a knife mill (Biotech, model B-602) (A 11 basic-IKA Works, Brazil) and stored in batches of 100 g dry mass in airtight plastic bags.

2 Lipid extractions in macroalgae

Reagents

For extraction and identification of fatty acids, the FAME 37 Mix reagent (Sulpeco, Belefonte, Pennsylvania, USA) was used as the analytical standard and nonadecanoic acid (C19: 0; Sigma Aldrich, St Louis, Missouri, USA) as internal standard. A gas chromatograph coupled to a flame ionization detector (GC-FID) (Tang and Row, 2013) was used to identify the fatty acids and a quantitative analysis was performed. All reagents that are used had an analytical grade.

Lipid extraction and fatty acid methyl esters preparation

Extraction of fatty acids followed the modified procedure of Bligh and Dyer (1959). To 1 g of the dried algae biomass was added 30 mL of chloroform/methanol (1:2 v/v) and 10 mL of an aqueous solution of sodium sulfate (1.5%, w/v). Subsequently, the mixture was agitated at room temperature, at 2500 rpm for 30 min and after this period, the organic phase (19.8 mL) was retrieved and the solvent was evaporated in a rotary evaporator (Buchi, Switzerland) with a V-700 vacuum pump and a B-741 cooler. In order to enable the analysis by GC, the lipids were converted to their respective fatty acid methyl esters (FAME) using boron trifluoride, as reported as method “B” of Moss (Moss, Lambert and Merwin, 1974). Briefly, 6 mL of a methanolic solution of potassium hydroxide (2%, w/v) were added to a 50 mL flask that contained the previous extracted lipids, under agitation and reflux at 80°C for 8 min. Afterwards, 7 mL of a methanolic solution of boron trifluoride (Aldrich; 14%, v/v) was added, and the solution was stirred under reflux for 2 min. The resulting FAME mixture in hexane was subjected to GC-FID detection.

Chromatographic Procedures

The quantitative analysis were performed in a gas chromatograph with a flame ionization detector - GC/FID 2010, with a split/splitless injector, autoinjector AOC-20i (Shimadzu Corporation, Kyoto, Japan), and capillary column Elite-Wax (30 m × 0.25 mm I.D. x df 0.25 µm; Supelco). The injections were performed in a 1:25 ratio, and hydrogen was used as carrier gas under a constant flow mode at 1.2 mL / min. The injector was heated to 250°C and the FID operated at 250°C. The initial temperature program of the oven at 100°C, remaining at this temperature for 0.5 min, and increasing in the ratio of 7°C / min to 200°C after increasing in the ratio of 5°C / min to 202.6°C, remaining 2 min at this temperature. It was then increased the ratio of 5°C / min to 222.9°C remaining for 2 min, and then raising the ratio of 5°C / min to 230°C remaining for 10 min (Santos *et al.*, 2017). The analyses were conducted at the Federal University of Pelotas (Laboratory of Lipidomics and Bioorganic).

3 Determination of Cytotoxicity

Cell Culture

This study was performed using human bladder cancer cell line T24 (EGFR-expressing human bladder carcinoma cell line) obtained from Rio de Janeiro Cell Bank (PABCAM, Federal University of Rio de Janeiro, Brazil). The transitional cell carcinoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin/streptomycin at 37 ºC and 5% of CO_2 in a humidified atmosphere incubator. All experiments were performed using cells in the logarithmic growth phase and the results were obtained by averaging three independent experiments performed in

triplicate for each experiment. The IC_{50} (concentration that inhibits 50% of cell growth) was also calculated using GraphPad Prism 7.0 Software. The drug vehicle DMSO was calculated to never exceed 0.5% per well.

Cell Viability Assay

The viability of the T24 cell line was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide). The cells were treated with different concentrations of FA (10–200 µg/mL) from the macroalgae *M. laminarioides* in vegetative phase for 24 and 48 h. These components were dissolved in dimethyl sulfoxide (DMSO) and added to the medium supplemented with 10% FBS (v/v) at the desired concentrations. The final DMSO concentration in the medium did not exceeded 0.2% (v/v). Control groups that were treated with an equivalent volume of the solvent or containing only the medium were also included. Thereafter, the incubation medium was removed and 180 µL of medium and 20 µL MTT (5 mg MTT/mL solution) were subsequently added to each well. The plates were incubated for an additional 3 h, and the medium was discarded. DMSO was added to each well, and the formazan was solubilized on a shaker for 5 min at 100 rpm. The absorbance was read on a microplate reader (Victor X5, PerkinElmer, USA) at a test wavelength of 492 nm. Cell inhibitory growth was determined as follows: cell growth inhibition ratio = (1 – $Abs_{492 treated\ cells}/Abs_{492 control\ cells}$) x 100%. For the control group of cells, DMSO and FA were not added. All the observations were validated by at least two independent experiments in triplicate for each experiment.

LIVE/DEAD Assay

The LIVE/DEAD Cell Viability Assay (InvitrogenTM, Carlsbad, CA, USA) was performed according to the manufacturer's instructions for the most significant result observed from the MTT assay. Briefly, the cells were treated with 200 µg/mL of FA extracted from macroalgae *M. laminarioides* for 48 h. The live cells were able to take up calcein and could be analyzed by green fluorescent light emission (488 nm). Ethidium bromide homodimer diffuses through the permeable membranes of dead cells and binds to DNA. Dead cells could be detected using a red fluorescent signal (546 nm). The LIVE/DEAD assay was analyzed using an Olympus IX71 fluorescence microscope (Olympus Optical Co.) with multicolor imaging. The number of red (dead) and green cells (live) in a total of 300 cells was counted in triplicate for each sample. Viability was expressed as the average percentage of viable cells. This experiment was repeated two times.

DAPI Assay

Apoptosis was detected using DAPI (4',6-diamidino-2-phenylindole) staining, which forms a fluorescent complex with double-stranded DNA. Cells seeded in a 96-well plate were treated with 200 µg/mL of FA extracted from *M. laminarioides* macroalgae. After incubation, the cells were washed three times in phosphate-buffered saline (PBS) and fixed with 1 mL methanol solution at room temperature for 10 min. The fixed cells were then washed with PBS and stained with a DAPI solution at room temperature in the dark. The nuclear morphology of the cells was examined by fluorescence microscopy with an Olympus IX71 (Olympus Optical Co.).

4 Statistical analyses

All data were previously analyzed to evaluate the distribution and presented as mean ± standard error of the mean (SEM). Evaluation of the results regarding the FA quantification and MTT were performed applying the two way analysis of variance (ANOVA), followed by the Bonferroni's *post hoc* test. LIVE/DEAD and DAPI assays were alalyzed using one-way ANOVA, followed by Tukey test. For all tests weew using a statistical program (GraphPad Prism 7 Software Inc., San Diego, CA, USA). The limit of statistical significance was set at $p < 0.05$.

Results

The concentrations of FAs found in the macroalgae *M. laminarioides* in winter and summer for three distinct developmental phases are shown in Table 1. The values are expressed as % of molar FAs. In all, 19 saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids were identified. They were found in winter and summer and in the three developmental phases: myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), palmitoleic acid (C16:1), oleic acid (C18:1n9c), nervonic acid (C24:1), linoleic acid (C18:2*n*6c), linolenic acid (C18:3*n*3), γ–linolenic acid (C18:3*n*6), cis-8,11,14-eicosatrienoic acid (C20:3*n*6), arachidic acid (C20:4*n*6), cis-5,8,11,14,17-eicosapentaenoic acid (C20:5*n*3), behenic acid (C22:0), linoletal acid (C18:2*n*6t), cis-11,14-eicosadienoic acid (C20:2). It was found only in summer in the tetraesporophytic and cystocarpic phases pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0). Cis-11-eicosenoic acid (C20:1) was found in the vegetative phase in winter and in the tetraesporophytic and cystocarpic phases in the summer. Arachidic acid (C20:0) was detected in the tetraesporophytic phase in summer and cystocarpic in winter. The most representative percent fatty acids in the specimens were C16:0, C18:1*n*9c, C20:4*n*6 and C20:5*n*3.

Table 1. Fatty acid composition determined by GC-FID of the *M. laminarioides* macroalgae in the vegetative, tetraesporophytic and cystocarpic phases, in winter and summer.

Fatty Acid [% total of fatty acids]	Concentration (%)					
	Vegetative		Tetraesporophytic		Cystocarpic	
	Winter	Summer	Winter	Summer	Winter	Summer
Saturated Fatty Acids (SFA)						
Myristic acid (C14:0)	2,26 ± 0,05	2,94 ± 0,21	2,73 ± 0,62	2,76 ± 0,10	1,84 ± 0,36	3,66 ± 0,24
Pentadecanoic acid (C15:0)	-	-	-	0,31 ± 0,01	-	0,33 ± 0,04
Palmitic acid (C16:0)	25,64 ± 1,17	29,36 ± 0,60	26 ± 0,15	28,68 ± 1,08	26,10 ± 0,46	30,19 ± 0,37
Heptadecanoic acid (C17:0)	-	-	-	1,13 ± 0,19	-	1,52 ± 0,29
Stearic acid (C18:0)	3,67 ± 0,15	1,59 ± 0,03	4,35 ± 1,18	1,99 ± 0,32	3,79 ± 0,15	3,06 ± 1,19
Arachidic acid (C20:0)	-	-	-	0,67 ± 0,10	0,56 ± 0,13	-
Behenic acid (C22:0)	-	0,90 ± 0,18	-	0,98 ± 0,22	-	0,91 ± 0,09
ΣSFAs	31,57±0,97	34,79 ± 0,53	33,08 ± 0,32	36,52 ± 0,89	32,29 ± 0,41	39,67 ± 0,41
Monounsaturated Fatty Acids (MUFA)						
Palmitoleic acid (C16:1)	2,02 ± 0,15	3,69 ± 0,28	2,28 ± 0,09	5,34 ± 0,33	2,48 ± 0,26	5,39 ± 0,11
Oleic acid (C18:1*n*9c)	8,40 ± 0,63	9,26 ± 0,5	9,32 ± 1,59	10,27 ± 0,30	12,14 ± 4,48	11,30 ± 0,70
Cis-11-Eicosenoic acid (C20:1)	0,57 ± 0,07	-	-	0,38 ± 0,02	-	0,54 ± 0,18
Nervonic acid (C24:1)	0,72 ± 0,31	0,84 ± 0,11	1,13 ± 0,30	0,99 ± 0,37	0,57 ± 0,01	0,87 ± 0,23
ΣMUFAs	11,71 ± 0,50	13,79 ± 0,42	12,73 ± 1,21	16,98 ± 0,31	15,19 ± 3,62	18,10 ± 0,49
Polyunsaturated Fatty Acids (PUFA)						
Linolelaidic acid (C18:2*n*6t)	-	2,3 ± 0,33	-	2,35 ± 0,39	-	1,40 ± 0,11
Linoleic acid (C18:2*n*6c)	2,62 ± 1,29	4,19 ± 0,14	3,82 ± 1,42	5,04 ± 0,20	5,64 ± 4,28	4,85 ± 1,06
Linolenic acid (C18:3*n*3)	0,92 ± 0,26	5,05 ± 0,34	1,22 ± 0,04	6,32 ± 0,10	0,28 ± 0,04	3,40 ± 0,17
γ-Linolenic acid (C18:3*n*6)	1,15 ± 0,28	0,86 ± 0,06	1,07 ± 0,12	0,57 ± 0,03	0,13 ± 0,01	0,80 ± 0,38
Cis-11,14-Eicosadienoic acid (C20:2)	-	2,87 ± 0,14	-	4,17 ± 0,15	-	2,69 ± 0,24
Cis-8,11,14-Eicosatrienoic acid (C20:3*n*6)	1,32 ± 0,43	2,04 ± 0,18	1,09 ± 0,28	1,62 ± 0,37	1,42 ± 0,20	1,49 ± 0,43
Arachidonic acid (C20:4*n*6)	16,4 ± 0,44	17,37 ± 0,07	15,32 ± 1,45	14,29 ± 0,80	11,82 ± 2,07	11,70 ± 0,45
Cis-5,8,11,14,17-Eicosapentaenoic acid (C20:5*n*3)	34,62 ± 2,30	17,05 ± 0,42	33,21 ± 0,32	13,24 ± 0,07	33,23 ± 5,82	15,97 ± 0,46
ΣPUFAs	55,03 ± 1,66	52,07 ± 0,23	55,73 ± 0,71	47,6 ± 0,34	52,52 ± 4,61	42,30 ± 0,47
ΣPUFAs n-3	35,54 ± 2,25	22,10 ± 0,40	34,43 ± 0,31	19,56 ± 0,08	33,51 ± 5,77	19,37 ± 0,41
ΣPUFAs n-6	21,49 ± 0,53	26,76 ± 0,11	21,3 ± 1,32	23,87 ± 0,59	19,29 ± 2,53	20,24 ± 0,57

Data expressed in mol %. The values are the average ± standard deviation of three independente experiments (n=3 replicates).

In relation to the SFA (Figure 1a-1g), there was a higher concentration of C14:0 ($p < 0.01$), C16:0 and C22:0 ($p < 0.0001$) in summer compared to winter in the vegetative phase, in contrast to that observed with C18:0 ($p < 0.0001$). In the tetraesporophytic phase, higher amounts of C15:0, C16:0, C17:0, C20:0 and C22:0 ($p < 0.0001$) were found in the summer compared to winter, contrary to that found in C18:0 ($p < 0.0001$). On the other hand, the cystocarpic phase presented lower percentages of C14:0, C15:0, C16:0, C17:0 and C22:0 ($p < 0.0001$) in winter when compared to summer. Particularly, C20:0 was detected in the winter only in the cystocarpic phase, which made its quantity larger in relation to the vegetative and tetraesporophytic phase ($p < 0.0001$). In summer, higher concentrations of C15:0, C17:0 and C20:0 ($p < 0.0001$) were found in relation to winter, as opposed to C22:0 ($p < 0.0001$). In this season there were higher percentages of C14:0 ($p < 0.01$), C15:0, C17:0, C18:0, C20:0 ($p < 0.0001$) in the cystocarpic phase in relation to the vegetative phase, in contrast to C22:0 ($p < 0.0001$). The amounts of C14:0, C22:0 ($p < 0.0001$), C16:0 ($p < 0.01$), C17:0 ($p < 0.001$) and C20:0 were higher in the cystocarpic phase compared to the tetraesporophytic phase in the summer.

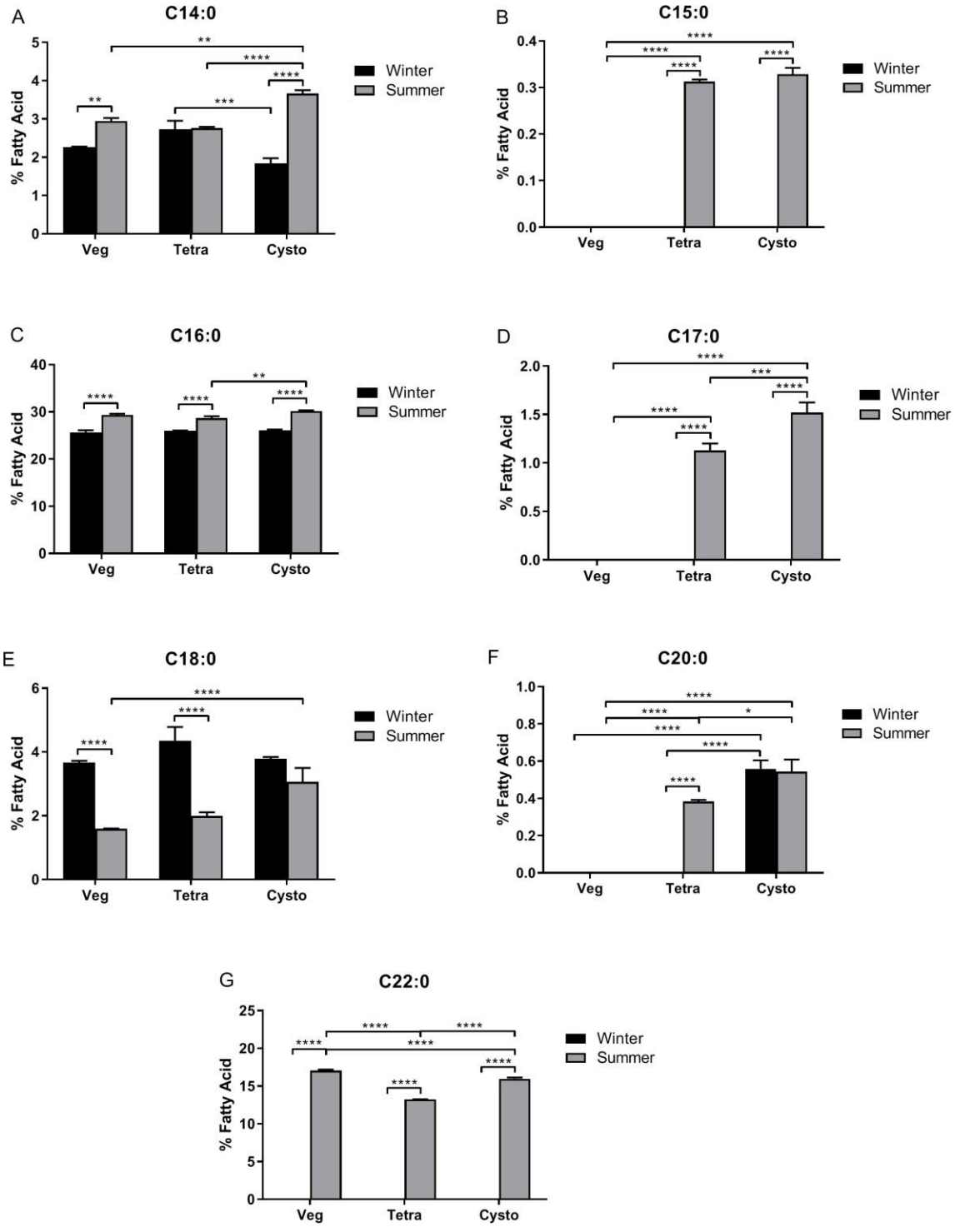

Figure 1. Saturated fatty acids (SFAs) of the *M. laminarioides* macroalgae in the vegetative, tetraesporophytic and cystocarpic phases in winter and summer. Data expressed as mol %. Brackets indicate differences between groups. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Among the MFA (Figure 2a-2d) found in the macroalgae *Mazzaella laminarioides*, C16:1 and C20:1 presented higher concentrations in the summer compared to winter in the vegetative, tetraesporophytic and cystocarpic phases ($p < 0.0001$). In winter, the amounts of C20:1 between the vegetative and tetraesporophytic phases were smaller ($p < 0.0001$) and C24:1 higher ($p < 0.05$), respectively. At this time of year, the percentages of the vegetative phase of C16:1 and C18:1n9c were lower ($p < 0.01$ and < 0.05) than cystocarpic, while C20:1 were higher ($p < 0.0001$). In the summer, larger amounts of C16:1 were observed in both the tetraesporophytic and cystocarpic phases in relation to the vegetative phase ($p < 0.0001$), while C20:1 presented a higher concentration in the tetraesporophytic phase and lower in the cystocarpic phase in relation to the vegetative phase ($p < 0.0001$). At this time of year, there was a lower percentage of C20:1 in the cystocarpic phase compared to the tetraesporophytic ($p < 0.0001$).

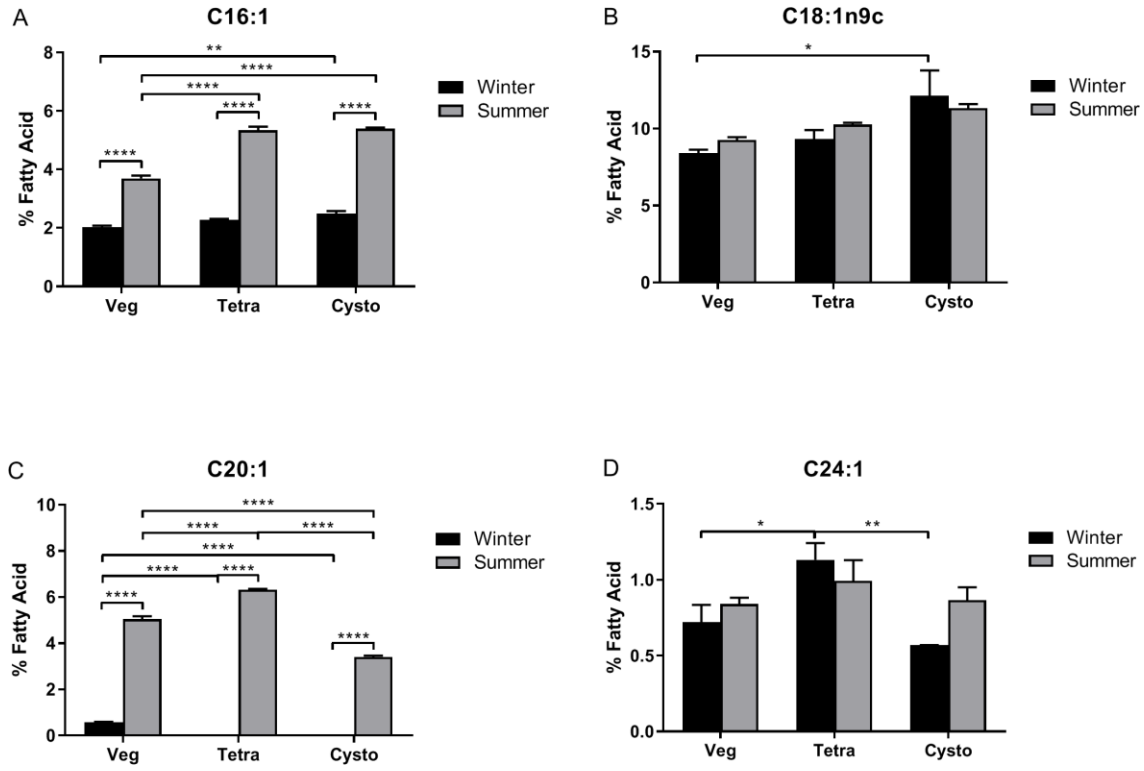

Figure 2. Monounsaturated fatty acids (MUFAs) of the *M. laminarioides* macroalgae in the vegetative, tetraesporophytic and cystocarpic phases in winter and summer. Data expressed as mol%. Brackets indicate differences between groups. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

The PUFA content (Figure 3a-3h) varied considerably both in the seasons and in the reproductive phases of the alga studied. In the vegetative phase, higher concentrations of C18:2*n*6t, C20:2 ($p < 0.0001$) and C20:3*n*6 ($p < 0.01$) were found in summer in relation to winter, while the opposite occurred with C18:3*n*3 and C20:5*n*3 ($p < 0.0001$). In the tetraesporophytic phase, it was possible to detect higher amounts of C18:2*n*6t and C20:2 ($p < 0.0001$) in summer compared to winter, while the reverse occurred with C18:3*n*3, C20:5*n*3 ($p < 0.0001$), C18:3*n*6 ($p < 0.001$). In the cystocarpic phase, the highest percentages of C18:2*n*6t, C18:3*n*6, C20:2 (p <

0.0001) were observed in summer in relation to winter, whereas C18:3*n*3 and C20:5*n*3 had higher summer concentrations ($p < 0.001$ and $p < 0.0001$, respectively). In winter, there was no difference between the vegetative and tetraesporophytic phases. However, a less quantity was observed in the cystocarpic phase of C18:3*n*3, C18:3*n*6 and C20:4*n*6 in relation to vegetative ($p < 0.0001$), and in relation to the tetraesporophytic phase ($p < 0.01$, $p < 0.0001$ and $p < 0.0001$, respectively). In the summer, the C20:2 concentrations were higher in the tetraesporophytic phase than in the vegetative phase ($p < 0.0001$), while the C20:4*n*6 concentrations were lower ($p < 0.001$). At this season, the cystocarpic phase showed lower amounts of C18:2*n*6t, C20:4*n*6 ($p < 0.0001$) and C20:3*n*6 ($p < 0.05$) in relation to the vegetative phase and C18:2*n*6t, C18:3*n*3 and C20:2 ($p < 0.0001$) in relation to the tetraesporophytic phase.

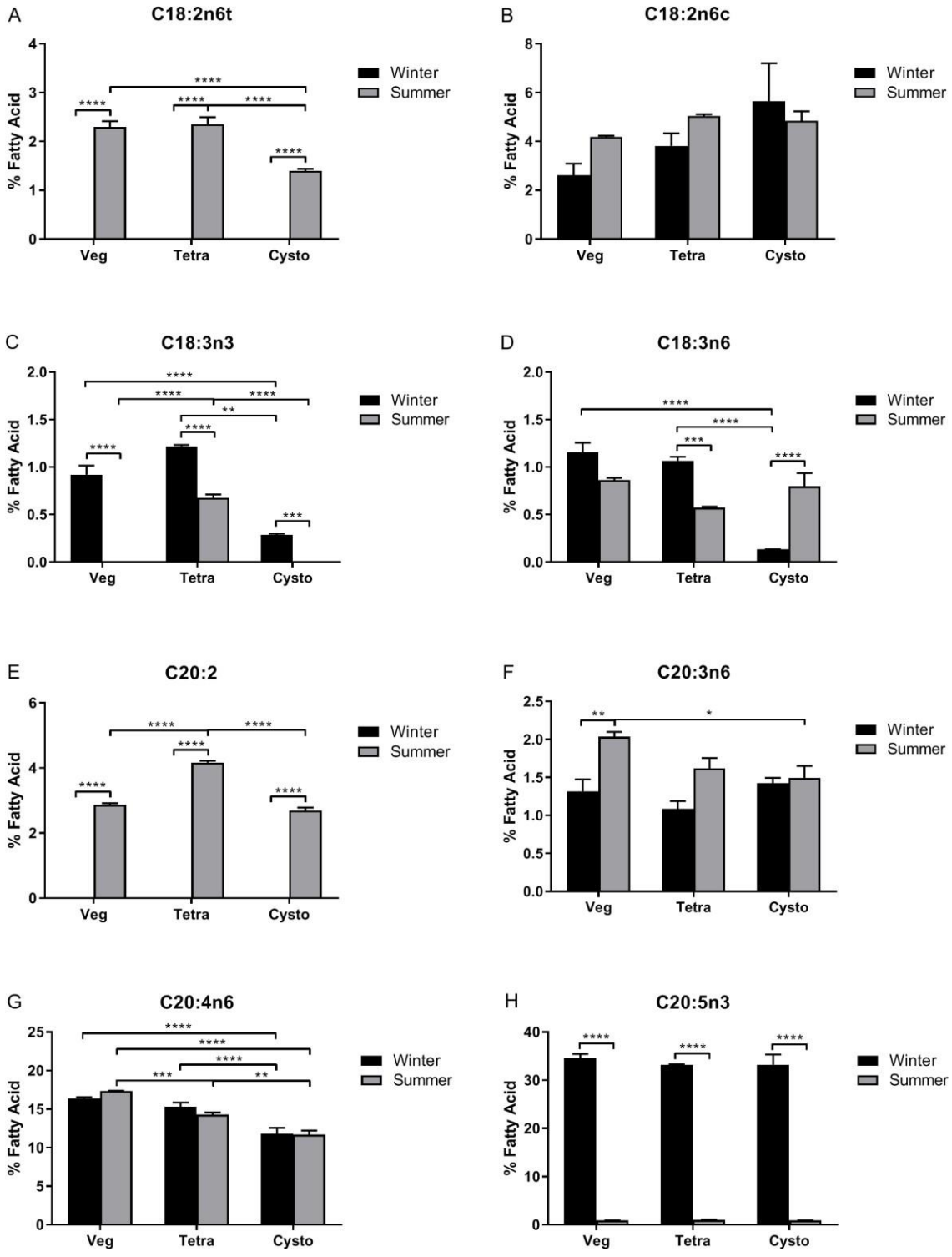

Figure 3. Polyunsaturated fatty acids (PUFAs) of the *M. laminarioides* macroalgae in the vegetative, tetraesporophytic and cystocarpic phases in winter and summer. Data

expressed as mol%. Brackets indicate differences between groups. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

FA extracts of *M. laminarioides* were able to reduce T24 cells viability showing cytotoxic potential against BC cells (Figure 4). The results demonstrated that after 24 h of exposure to concentrations of 10, 25, 50, 100 and 200 µg/mL of FAs presented inhibitory ratio of 7.65, 20.35, 24.04, 29.48 and 61.59 %, respectively. In this period, the inhibitory ratio of 10 µg/mL FA was lower than observed in 25, 50, 100 and 200 µg/mL FA ($p < 0.05$). There was no difference in 50 µg/mL FA when compared to 25 µg/mL FA ($p > 0.05$). The 100 µg/mL FA presented higher inhibitory ratio than 10, 25, and 50 µg/mL FA, but lower than 200 µg/mL ($p < 0.05$). After 48 h, the inhibitory ratio was ranged to 12.18, 23.50, 31.67, 38.52 and 45.44 % for the 10, 25, 50, 50 and 200 µg/mL FA concentrations. It could be observed a dose-dependent pattern, with increased inhibitory ratio when the dosis were higher. In a comparison with periods, there were differences in all concentrations. At 24 h, there was lower inhibitory ratio in 10, 25, 50 and 100 µg/mL FA concentration when compared to respective ones at 48 h ($p < 0.05$). In 200 µg/mL FA concentration, was observed lower inhibitory ratio in 48 h when compared to 24 h.

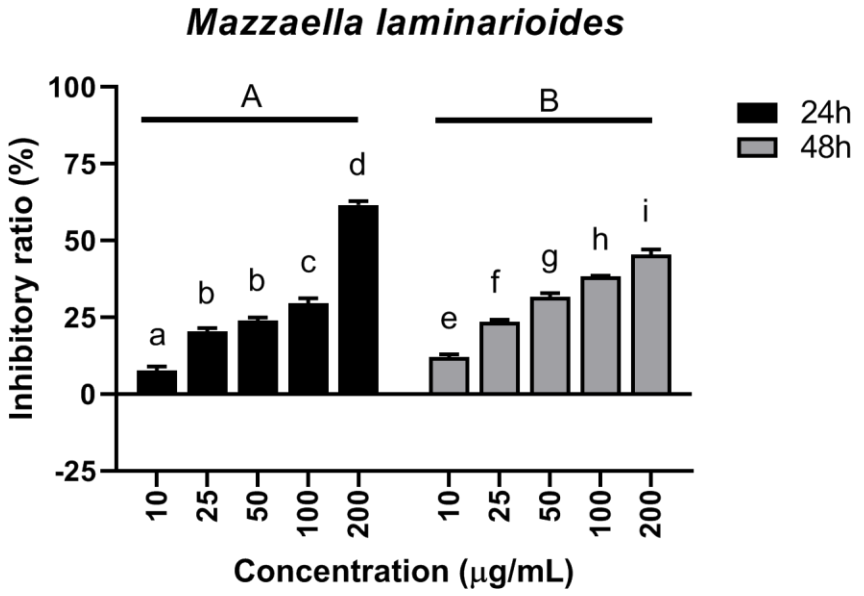

Figure 4. Effects of different concentrations of the combination of FAs extracted from *M. laminarioides* at 24 and 48 h in inhibiting T24 cells. Cytotoxicity was assessed by MTT assay. Data are expressed as the mean ± SEM of a representative experiment performed in triplicate (n = 3). Significance was considered at $p < 0.05$ (Tukey test). Capital letters indicate differences between the different times. The lowercase letters indicate differences between the different concentrations.

With approximately 50 % of inhibition rate at 48 h, 200 µg/mL of FA extracts of *M. laminarioides* were used to LIVE/DEAD assay. Were observed higher amounts of dead cells in FA extracts treated cells when compared to control group (16.14 vs 3.21 %, respectively) ($p < 0.05$) (Figure 5B). However, DAPI assay did not show statistical differences in apoptotic ratio between FA extracts of *M. laminarioides* and control group (6.32 and 3.56 %, respectively) ($p > 0.05$) (Figure 5C).

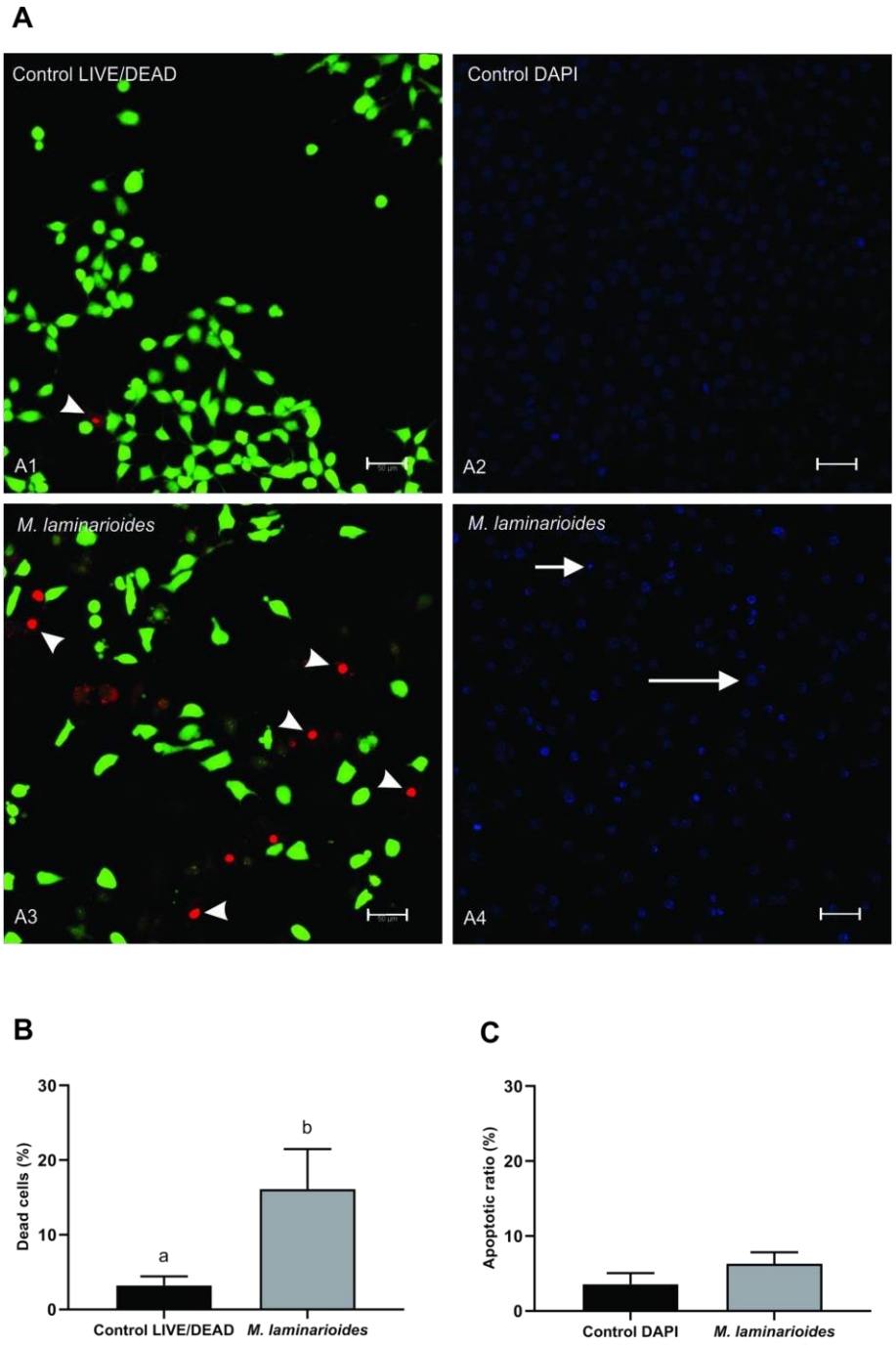

Figure 5. T24 cells were treated with 200 μg/mL FAs from *M. laminarioides* for 48 h (**A**). On the left side, photomicrograph of cell deaths was estimated by LIVE/DEAD assay (green/red) in Control (**A1**) and treated cells (**A3**). The arrow heads indicate dead cells (red), and green cells indicate live cells. On the right side, photomicrograph of cell apoptosis assessed by DAPI staining (blue) of Control (**A2**)

and treated cells (**A4**). The short arrow indicates apoptotic cell (condensed or fragmented nuclei) and the long arrow indicates live cells. All images were obtained using an Olympus IX71 fluorescence microscope (Olympus Optical Co., Tokyo, Japan). (**B**) The graphic shows the mean ± SEM of the dead cells in three different areas of the plate. (**C**) The graphic presents the mean ± SEM of the apoptotic ratio in three different areas of the plate. Lowercase letters indicate statistical differences ($p < 0.05$). The scale bar indicates 50 µm.

Discussion

Some studies have emphasized the importance of evaluating the impact of seasonality on the variability of FAs present in macroalgae (Nelson, Phleger and Nichols, 2002; Renaud and Luong-Van, 2019; Kendel *et al.*, 2013). It is important to note in the present study that the concentration of FAs in the different developmental phases during winter and summer of the macroalga *M. laminarioides* showed variability. Following our results, the greatest differences were found mainly according to the season that the FA extracts were obtained. This study appears to be the first to evaluate the influence of the seasons of the year on the different reproductive phases in the amount of FAs of the Chilean Sub-Antarctic macroalgae *M. laminarioides*. Although the factors that may influence the FA profiles in red macroalgae are not yet completely elucidated, it is important to mention that the intertidal region is subject to wide environmental fluctuations such as solar radiation or disecation that may alter the macroalgae metabolism (Dudgeon, Davison and Vadas, 1990; Kübler and Davison, 1993; Williams and Dethier, 2005). Hence, the biochemical plasticity is closely linked to the amount of light necessary to do the photosynthesis, and this factor is determinant for macroalgae survival and

reproduction (Flores-Molina *et al.*, 2014). Under natural conditions, the production of lipids by these organisms is extremely important, because polar lipids integrate the structural components of the membrane in the form of phospholipids and glycolipids (Gurr, Harwood and Frayn, 2002). In this context, neutral lipids such as triacylglycerols (TAGs) are present and have the role of reserving energy sources (Guschina and Harwood, 2009) that prove useful for survival in challenging environmental conditions such as winter in regions of high latitudes (Mansilla and Ávila, 2011).

Important compounds in the lipid storage process, polyunsaturated fatty acids (PUFA) have a chain of 16 to 20 carbons. In relation to saturated fatty acids (SFA), it has a higher melting temperature than unsaturated ones. This seems to be a characteristic in subtropical ecosystems and cold environments, as larger amounts of PUFA and SFA can help to control the stiffness and permeability of cell membrane (Gómez *et al.*, 2009). Our results showed C14:0, C16:0 and C18:0 as the most abundant SFAs, particularly C16:0, which presented mean concentrations ranging from 25.64% to 30.19%. These findings are in agreement with previous studies that point to C16:0 as the most representative fraction of SFA in algae from the Rhodophyta (Nelson, Phleger and Nichols, 2002; Hanson, Hyndes and Wang, 2010). For comparison purposes, in red algae from Polar Regions, the concentration of C16:0 varied between 20-30% (Graeve *et al.*, 2002). These parameters reinforce the highly representative aspect of the Sub-Antarctic environment in the metabolism of the macroalga studied.

Monounsaturated fatty acids (MUFA) play an important role in the energetic and physiological metabolism in cell membranes. In low-temperature or seasonally influenced ecosystems, the adaptation of some species of algae depends on the

ability to efficiently store energy through carbon-rich compounds (Hu *et al.*, 2008). Our study brings a larger number of MUFA found during the summer, with a mean concentration varying from 11.71% to 18.10%, with C18:1n9c being the most representative FA in this group. Other authors found similar percentage of MUFA in red macroalgae (Nelson, Phleger and Nichols, 2002; Hanson, Hyndes and Wang, 2010). It has been proposed the metabolic role that MUFA play in the physiological adaptations in cell membranes (Stevenson, Bothwell and Lowe, 1996).

The central role that PUFA have in the permeability and fluidity of algal cell membranes is well established (Gombos, Wada and Murata, 1994; Guschina and Harwood, 2009). Rhodophyta algae are able to synthesize PUFA and are the most representative long chain fatty acids of 18 and 20 carbons (Galloway *et al.*, 2012). In this study, PUFA represented the largest group of fatty acids present in *M. laminarioides*, with average concentrations ranging from 42.30% to 55.03%. It is important to mention that the PUFA with the highest proportional amount, C20:5*n*3, showed greatest reduction of its concentration in summer, while curiously the concentrations of most other PUFAs increased during this period. Importantly, our results show a higher concentration of PUFAs compared to found in a previous study in algae of the same phylum. Data from literature pointed to a concentration ranging from 17.96 to 32.10% of PUFA in other red seaweeds (Hanson, Hyndes and Wang, 2010). Our results suggest a compensatory mechanism directly related to PUFA on the control of cell membrane fluidity and permeability in situations of thermal amplitude and variation of luminosity throughout the year, especially in a Sub-Antarctic region.

Long chain fatty acids of types *n*-3 and *n*-6 as C20:5*n*3 and C20:4*n*6, respectively, are considered essential fatty acids (Sargent and Whittle, 1981) whose quantities

produced by animal organisms are insufficient for the physiological requirements (Kanazawa, Teshima and Ono, 1979). In the biochemical scope, it is important to highlight that C20:5*n*3 acts as a precursor to eicosanoids, which are important compounds involved in inflammatory processes (Sargent, Tocher and Bell, 2002). In addition, C20:4*n*6 FA has been associated with the development and maturation of the central nervous system (Bell *et al.*, 1995). Positive effects of *n*-6 FA intake on human health and prevention of heart disease have been reported (Kumar *et al.*, 2008; Patterson *et al.*, 2012). As can be seen, the macroalgae *M. laminarioides* proved to be an important source of these compounds, especially if obtained in winter. These observations reinforce the biological aspect of the use of this macroalga as a nutritional or pharmacological source, since algae from cold environments have been prospected for this purpose (van Ginneken *et al.*, 2011; Pereira *et al.*, 2012). As observed in our results, the reproductive status did not exert high impact on the FA profile. Hence, we considered appropriated to carry out the biological assays in the vegetative phase during the winter. In this condition, it is possible to obtain a considerable biomass, and the collection of material does not compromise the bioavailability and reproduction of this seaweed in nature because these macroalgae are also easily cultivable in the laboratory.

Many studies have shown cytotoxicity induced by FA in some types of cancer, such as skin (Rhodes *et al.*, 2003), colon (Kato, Kolenic and Pardini, 2007), prostate (Gu *et al.*, 2013), and breast cancer (Pacheco *et al.*, 2018). To our knowledge this is the first time that a lipid fraction of *M. laminarioides* have been tested in cancer cells. Our results showed that when the FAs concentration was increased, the suppressor effect of cell proliferation became more apparent. According to the MTT assay, the extracted FA exhibited increased cytotoxicity in T24 BC cells in a dose-dependent

manner. Bioactivity of seaweed extracts have been studied in melanoma cells, to evaluate and demostrate cytotoxic activity (Rocha *et al.*, 2007). In a recent study about the cytotoxic effects of microalgae extracts on human gastric cancer cell line, Shakeri (Shakeri *et al.*, 2017) showed an IC_{50} value of 6170 µg/mL for SFA-rich oil and 1260 µg/mL for PUFA-rich oil. In comparison, the IC_{50} obtained by the present study was 30.85-fold lower for the SFA-rich oil and 6.3-fold lower for the PUFA-rich oil obtained by the mentioned author. Some studies on breast cancer cells have shown that *n*-3 PUFA intake could reduce cell growth, tumor volume and preventing metastasis, by different mechanisms such as gene expression and signal transduction, changes in estrogen metabolism, alterations in the production of reactive oxygen species (ROS), suppression of neoplasic transformation, the inhibition of cell growth and increased apoptosis (Larsson *et al.*, 2004; Wannous *et al.*, 2013).

Tumor cells have uncontrolled cell growth and limited capacity to die by apoptosis. Studies have pointed that resistance to apoptosis is one of the main characteristics of the malignant tumors (Okada and Mak, 2004). The DAPI-staining results suggested that FA from *M. laminarioides* induced apoptosis in T24 BC cells at the tested concentration. In our findings, the cytotoxic effect was higher when FAs were administrated in higher concentrations but was not followed by an increase in apoptosis rate. One possible explanation is an autophagy mechanism exert by *n*-3 PUFA, with a synergic effect of toxicity against T24 cells. This hypothesis is in accordance of Kim (Kim *et al.*, 2018), who found an increase of the AMPK level, thus decreases in mTOR activity and phosphorylated Akt levels after *n*-3 PUFA administration in glioblastoma cells. Jing (Jing *et al.*, 2011) obtained earlier a similar result, evidenced m-TOR inhibition through AMPK activation after docosahexaenoic

acid (DHA) administration. Besides that, in a study in human RT112 urinary bladder cancer cells, DHA in concentrations that do not affect cell proliferation inhibits the invasion of this cell line, therefore, reducing the metastatic potential (D'Eliseo *et al.*, 2012). As concerns to influence of cytotoxic activity associated to reproductive status and season of year in FA-derived from *M. laminarioides*, this report represents the first description in T24 BC cell lineage. The effect observed may be further well explored in order to investigate the combined natural and synthetic therapies, and thus minimize the side effects typical of the conventional chemotherapeutic treatment.

Conclusions

The lipid extracts of the seaweed *M. laminarioides* present variable amounts of FAs according the season of year or reproductive status. The most representative fraction of PUFAs in lipid extracts was obtained in vegetative phase in winter. Higher concentrations of lipid extracts could lead to a cytotoxic effect against T24 BC cells by an apoptotic-independent manner. These findings classify and prospect this macroalga as an important source of FA, especially PUFA, with significative biotechnological value due the cytotoxicity against BC cells.

Acknowledgements

This study had financial and logistic support from the Brazilian Antarctic Program (PROANTAR/MCT/CNPq-No. 23/ 2009; 64/2013) and Brazilian Navy. The authors

are grateful for the financial and fellowship support from the Brazilian research funding agencies Foundation for Research Support of the State of Rio Grande do Sul (FAPERGS), Foundation for Research Support of the State of São Paulo (FAPESP), National Council for Scientific and Technological Development (CNPq), and National Program for Post-Doctoral Studies - Coordination for Improvement of Higher Level Personnel (PNPD/CAPES). A. M. Thanks to Proyecto Conicyt PIA Apoyo CCTE AFB170008". Instituto de Ecología y Biodiversidad (IEB) and FONDECYT Program grant 1180433.

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6. CONCLUSÕES

As fucoidanas exibem seletivamente atividade antitumoral contra diversas linhagens celulares. Além disso, as ações em múltiplas frentes, como antiproliferativa, antiangiogênica e antimetastática tornam as fucoidanas moléculas bioativas bastante promissoras. Somado a isso, os achados recentes na literatura onde a administração de fucoidanas promove uma maior tolerância aos quimioterápicos convencionais junto à redução dos efeitos colaterais tornam as fucoidanas moléculas altamente atraentes do ponto de vista biotecnológico na terapêutica antineplásica.

Os extratos lipídicos das algas marinhas de *M. laminarioides* apresentam quantidades variáveis de FAs de acordo com a estação do ano ou o estado reprodutivo que se encontravam. A fração mais representativa de PUFAs nos extratos lipídicos foi obtida na fase vegetativa no inverno. Concentrações mais altas de extratos lipídicos podem levar a um efeito citotóxico contra células T24 de BC de maneira independente de apoptose. Esses achados classificam e prospectam essa macroalga como uma fonte importante de FAs, principalmente PUFA, com valor biotecnológico significativo devido à citotoxicidade contra células de BC.

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