UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA E FISIOLOGIA

WERLAYNE MENDES DE SANTANA

POTENCIAL FARMACOLÓGICO DA MACROALGA *Gracilaria birdiae* OBTIDA DE DIFERENTES FONTES.

Recife
2015

WERLAYNE MENDES DE SANTANA

POTENCIAL FARMACOLÓGICO DA MACROALGA *Gracilaria birdiae* OBTIDA DE DIFERENTES FONTES.

Tese apresentada ao Programa de Pós-Graduação em Bioquímica e Fisiologia da Universidade Federal de Pernambuco como pré-requisito para a obtenção do grau de doutor em Bioquímica e Fisiologia.

Orientador: Prof. Dr. Ranilson de Souza Bezerra

Co-orientador: Prof. Dr. Hugo Alexandre Oliveira Rocha

Colaborador: Prof. Dr. Dárlio Inácio Alves Texeira

Recife

2015

Catalogação na Fonte:
Bibliotecário Bruno Márcio Gouveia, CRB-4/1788

Santana, Werlayne Mendes
Potencial farmacológico da macroalga *Gracilaria birdiae* obtida de diferentes fontes / Werlayne Mendes Santana. – Recife: O Autor, 2015.

109 f.: il.

Orientadores: Ranilson Souza Bezerra, Hugo Alexandre Oliveira Rocha
Tese (doutorado) – Universidade Federal de Pernambuco. Centro de Biociências. Programa de Pós-graduação em Bioquímica e Fisiologia, 2015.
Inclui referências e anexos

1. Algas 2. Farmacologia I. Bezerra, Ranilson Souza (orient.) II. Rocha, Hugo Alexandre Oliveira (coorient.) III. Título.

579.8 CDD (22.ed.) UFPE/CB-2017-461

POTENCIAL FARMACOLÓGICO DA MACROALGA *Gracilaria birdiae* OBTIDA DE DIFERENTES FONTES.

Tese apresentada ao Programa de Pós-Graduação em Bioquímica e Fisiologia da Universidade Federal de Pernambuco como pré-requisito para a obtenção do grau de doutor em Bioquímica e Fisiologia.

Aprovado em 27/ 02 / 2015**.**

BANCA EXAMINADORA

_____

Prof. Dr. Ranilson de Souza Bezerra/ UFPE

_____

Prof. Dr. Leandro Silva Costa/ IFRN

_____

Profa. Dra. Juliana Ferreira dos Santos/ UAST

_____

Profa. Dra. Juliett de Fátima Xavier da Silva/ UFAL

_____

Dra. Renata Cristina da Penha França/ UFPE

Senhor
Eu sei que tu me sondas
Sei também que me conheces
Se me assento ou me levanto
Conheces meus pensamentos
Quer deitado ou quer andando
Sabes todos os meus passos
E antes que haja em mim palavras
Sei que em tudo me conheces

Senhor, eu sei que tu me sondas
Senhor, eu sei que tu me sondas
Senhor, eu sei que tu me sondas
Senhor, eu sei que tu me sondas

Deus, tu me cercaste em volta
Tuas mãos em mim repousam
Tal ciência é grandiosa
Não alcanço de tão alta
Se eu subo até o céu
Sei que ali também te encontro
Se no abismo está minh'alma
Sei que aí também me amas

Senhor, eu sei que tu me sondas
Senhor, eu sei que tu me sondas
Senhor, eu sei que tu me sondas
Senhor, eu sei que tu me sondas

Senhor, eu sei que tu me amas
Senhor, eu sei que tu me amas
Senhor, eu sei que tu me amas
Senhor, eu sei que tu me amas

O Tamanho das Pessoas

Os Tamanhos variam conforme o grau de envolvimento…

Uma pessoa é enorme para você, quando fala do que leu e viveu, quando trata você com carinho e respeito, quando olha nos olhos e sorri destravado.

É pequena para você quando só pensa em si mesma, quando se comporta de uma maneira pouco gentil, quando fracassa justamente no momento em que teria que demonstrar o que há de mais importante entre duas pessoas: a amizade, o carinho, o respeito, o zelo e até mesmo o amor.

Uma pessoa é gigante para você quando se interessa pela sua vida, quando busca alternativas para o seu crescimento, quando sonha junto com você.

E pequena quando desvia do assunto.

Uma pessoa é grande quando perdoa, quando compreende, quando se coloca no lugar do outro, quando age não de acordo com o que esperam dela, mas de acordo com o que espera de si mesma.

Uma pessoa é pequena quando se deixa reger por comportamentos clichês.

Uma mesma pessoa pode aparentar grandeza ou miudeza dentro de um relacionamento, pode crescer ou decrescer num espaço de poucas semanas.

Uma decepção pode diminuir o tamanho de um amor que parecia ser grande.

Uma ausência pode aumentar o tamanho de um amor que parecia ser ínfimo.

É difícil conviver com esta elasticidade: as pessoas se agigantam e se encolhem aos nossos olhos. Nosso julgamento é feito não através de centímetros e metros, mas de ações e reações, de expectativas e frustrações.

Uma pessoa é única ao estender a mão, e ao recolhê-la inesperadamente, se torna mais uma.

O egoísmo unifica os insignificantes.

Não é a altura, nem o peso, nem os músculos que tornam uma pessoa grande…

É a sua sensibilidade, sem tamanho…

W.Shakespeare

AGRADECIMENTOS

Agradeço diariamente a Deus por me encobrir de bênçãos, por ser minha força e companhia em cada momento de alegria, conquista, tristeza, fraqueza e saudade.

A minha família, meu chão, fonte de inspiração e garra, especialmente a minha mãe Fátima Santana, minha fonte incansável de motivação e fé em Deus, obrigada pelo o seu amor, por tudo. Meu pai Edmundo Santana, pelas as suas bênçãos, que no auge do seu silêncio, eu sei que me acalentava. Minhas irmãs Werlanne e Edlayne Santana, meus amores, que mesmo distante, eu sei o quanto estavam presente me dando força para seguir em frente, mesmo que o corpo e a mente não conseguissem mais. E você minha princesa, meu amor maior Yule Maria, que no silêncio de sua chegada me fez perceber que mesmo que ganhemos o mundo, nossa maior riqueza é, e sempre será nossa família.

Aos meus tios, tias, primos e primas que apesar da distância estão sempre torcendo por mim.

A você Henrique que mesmo na escuridão dos teus medos, me fez acreditar que por mais que o chão me faltasse eu podia ir adiante, pois os obstáculos aparecem para nos fortalecer. Obrigada por ser tão especial e esta sempre do meu lado, mesmo que às vezes tão distante.

Aos meus amigos Olávio e Robson pelo carinho e maravilhosa convivência durante todos esses anos.

Allana e tia Conceição obrigada por tudo que fizeram por mim nesse momento.

Ao meu amigo Rafael David pelo carinho compartilhado, pelo ombro amigo, por todos os momentos vividos.

As minhas queridas amigas Helane, Juliana, Juliett, Renata e a família de vocês, obrigada por serem tão especial e estarem sempre ao meu lado me dando leveza para enfrentar as dificuldades do dia a dia.

Agradeço ao meu orientador prof. Dr. Ranilson Bezerra pela oportunidade oferecida, pela atenção e auxílio prestado durante todos esses anos.

Ao querido prof. Dr. Hugo Alexandre, obrigada por abrir as portas do seu laboratório e me proporcionar à oportunidade de novos conhecimentos.

Ao prof. Dr. Dárlio Teixeira pela ajuda nesse trabalho.

A todos que integram o LABENZ: Ana Cláudia, Andreia, Cybelle, Amália Medeiros, Augusto Freitas, Caio Assis, Cleópatra Silva, Cyndy Mary, Daniela Campeche, Daniele Matias, Dárlio Teixeira, Diego Buarque, Douglas Holanda, Fábio Marcel, Flávia Thuanne, Guilherme Firmino, Helane Costa, Ian Porto, Janilson Felix, Jéssica Vasconcelos, Juliana Interaminense, Juliana Santos, Juliett Xavier, Kaline Catiely, Karina Ribeiro, Karollina Lopes, Kelma Souza, Lidiane Cristina, Liliane Moreira, Luiz Swintiskas, Marina Marchuschi, Marlyete Chagas, Milena Márcia, Natália Albuquerque, Paula Rayane, Rafael David, Raquel Pereira, Renata Cristina, Renata Nascimento, Robson Coelho, Ruy Tenório, Talita Espósito, Thiago Cahú, Vagne Melo, pela amizade e carinho ao longo destes anos.

As pessoas maravilhosas que tive a oportunidade de conhecer no laboratório BIOPOL: Gabriel Fidelis, Moacir, Joanna, Jailma, Pablo, Rafael Camara, Ruth, Raniere, Vinicius,

Maxsuell, Rony Lucas, Leandro, Marília, Monique, Ajax, Larisse, Talita, Mônica, Almino Afonso, Jéssica, Ana Karinne, Yara, obrigada pela deliciosa convivência e ajuda no desenvolvimento deste trabalho.

Ao grupo Macroalgas: Rayane, Júlio, Lucas, Nailton, Cintia, a todos aqueles que não citei, obrigada pela a ajuda no desenvolvimento desse trabalho.

Aos professores do programa de pós-graduação e a aqueles professores convidados da UFPE, por me darem oportunidade de aumentar meus conhecimentos. Aos meus colegas de turma, que mesmo nas dificuldades enfrentadas tivemos a oportunidades de ter novas experiências.

Aos funcionários do departamento de Bioquímica da UFPE, Albérico do Espírito Santo, Miron Oliveira, João Virgínio, Fredson e Helena.

A UFPE, ao Programa de Pós-Graduação em Bioquímica e fisiologia, ao departamento de bioquímica e a UFRN, pela oportunidade de concluir essa Pós-graduação, assim como as agências financiadoras, CAPES e CNPq.

Prof. Dr. Maulori Cabral obrigada por tantos ensinamentos em tão pouco tempo, feliz daquele que tem a oportunidade de lhe conhecer.

A Banca de qualificação (Renata França e Helane Costa) e aos avaliadores deste trabalho pelas sugestões e enriquecimento do trabalho.

A TODOS aqueles que eu tive a oportunidade de conhecer e conviver por todos esses anos e não citei aqui, mas que estarão sempre em minha memória, o meu sincero agradecimento.

A todos, o meu muito obrigada!

RESUMO

As algas são importantes fontes de compostos essenciais para a nutrição humana. Este valor nutricional elevado é devido à presença de proteína, fibra dietética, ácidos graxos essenciais, carotenóides, vitaminas e minerais. Além de sua importância econômica nas indústrias de alimentos, farmacêutica e de cosméticos, com numerosas aplicações, as algas têm sido pesquisadas por apresentar uma alta variedade de metabólitos secundários e compostos biologicamente ativos. Com o objetivo de investigar o potencial farmacológico da macroalga vermelha *Gracilaria birdiae* foi caracterizado quimicamente o extrato bruto desta espécie obtidos de três diferentes fontes, (GbD) à deriva (arribada), (GbBN) banco natural, (GbF) cultivo de algas para avaliação do mecanismo de atividade antioxidante, para a inibição de cristais de oxalato de cálcio e os efeitos antiproliferativos em células tumorais *in vitro*. Os resultados apontaram grande potencial antioxidante nas três fontes. O extrato GbF apresentou maior atividade sequestradora de íons superóxido. GbNB e GbF apresentaram maior atividade quelante de ferro, enquanto que GbNB teve maior atividade quelante de cobre. A concentração de sulfato foi de 2,3% para GbD, 1,79% para o GbNB e 5,9% para GbF. O percentual do açúcar total foi: 32,49% (GbD), 26,44% (GbNB) e 24,73% (GbF). Além disso, Os níveis de proteína foram de 0,12%, 0,07% e 0,26% para GbD, GbF e GbNB, respectivamente. O conteúdo fenólico foi de 0,53% para GbD, enquanto que o GbNB apresentou 0,30% e GbF foi de 0,78%. Quanto aos cristais de oxalato de cálcio, foi observado efeito sobre a morfologia e a quantidade dos cristais mono, di e de tri-hidratados, usando citrato de sódio como controle. A presença de GbF promoveu uma maior formação de cristais de oxalato de cálcio dihidratado de pequeno tamanho, forma que é menos agressiva. Além disso, o extrato da *G. birdiae* das três fontes observadas após 24 horas de incubação não foi citotóxico para células renais humanas (HEK-293). Quanto ao efeito antiproliferativo nas células tumorais, o extrato GbD inibiu as células A549 em 40% após 48 e 72 horas de incubação, enquanto que nas células 786, os extratos GbD, GbNB e GbF foram representativos após 48 horas de incubação. O extrato GbD inibiu a proliferação das células (HELA) em 30% após 48 e 72 horas de incubação, enquanto que os extratos GbD, GbNB e GbF inibiram as células B16-F10 em 10% após 72 horas de incubação. Em conclusão, *G. birdiae* apresentou atividades antioxidante, anti-proliferação de células tumorais e de inibição de oxalato de cálcio. Contudo, ensaios in vivo devem ser realizados para verificar a eficácia dos extratos de macroalgas nos aspectos estudados.

Palavras-chave: algas, *Gracilaria birdiae*, algas vermelhas

ABSTRACT

Algae are important sources of essential compounds for human nutrition. This high nutritional value is due to the presence of protein, dietary fiber, essential fatty acids, carotenoids, vitamins and minerals. In addition to its economic importance in the pharmaceutical, food and cosmetic industries, with several applications, algae have been researched for presenting a high variety of secondary metabolites and biologically active compounds. In order to investigate the pharmacological potential of red macroalgae *Gracilaria birdiae*, the crude extract of this species obtained from three different sources (GbD - drift, GbNB - natural bank and GBF - algae cultivation) was chemically characterized concerning the mechanism of antioxidant activity for the inhibition of calcium oxalate crystals and antiproliferative effects in tumor cells *in vitro*. The results showed great potential antioxidant in the three sources. The GbF extract showed the highest scavenging activity of superoxide ions. GbNB and GbF were more effective in iron chelating and GbNB was more active concerning chelating activity of copper. The sulfate concentration was 2.3% for GbD, 1.79% to 5.9% for GbNB and GbF. The percentage of total sugar were 32.49% (GbD), 26.44% (GbNB) and 24.73% (GbF). Protein levels were 0.12%, 0.07% and 0.26% for GbD, GbF and GbNB, respectively. The phenolic content was 0.53% for GbD, the GbNB 0.30% and 0.78% GbF presented. As for the calcium oxalate crystals was observed effect on the amount of morphology of the mono, di and trihydrate crystals by using sodium citrate as a control. The presence of GbF increased formation of calcium oxalate dihydrate crystals of small size, less aggressive. In addition, the three sources *G. birdiae* extract was not cytotoxic for human kidney cells (HEK-293) after 24 hours of incubation. Regarding the effect antiproliferative in A549 tumoral cells, the GbD extract inhibited about 40% of proliferaion after 48 and 72 hours of incubation, whereas the extracts of GbD, GbNB and GbF were representative in the 786 cells, after 48 hours of incubation. In HELA cells GbD extract inhibited the proliferation in 30% after 48 and 72 hours of incubation. Moreover, B16-F10 cells were inhibite (10%) by the GbD extracts GbNB and GbF after 72 hours of incubation. In conclusion, *G. birdiae* demonstrated antioxidant and antitumoral and as calcium oxalate inhibitor. However, in vivo studies are needed to understand the effects of algae extracts in these stuied aspects.

Keywords: algae, *Gracilaria birdiae*, red seaweed.

LISTA DE ILUSTRAÇÕES

CAPITULO 1: Antioxidant activity of crude extracts from the red seaweed *Gracilaria birdiae*.

CAPITULO 2: Evaluation of crude extracts from the red seaweed *Gracilaria birdiae* as inhibitors of the formation of Calcium Oxalate Crystals

CAPITULO 3: Antiproliferative activity of macroalgae *Gracilaria birdiae* against tumor cell lines Hela, A549 and B16-F10 and normal cells MRC5

LISTA DE TABELAS

LISTA DE ABREVIATURAS

µL Microlitros;

786-0 Linhagem de células derivadas de adenocarcinoma renal;

A549 Linhagem de células carcinoma de pulmão humano;

AA Ácido ascórbico;

B16-F10 Linhagem de células de melanoma murino;

$CaCl_2$ Cloreto de cálcio;

CAT Capacidade antioxidante total;

COM Oxalato de cálcio monohidratado;

COD Oxalato de cálcio dihidratado;

COT Cristais de oxalato de cálcio trihidratados;

DMEM Meio eagle modificado por Dulbecco;

EAA Equivalente de ácido ascórbico;

EDTA Ácido etilenodiaminotetracético;

g Grama;

GbD Extrato da macroalga *Gracilaria birdiae* arribada (à deriva);

GbF Extrato da macroalga *Gracilaria birdiae* cultivada;

GbNB Extrato da macroalga *Gracilaria birdiae* de banco natural;

HeLa Linhagem de células de carcinoma cervical humano;

Hek-293 Linhagem de células embrionárias sádias humanas de rim;

min minuto;

mL mililitros;

MRC5 Linhagem de células sadias humanas de pulmão;

MTT Brometo de 3-(4,5-dimetilazol-2-il)-2,5-difenil tetrazólio;

NBT Nitroazul de tetrazólio;

Ox Oxalato;

PS Polissacarídeos Sulfatados;

RPMI Meio de cultura sintético complexo criado pelo Roswell Park Memorial Institute;

RNS Espécies reativas de nitrogênio;

ROS Espécies reativas de oxigênio;

SFB Soro fetal bovino;

Sulf Sulfato;

SUMÁRIO

1. INTRODUÇÃO

No Brasil, a extensão costeira entre as zonas das marés, com cerca de 8.500 Km, é representada pela presença significativa das algas (micro e macroalgas). Sendo organismos marinhos que despertam interesse em especial por apresentarem componentes com grande potencial biotecnológico, como polifenois, carotenoides, pigmentos, enzimas e polissacarídeos (GIULIETTI et al., 2005; PEREIRA et al., 2014).

A região costeira compreendida entre os estado do Ceará ao Rio de Janeiro abriga a flora algal mais diversificada do país. No litoral nordestino, principalmente entre os estados do Ceará e da Paraíba, a coleta de algas vermelhas é uma atividade de grande porte econômico, executadas para fins comerciais (*Gracilariae* e *Hypnea*), (VIDOTTI; ROLLEMBERG, 2004; RODRIGUES, 2006; OLIVEIRA et al., 2010).

A alga é um produto que pode ser inserido na alimentação humana e preparos de animais, utilizado como fertilizantes e adubos na agricultura e no uso medicinal através da extração de compostos bioativos (HOLDT e KRAAN, 2011). Rocha et al., (2004) cita alguns desses composto bioativos, como: galactanas sulfatadas, extraído das algas vermelhas (filo Rhodophyta), fucanas (homofucanas) e os fucoidanos (heterofucanas) das algas marrons (filo Ochrophyta), e a extração de polissacarídeos sulfatados heterogêneos, ricos em galactose, manose, xilose, arabinose, glicose e/ou ácidos urônicos, nas algas verdes (filo Chlorophyta).

Os polissacarídeos são macromoléculas solúveis em água, formados por um ou diversos tipos de monossacarídeos, ou seja, polímeros de carboidratos que desempenha funções de espessantes, gelificante, emulsificante e hidratante (RINAUDO, 2008).

De acordo com Prajapati et al. (2014) descreve que os polímeros naturais geralmente são moléculas não-tóxicos, biocompatíveis, biodegradáveis, de baixo custo, não causam danos ao meio ambiente e são encontrados em abundância na natureza. Logo, apresentam favoráveis condições de aplicabilidade na indústria alimentícia e farmacêutica.

Dentre os polímeros naturais, o ágar é um polissacarídeo sulfatado da família de galactanos presente na parede celular das algas vermelhas do filo Rhodophyta e sua quantidade e a qualidade variam de acordo com a espécie (CARVALHO & ROQUE, 2000). O mercado do ágar gera um aumento na demanda da matéria prima para sua produção, logo, observa-se uma crescente exploração dos estoques naturais do gênero *Gracilaria*. Nesse contexto, investir no cultivo de algas pode ser uma alternativa para reduzir a pressão sobre os estoques naturais e consequentemente oportunizar a geração de renda, promovendo um desenvolvimento para comunidade local (MARINHO-SORIANO, 2005).

De forma geral pode-se dizer que as algas além de apresentar importância econômica na indústria farmacêutica, alimentícia e cosmética, têm sido alvo de pesquisas por serem fontes de muitos metabólitos secundários e compostos biologicamente ativos (CARDOZO et al, 2011; TONON et al., 2011). Almeida et al. (2011), cita algumas atividades biológicas das algas com propriedades antioxidantes, antiinflamatórias, imunomodulatórias, antivirais e antimicrobianas, entre outras.

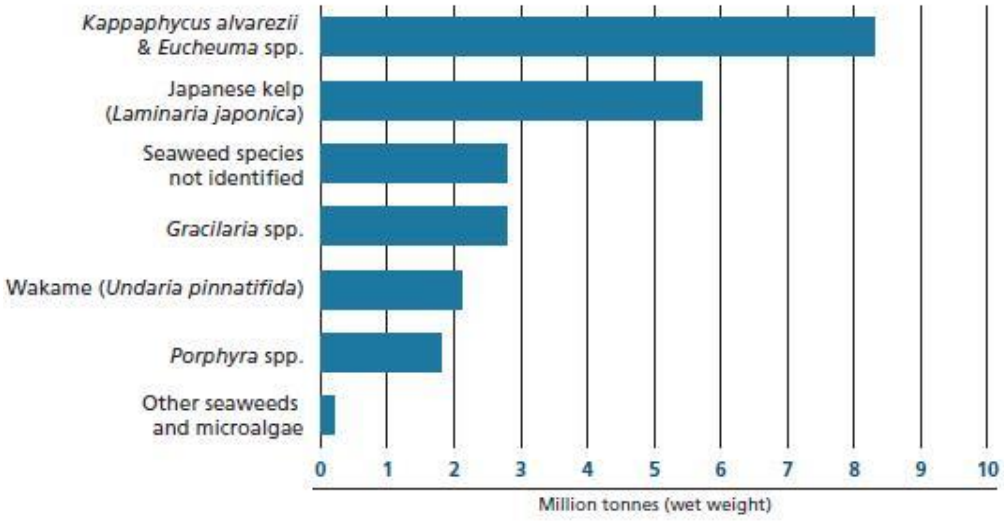
Sendo assim, o estudo sobre o potencial biotecnológico das algas obtidas através de estoques naturais, algas arribadas e cultivos pode fornecer informações importantes sobre a qualidade dessas fontes, para futuras aplicações industriais, bem como proporcionar maior respaldo para sustentabilidade econômica, social e ambiental de ecossistemas marinhos.

2. REVISÃO DA LITERATURA

2.1. Produção de macroalgas marinhas

Nos últimos anos, a produção aquícola global atingiu um recorde, totalizando 90,4 milhões de toneladas em 2012, incluindo 23,8 milhões de toneladas de algas. Só a China produziu 13,5 milhões de toneladas de algas aquáticas em 2012 (FAO, 2014). De acordo com os dados da FAO, em 2012, a produção de algas oriunda da captura totalizou cerca de 1,1 milhão de toneladas. Ainda em relação às estatísticas da FAO, o cultivo de algas aquáticas está centrado em aproximadamente 37 espécies ou grupos de espécies. Estas algas cultivadas podem ser categorizadas em 7 grupos de acordo com a natureza e utilização prevista (Figura 1).

Figura 1: Produção aquícola mundial de algas cultivadas em 2012



Fonte: FAO, 2014

Cerca de 25 milhões de toneladas de algas são colhidas anualmente para uso na indústria alimentícia, cosméticos e fertilizantes. A biomassa algal é processada e comumente utilizada na extração de agentes espessantes ou usada como aditivos nutricionais (FAO, 2014).

2.2. Cultivo de macroalgas marinhas

A coleta predatória de algas nativas é uma pratica que existe desde a década de 70. Dessa forma, as populações de macroalgas oriundas de estoques naturais vêem diminuindo em todos os bancos naturais onde ocorre este processo (TEIXEIRA et al. 2000; TEIXEIRA, 2004).

O cultivo de macroalgas marinhas tem sido realizado há séculos em países orientais, principalmente devido ao grande apelo popular, onde são amplamente utilizadas na culinária local. Alimentos como “nori”, “kombu”, “wakame”, amplamente populares em países como Japão, China e Coréia do Sul, encontram-se em processo de incorporação aos hábitos alimentares ocidentais (LÉPEZ et al., 2004).

Para realizar o cultivo de algas em grande escala, faz-se necessário conhecer a região para avaliar a espécie que melhor se adapte em condições de cultivo e consequentemente, estabelecer uma prática de manejo para tornar um produto economicamente atrativo e viável. Contudo, sabe-se que algumas algas são coletadas no ambiente natural e manejos inadequados podem provocar a degradação dos bancos naturais e dos substratos de fixação das mesmas. Nesse sentido, é interessante investir na maricultura como uma inciativa de sustentabilidade inclusive, alguns resultados de sucesso foram encontrados em cultivos com as espécies *Laminaria japônica* e *Undaria pinnatifida* (Phaeophyta), *Eucheuma* spp., *Gracilaria* spp. e *Porphyra* spp. (Rhodophyta) e *Monostroma* sp (Chlorophyta) (TEIXEIRA et al. 2000; TEIXEIRA, 2004).

No Brasil, a produção de algas em escala de produção comercial, já está sendo desenvolvida em algumas comunidades tradicionais dos estados nordestinos, usando técnicas de “longlines” já testadas, aprovadas e adaptadas às diferentes localidades litorâneas. As iniciativas citadas têm ocorrido no Ceará, no Rio Grande do Norte, na Paraíba e em Pernambuco. Já no Ceará, existe um cultivo extensivo em escala comercial localizado na comunidade de Flecheiras – Trairi e outro na comunidade de Barrinha em Icapuí. No estado do Rio Grande do Norte a comunidade de Rio do fogo também já está comercializando macroalgas marinhas cultivadas. Em Pernambuco foram realizados treinamentos sobre cultivo de macroalgas marinhas na Praia de Pau Amarelo, no Município de Paulista, e na Ilha de Itamaracá. Estes treinamentos ocorreram de 2007 à 2009 (TEIXEIRA et al. 2009).

Os cultivos citados desenvolvidos no Ceará, Paraíba e em Pernambuco usaram estruturas do tipo ‘’long-lines’’ usando cordas de polietileno, bóias como estruturas de flutuação e ancoras ou ‘’garatéias’’ como estrutura de fixação (TEIXEIRA, 2009; MIRANDA, 2006).

Mesmo apresentado potencial de produção e experiências positivas registradas no cultivo de algumas espécies, sabe-se que o desenvolvimento da maricultura em escala comercial no Brasil não foi efetivada devido a falta de conhecimento específicos em relação à biologia e ecologia das espécies, sendo este, um dos motivos do insucesso desses cultivos (OLIVEIRA, 1998; URSI & PLASTINO, 2001).

2.3. Espécie estudada

A espécie estudada, *Gracilaria birdiae*, trata-se de uma macroalga explorada comercialmente pelas comunidades pesqueiras no Brasil, distribuindo-se ao longo da região litorânea do Nordeste brasileiro estendendo-se até a região Norte do Espírito Santo (BEZERRA, et al., 2010; MARINHO-SORIANO, 2010).

Sistemática Filogenética do *Gracilaria birdiae* (Fonte: Plastino e Oliveira, 2002)

A macroalga marinha vermelha, *G. birdiae* ocupa a seguinte posição sistemática:

Filo: Rhodophyta
Subfilo: Eurhodophytina
Classe: Florideophyceae
Subclasse: Rhodymeniophycidae
Ordem: Gracilariales
Família: Gracilariaceae
Gênero: *Gracilaria*
Espécie: *Gracilaria birdiae*

As populações de *Gracilaria* ocorrem na maioria dos mares tropicais e temperados no mundo e em regiões tropicais, apresentam maior produtividade no inverno (OLIVEIRA & PLASTINO, 1994). Esta macroalga é comumente encontrada sobre substratos rochosos intertidais ou subtidais, estando presente em todos os períodos do ano (GUIRY, 2011). Entretanto, a colheita excessiva de algas do gênero tem tido consequências negativas sobre a sustentabilidade deste recurso natural (BEZERRA et al., 2010 MARINHO-SORIANO, 2010).

Quanto ao ciclo de vida, o gênero *Gracilaria* (Gracilariales, Rhodophyta) apresenta um histórico de vida trifásico, no qual a fase gametofítica e a tetrasporofítica são isomórficas e independentes. Embora isomórficas, é possível que existam diferenças fisiológicas entre essas fases, como já reportado para algumas espécies (AGUILAR-ROSAS et al., 1993; GUIMARÃES, 2000; URSI & PLASTINO, 2001; BARUFI, 2004; PLASTINO, 2004; COSTA, 2005; ESPINOZA-AVALOS, 2005; URSI, 2005; FERREIRA, 2008).

Gracilaria é um dos gêneros com grande representatividade de algas vermelhas, com cerca

de 100 espécies reconhecidas (BELLORIN, 2002). Nas últimas décadas, as algas gracilarióides têm sido bastante utilizadas como principal matéria prima na produção de ágar-ágar do mundo (OLIVEIRA et al. 2000), sendo de importância para as industrias alimentícia, de ração, fertilizantes, produtos medicinais e produção de biogás (OLIVEIRA & PLASTINO, 1994).

O ágar é um produto retirado de algas vermelhas que apresenta características físicas gelatinosa devido as suas estruturas da parede celular formado por microfibrilas de celulose e mucilagem, o que favorece sua utilização em diversos processos como na indústria alimentícias na produção de chocolates e pudins, assim como, componente de formação do meio de cultura, para análises microbiológicas (WERLINGER et al. 2004). As principais fontes de ágar estão nas espécies de Gracilaria spp. (53%) e Gelidium (44%), em menor quantidade este produto é extraído da Gelidiella e Pterocladia (3%) (MCHUGH, 2001). O conteúdo de ágar em muitas espécies corresponde aproximadamente 20 a 30% da biomassa seca (CHAOYUAN, 1990).

2.3.1. Bioecologia e importância da *Gracilaria birdiae*

Gracilaria birdiae apresenta uma estrutura bastante ramificada, com talos compridos e cilíndricos de coloração vermelho-vinácea (Figura 3). Sua estrutura vegetativa compreende um talo ereto de forma cilíndrica com 2,3 mm de diâmetro, podendo alcançar até 46 cm de altura. A partir da estrutura cilíndrica surgem ramificações pseudodicotômicas irregulares, frequentemente unilaterais e de segunda ordem. A fixação ao substrato ocorre por um pequeno apressório discoide (PLASTINO & OLIVEIRA, 2002).

A espécie *Gracilaria* (Gracilariales, Rhodophyta) apresenta bom desempenho de crescimento e ampla tolerância aos estresses ambientais (DE CASABIANCA et al., 1997), por isso têm despertando interesse para fortalecer conhecimentos sobre melhores condições de cultivo, bem como a seleção de linhagens mais adequadas à maricultura (ZHANG & VAN DER MEER 1987).

Figura 2: Aspecto geral da população de *Gracilaria birdiae*

Fonte: (Araujo, 2005)

Vários estudos têm sido realizados comprovando a presença de importantes atividades biológicas em extratos de algas *G. birdiae*. Almeida et al. (2011) relatam que extratos de algas do gênero *Gracilaria* têm apresentado atividade antibacteriana, antiviral, antifúngica, anti-hipertensiva, citotóxica, espermicida, embriotóxica e antiinflamatória. Souza et al. (2011) investigaram as propriedades antioxidantes dos extratos etanólico e metanólico de *G. birdiae* coletada na costa Atlântica nordeste do Brasil (Ceará) e concluíram que a referida alga pode ser utilizada como fonte de antioxidantes naturais.

2.4. Atividade antioxidante de algas

2.4.1 Radicais livres e antioxidantes

Um radical livre é definido como uma espécie de existência independente que contém um ou mais elétrons desemparelhados sozinhos num orbital atômico ou molecular (HALLIWELL, 1996). Os radicais podem ter carga positiva, negativa ou neutra. Eles são formados como intermediários necessários em uma variedade de reações bioquímicas normais, mas quando gerados em excesso ou não devidamente controlados, os radicais podem causar danos em uma ampla gama de macromoléculas. Uma característica proeminente de radicais é que possuem uma reatividade química muito alta, o que explica não só as suas atividades biológicas normais, como também os danos celulares (CUZZOCREA et al., 2001).

Existem vários tipos de radicais, mas aqueles de maior preocupação em sistemas biológicos são os derivados do oxigênio e conhecidos coletivamente como espécies reativas de oxigênio (HALLIWELL, 1992). O oxigênio tem dois elétrons desemparelhados em orbitais separados em sua camada externa. Esta estrutura eletrônica faz com que o oxigênio seja especialmente suscetível à formação de radicais. No organismo, essas espécies químicas em excesso podem reagir com moléculas importantes, e por isso são capazes de inibir ou modificar a função dessas moléculas e, consequentemente, provocar inúmeras desordens fisiológicas (CUZZOCREA et al., 2001). Por exemplo, ao reagir com o DNA os radicais livres podem modificar as bases púricas e pirimídicas que o compõem, gerando mutações ou inativando genes importantes. Já com proteínas, as espécies reativas são capazes de oxidar grupos sulfidrilas (-SH), as pontes dissulfeto (S-S), alterando a conformação e consequentemente a função das proteínas. Além disso, pode haver a oxidação de ácidos graxos poliinsaturados de membranas celulares, proporcionando mudanças estruturais e funcionais de tais lipídios (VALKO et al., 2006).

Por causa dessa alta reatividade, inúmeras condições relacionadas ao envelhecimento e a doenças têm sido atribuídas à ação dos radicais livres. Doenças neurodegenerativas como mal de Parkinson, mal de Alzheimer e esclerose amiotrófica lateral (BARNHAM; MASTERS; BUSH, 2004), diabetes tipo 2, aterosclerose (KANETO et al., 2010) e câncer (VALKO et al., 2006) são exemplos de condições derivadas diretamente ou indiretamente da ação destas espécies reativas.

É importante ressaltar que os radicais livres não trazem apenas malefícios. Se produzidos de uma forma bem regulada, os ROS e RNS têm importante função nos sistemas biológicos. Por exemplo, o óxido nítrico (NO), que é uma espécie reativa de oxigênio, quando produzido pelas células endoteliais é essencial para a regulação do relaxamento e proliferação de células musculares lisas vasculares, a adesão dos leucócitos, a agregação de plaquetas, a angiogênese, a trombose, o tônus vascular e a hemodinâmica. Além disso, o NO produzido por neurônios serve como um neurotransmissor. Já aquele gerado por macrófagos ativados é um importante mediador da resposta imune (FANG et al., 2002). Outros processos que os radicais livres participam são: Desintoxicação de xenobióticos pelo citocromo P450 (enzimas de oxidação) e apoptose de células estéreis ou com defeito (DEVASAGAYAM, et al., 2004).

O conjunto das substâncias que neutralizam os efeitos biológicos danosos dos ROS/RNS são chamados de antioxidantes, os quais podem ser enzimáticos e não-enzimáticos. Segundo Valko (2006), um antioxidante ideal deve ser capaz de sequestrar radicais livres, quelar metais de transição, interagir com outros antioxidantes, e ser absorvido, além de trabalhar tanto em soluções

aquosas como em domínios de membrana celular. No entanto, as substâncias até então descobertas que atuam como antioxidantes possuem apenas uma ou algumas dessas características (VALKO et al., 2007).

Em eucariotos, os antioxidantes enzimáticos mais eficientes compreendem a superóxido dismutase, a catalase e a glutationa peroxidase. A superóxido dismutase (SOD) catalisa a decomposição do superóxido formando H_2O_2 e O_2. A catalase e a glutationa dismutase removem o peróxido de hidrogênio (DEVASAGAYAM, et al., 2004). Os antioxidantes não enzimáticos compreendem o ácido ascórbico (vitamina C), o α-tocoferol (vitamina E), carotenóides, antioxidantes tióis (glutationa, tioredoxina e ácido lipóico), melatonina, dentre outros (VALKO et al., 2007; FERREIRA; MATSUBARA, 1997).

Apesar de o organismo humano possuir um potente sistema antioxidante e de reparo, não é possível combater todos os danos oxidativos. Desse modo, o organismo faz uso de antioxidantes exógenos fornecidos pela alimentação (SIMIC, 1988).

A indústria alimentícia faz uso de antioxidantes sintéticos, dentre os quais se destacam o hidroxianilose butilado (BHA), hidroxitolueno butilado (BHT), tert-butilhidroquinona (TBHQ) e o galato propil (PG) para garantir a qualidade de seus produtos dentro do período de validade. Estes antioxidantes são utilizados como aditivos alimentares, porém seu uso está sob regulação severa devido ao perigo para a saúde que os mesmos apresentam. O BHA e o BHT, por exemplo, têm sido suspeitos de causar danos no fígado e promover a carcinogênese (ITO; TURUSHIMA; TSUDA, 1985; QI et al., 2005a,). Assim, se torna essencial a descoberta e a utilização de antioxidantes naturais que possam proteger organismos dos radicais livres e retardar o progresso de muitas doenças crônicas, além de antioxidantes que possam ser utilizados na indústria alimentícia.

2.4.2. Atividade antioxidante das algas marinhas

A definição mais aceita para antioxidantes relata que estes são substâncias que, mesmo presentes em baixas concentrações em relação ao substrato oxidante, podem atrasar ou inibir as taxas de oxidação (SIES; STHAL, 1995). Vários componentes fisiológicos são conhecidos pela capacidade de inativar ou neutralizar radicais livres, dentre eles estão vários metabólitos secundários provenientes de produtos naturais (PIETTA, 2000; NORDBERG; ARNÉR, 2001).

O interesse inicial pelo estudo de substâncias com atividade antioxidante em algas surgiu no Japão, na busca de novos aditivos para alimentos, em substituição aos antioxidantes sintéticos,

como o hidroxianisol butilado (BHA) e o hidroxitolueno butilado (BHT) (ROCHA et al., 2007). Desta forma, a pesquisa por antioxidantes naturais em substituição aos antioxidantes sintéticos, é objeto de estudo de muitos pesquisadores. Dentro deste contexto, o fato da alga desidratada e armazenada por um longo período não sofrer oxidação, mesmo apresentando mais de 30% do total de seus ácidos graxos na forma poliinsaturada, despertou o interesse dos pesquisadores em relação ao seu mecanismo antioxidante (ROCHA et al., 2007). Assim, os antioxidantes de algas poderiam ser utilizados no combate aos readicais livres, incluindo àqueles relacionados à enfermidades, como os cálculos renais e alguns tipos de câncer.

2.5. Cálculos renais

2.5.1. Urolitíase

Urolitíase ou litíase urinária é uma condição fisiopatológica oriunda da formação de cálculos renais ou concreções no sistema urinário, sendo, portanto caracterizado como uma massa ou agregado cristalino sólido que se forma nos rins a partir de sais minerais presentes na urina. A urolitíase tem sido reconhecida como uma doença crônica à qual se associam custos econômicos substanciais e morbidade significativa e altas taxas de recorrência (PEARL et al, 2005). Cálculos são estruturas sólidas, resultantes da aglomeração de cristais, compostos por cristais inorgânicos e orgânicos, os quais podem estar amalgamados por proteínas. São derivados de uma alteração metabólica crônica do organismo provocando uma excreção aumentada de substâncias pela urina, como cálcio, oxalato, fosfato e/ou diminuição de excreção de substâncias inibidoras da cristalização, como o citrato (MOE, 2006).

A presença de cálculos renais acompanha a história da humanidade. Os cálculos em bexiga foram descobertos em múmias do Egito que remota 7.000 anos atrás (EKNOYAN, 2004). Existem registros em papiros feitos por babilônicos e egípcios, há 4.800 anos a.C, de dietas para o tratamento de doenças do trato urinário, incluindo os cálculos (LOPES E HOPPE, 2010).

A incidência anual de formação dos cálculos renais tem sido relatada como sendo em torno de 1500-2000 casos por milhão, e em 25% dos casos, as pessoas afetadas precisam remover ativamente os cálculos (TISELIUS, 2003).

A dieta é um fator extremamente importante na formação dos cálculos renais, pois a composição da urina está diretamente relacionada com a mesma. Assim, os cuidados com a alimentação têm como objetivo prevenir ou diminuir a recorrência de litíase renal (RIELLA e MARTINS 2001). A formação de cálculos renais pode aumentar em função de alguns fatores

nutricionais, tais como: ganho de peso e obesidade, excesso de sal na comida e o consumo reduzido de líquidos, dentre outros. Portanto, modificações na dieta podem ser feitas conforme os diversos fatores metabólicos que contribuem para a formação dos cálculos (ex: hipercalciúria, hiperoxalúria, hipocitratúria, entre outros) (CACHAT 2004).

Nos últimos anos, vem se desenvolvendo técnicas e pesquisas a fim de eliminar e ou prevenir os cálculos renais, pois, perante uma crise renal provocada pelos cálculos, o tratamento clinico comumente realizado ainda é a analgesia. Desse modo, os antiinflamatórios não-esteróides (AINEs) e as prostaglandinas (PGs) potenciam e modulam os mecanismos locais e centrais da dor (GOMES, et al., 2002).

Após a analgesia, o procedimento posterior dependerá do tamanho e localização do cálculo formado. O tratamento tradicional é a eliminação do cálculo espontaneamente, porém bloqueadores de canais de cálcio (nifedipina) podem ser utilizados, pois seus efeitos miorrelaxantes parecem aumentar a taxa de eliminação espontânea dos cálculos. Outro tratamento utilizado é a quemólise para a dissolução do cálculo de ácido úrico. Porém o bombardeamento por laser e procedimentos cirúrgicos são ainda a forma de tratamento mais utilizado para cálculos de tamanhos maiores ou com riscos críticos de obstrução mesmo sendo o método mais invasivo (KNOLL e PEARLE, 2013).

Estudos reportados na literatura trabalhos mostram que cálculos calcários, principalmente formados por oxalato de cálcio, são os mais frequentes na urolitíase, representando mais de 80% dos cálculos encontrados. Aqueles constituídos de ácido úrico representam cerca de 5-10%, seguido pelos constituídos de cistina, estruvita, e cálculos de urato ácido de amônio. A formação de diversos tipos de cálculos altamente incomuns está associado com a presença de xantina, 8-diidroxiadenina, matriz protéica e fármacos, por exemplo, indinavir e triantereno. Além disso, cálculos de origem mista não são raros e podem mostrar uma fisiopatologia subjacente bastante diferente do cálculo formado por um único componente, o que também pode modificar drasticamente o tratamento (MOE, 2006).

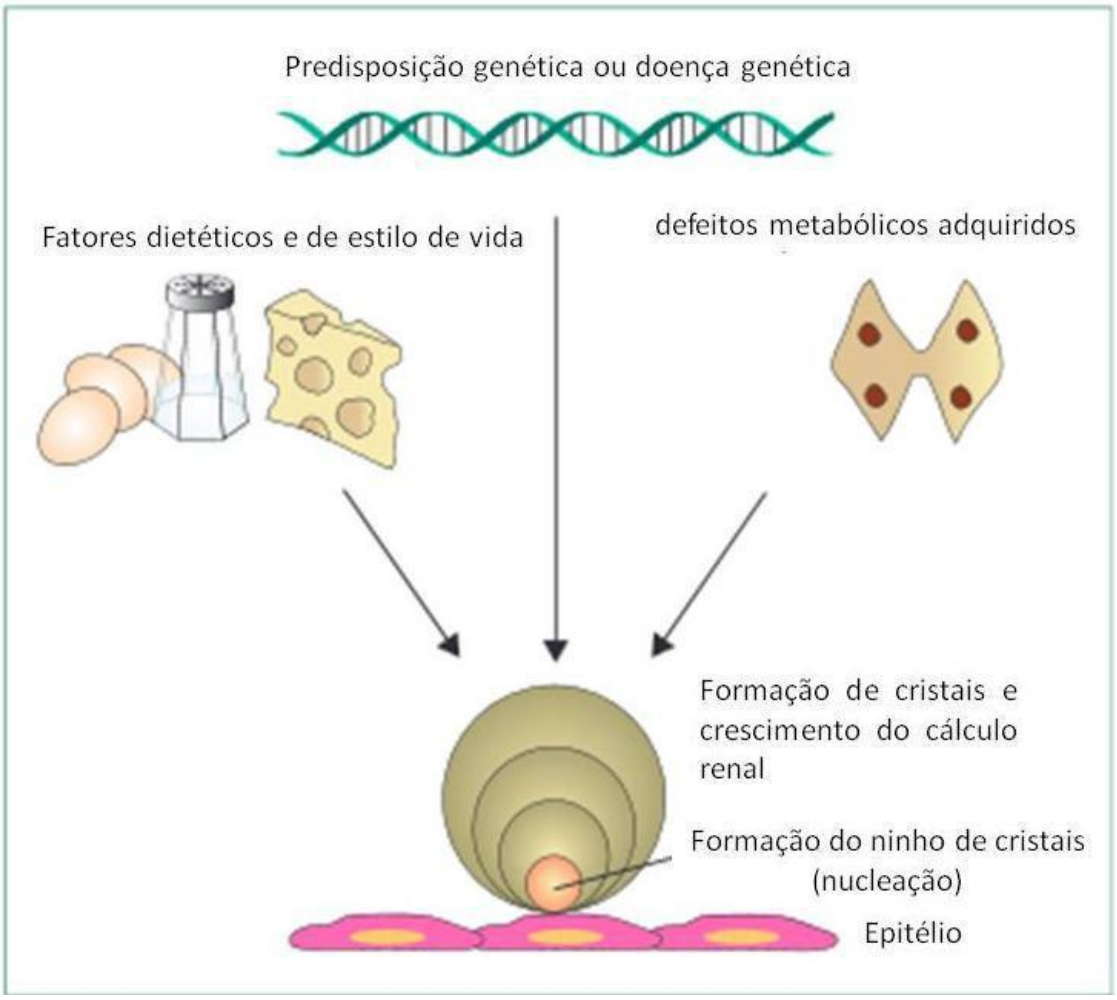
Nesse contexto, vários fatores podem estar relacionados à urolitíase sendo os principais: a herança genética, o sedentarismo, clima e/ou exposição a temperaturas elevadas, hábitos alimentares, entre outros (SELVAM, 2002). A literatura mostra ainda que há um risco de cerca de 10 a 15% de desenvolvimento de cálculos em países desenvolvidos e cerca de 25% nos que estão em desenvolvimento. Além disso, a probabilidade de recorrência é de 50% entre 5 a 10 anos e 75% em 20 anos e cerca de 80% dos cálculos renais ocorrem na maioria das vezes nos homens. (LOPES

E HOPPE, 2010).

2.5.2. Formação de cristais de oxalato de cálcio e estresse oxidativo

A formação do cálculo de oxalato de cálcio é resultado de um processo físico-químico compreendendo três fases, as quais são a nucleação, o crescimento e a agregação dos cristais. Para a formação de um cristal é primariamente necessária a supersaturação urinária, que é o estado no qual alguns sais estão dissolvidos na urina em concentrações muito maiores que as plasmáticas (KNOLL, 2010). Para tal, elevadas concentrações dos íons em uma solução levam ao agrupamento por meio de suas cargas, gerando cristais diminutos. Este processo pode ser homogêneo ou heterogêneo dependendo da composição da urina (RUSSEL, 1972). O processo homogêneo ocorre com o cristal formado servindo de substrato para a deposição de outros nanocristais semelhantes. Enquanto isso, o heterogêneo (figura 4), acontece como consequência da deposição dos cristais sobre um substrato, que pode ser formado por macromoléculas ou por outro cristal diverso quimicamente (GRASES et al., 1998). Finalmente, os cristais se atraem formando agregados que crescem até formar uma fase sólida (COE, FAVUS, 1997). Esse cristal em fase sólida, pode se ligar a proteínas e outras substâncias presentes na urina, originando-se, por sua vez, os cálculos.

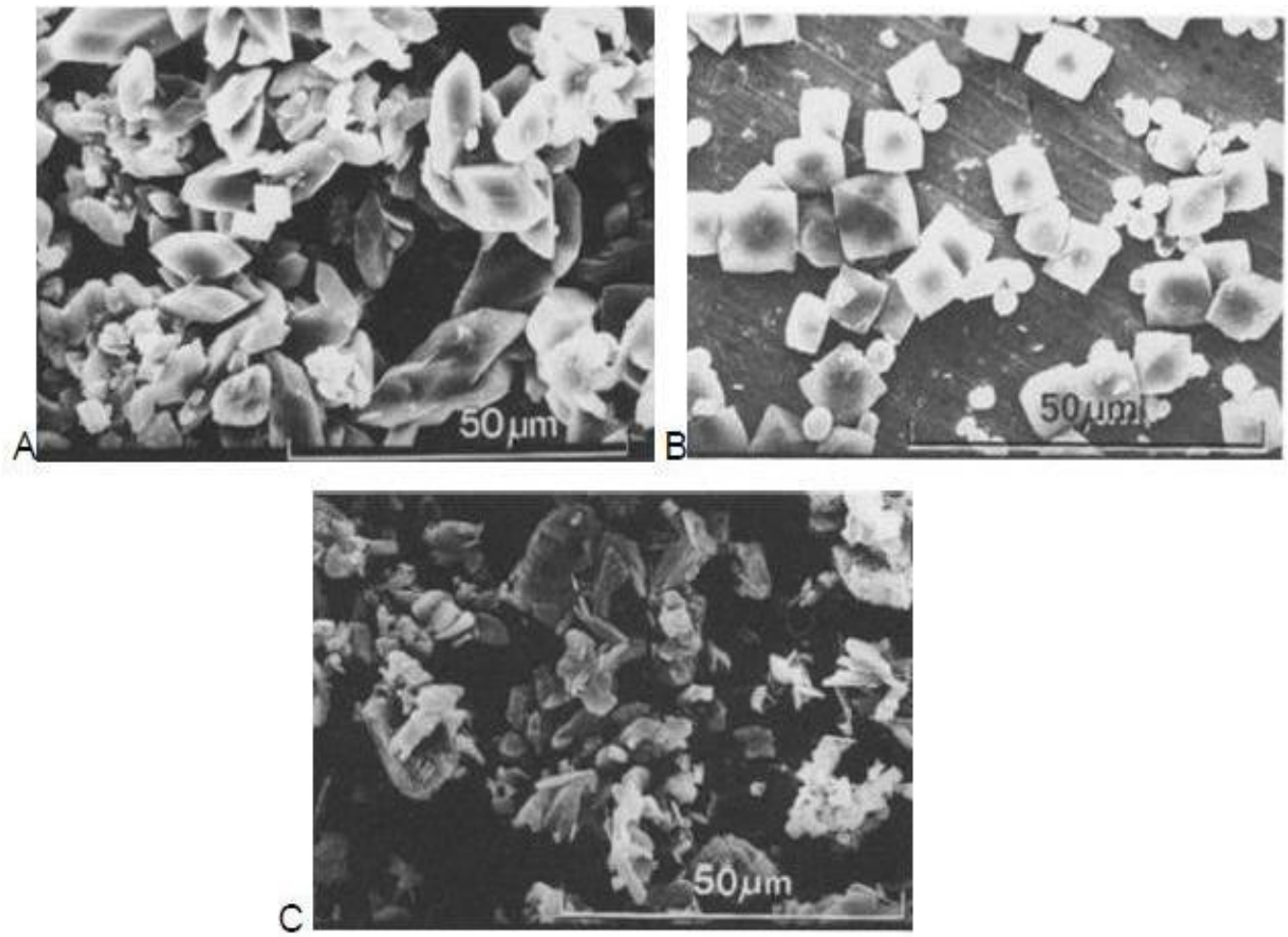
Figura 3: Patogênese dos cálculos de oxalato de cálcio nos rins. Três categorias de fatores (genética, metabólica e alimentar), em conjunto ou isoladamente para levar a formação do cálculo. O processo de nucleação heterogênea provavelmente precisa de um ninho iniciando no epitélio (I), que constitui o substrato para a cristalização e para o crescimento (II).



Fonte: MOE, 2006.

Os cristais de oxalato de cálcio desenvolvem-se em monohidratados (COM) (figura 5.A), dihidratados (COD) (figura 5.B) e trihidratados (COT) (figura 5.C). Os cristais de COM apresentam uma geometria de prisma tetragonal alongada, com superfície externa irregular, uma estrutura densa e elevada dureza, (YU et al. 2011). O COD são cristais termodinamicamente instáveis, visto que, em contato com líquido, se transformam gradualmente em COM (GRASES, 1998). Já o COT é raramente encontrado nos cálculos.

Figura 4: Imagens de Microscopia Eletrônica de Varredura de Cristais de Oxalato de Cálcio. (A) Monohidratado; (B) Dihidratado; (C) Trihidratado.



Fonte: GRASES et al., 1989

Um dos passos decisivos no processo da urolitíase é o tipo de cristal formado, uma vez que sua forma é determinante para a ligação ao epitélio renal e posterior formação do cálculo. Desse modo, a forma monohidratada (COM) por ter a morfologia termodinâmica mais estável dentre os três tipos é consequentemente aquela que tem mais facilidade de se ligar ao epitélio renal. Um fato importante relacionado a essa preferência é a carga de superfície, já que os cristais COM aderem à superfície da célula em sítios aniónicos específicos, os quais se encaixam perfeitamente a sua forma tetragonal alongada. Além disso, propriedade adesiva, ligação eletrostática e interações específicas são também outros fatores mediadores importantes para adesão desse cristal (FONG-NGERN, et al., 2011).

A ligação ao epitélio acontece preferencialmente nos lugares onde o epitélio renal está com as suas camadas protetoras (glicosaminoglicanos-GAG) danificadas ou destruídas. Esse dano está

intimamente relacionado ao estresse oxidativo celular e formação de radicais livres (SELVAM, 2002).

2.6. Câncer

O câncer é um dos problemas mais complexos que os sistemas de saúde mundial enfrentam e essa doença está prestes a se tornar uma das maiores causas de mortalidade nas próximas décadas. Segundo a Organização Mundial de Saúde (WHO, 2010) o número de casos de câncer no mundo deverá aumentar em 75% até 2030. E segundo esta mesma pesquisa, essa taxa pode ser ainda mais alta e chegar a 90% em países mais pobres (WHO, 2010; BRAY et al., 2004).

Para o tratamento do câncer, os três principais tratamentos atuais são a cirurgia, a radioterapia e a quimioterapia, cuja escolha depende do tipo de tumor e do estágio de seu desenvolvimento (Rang et al., 2004). Embora esses tratamentos sejam de grande valor, podem apresentar desvantagens e limitações, como complicações pós-cirúrgicas e toxicidade sistêmica. Por essa razão, pesquisas que buscam métodos alternativos e/ou complementares de tratamento estão em evidência e visam sempre ser mais eficientes em relação às terapias convencionais (ROTHENBERG et al., 2003).

Alguns estudos têm sugerido que biomoéculas de organismos marinhos têm atividade contra alguns tipos de câncer e em cálculos renais. Dentro deste contexto, foi avaliado o potencial frmacolóogico da alga G. birdiae frente a estas enfermidades, bem como a sua atividade antioxidante.

3. OBJETIVOS

3.1 Geral

Avaliar comparativamente o potencial farmacológico da macroalga vermelha *Gracilaria birdiae* arribada, de banco natural e de cultivo, visando contribuir para o conhecimento de ferramentas de organismos marinhos para o combate à enfermidades.

3.2. Específicos

- ✓ Obter extrato bruto das diferentes fontes (arribada, banco e cultivo) das algas *Gracilaria birdiae*;
- ✓ Determinar a composição química desses polissacarídeos extraídos;
- ✓ Determinar a atividade antioxidante dos extratos utilizando diferentes ensaios *in vitro*;
- ✓ Verificar o potencial dos extratos como agente inibidor de formação de cristais de oxalato de cálcio;
- ✓ Comparar o efeito dos extratos brutos das macroalgas coletadas sobre as atividades antiproliferativas de células tumorais.

CAPITULO 1

A ser submetido ao periódico

Molecules

(ISSN 1420-3049)

www.mdpi.com/journal/molecules

CAPÍTULO 1: Antioxidant activity of crude extracts from the red seaweed *Gracilaria birdiae*

Werlayne M. Santana[1]; Rafael B. G. Camara[2]; Douglas H. H. Andrade[1]; Rafael D. S. Azevedo[1]; Juliett F. X. Silva[3]; Lucas V. Silva[4], Dárlio I. A. Teixeira[4]; Hugo A. O. Rocha[2] and Ranilson S. Bezerra[1*]

[1] Laboratório de Enzimologia (LABENZ), Departamento de Bioquímica, Universidade Federal de Pernambuco (UFPE), Cidade Universitária, 50670-420, Recife-PE, Brasil; E-mails: werlaynemendes@gmail.com (W.M.S.); douglasvicencia@gmail.com (D.H.H.A.); rafaeldavidbio@gmail.com (R.D.S.A.); ransoube@uol.com.br (R.S.B.)

[2] Laboratório de Biotecnologia de Polímeros Naturais (BIOPOL), Departamento de Bioquímica, Centro de Biociências, Universidade Federal do Rio Grande do Norte (UFRN), Natal, Rio Grande do Norte 59072-970, Brasil; E-mails: rafael_bgc@yahoo.com.br (R.B.G.C.); hugo@cb.ufrn.br (H.A.O.R.);

[3] Universidade Federal de Alagoas, campus Penedo; E-mail: juliettpesca@yahoo.com.br (J.F.X.S.);

[4] Escola agrícola de Jundiaí, UFRN; E-mail: darlioteixeira@gmail.com (D.I.A.T)

*Corresponding Author:

Ranilson S. Bezerra

Laboratório de Enzimologia (LABENZ), Departamento de Bioquímica e Laboratório de Imunopatologia Keizo Asami (LIKA), Universidade Federal de Pernambuco, Cidade Universitária, 50670-420, Recife-PE, Brazil.

Tel, +55 81 21268540; Fax, +55 81 21268576

e-mail: ransoube@uol.com.br

Antioxidant activity of crude extracts from the red seaweed *Gracilaria birdiae*

Three different crude extracts from the red seaweed *Gracilaria birdiae* (Gb): (GbD – extract from drift, GbNB – extract from natural banks and GbF – extract from farmmed) collected from Natal city coast (Northeast of Brazil) were evaluated for their, antioxidant in vitro activities. All species collected showed antioxidant activities. This screening emphasized the great antioxidant potential (total capacity antioxidant, hydroxyl radical, superoxide radical, ferrous chelating and copper chelating) of three different sources from the red seaweed *Gracilaria birdiae*. That GbD and GbF samples showed a great total antioxidant capacity in comparison to the GbNB extract. Samples from GbF showed higher superoxide ion scavenging activity. The most active compounds of ferrous chelating capacity were from GbNB and GbF. On the other hand the most active compounds of copper chelating capacity was GbNB. Studies of the bioactivity of algal components contribute to the body of knowledge about marine-derived compounds and their use in the pharmaceutical and chemical industries.

Keywords: crude extracts, different sources, *Gracilaria birdiae*, antioxidant, red seaweed.

1. Introduction

In recent years, marine resources have attracted attention in the search for bioactive compounds to develop new drugs and healthy foods (Qi *et al.,* 2005). In particular, seaweeds are a very important and commercially valuable resource for food, fodder, soil conditioners and pharmaceuticals (Yang *et al.,* 2006). One of the most bioactive compounds it's a sulfated polysaccharides to exhibit many biological and physiological activities including anticoagulant, antiviral, antitumor, anti-inflammatory and antioxidant (De S.F-Tischer *et al.,* 2006; Becker *et al.,* 2007; Ye *et al.,* 2008; Souza *et al.,* 2012).

Marine red algae of the genus *Gracilaria* are a major agarophyte resource in the world and are cultivated for the phytocolloid industry or for integrated marine culture (Troell *et al.,* 2003). The red marine alga *Gracilaria birdiae* has a great economic impact in Brazil due to agar production (Plastino *et al.,* 2004).

Seaweed lives in a constant salt and hydrolytic stress environment. It is exposed to ultraviolet radiation and oxygen. These factors lead to formation of free radicals and other oxidants. However not observed severe oxidative damage in seaweed indicating that possess protective mechanisms mediated by enzymes or not enzymatic. Antioxidants are substances that can delay or prevent oxidation of cellular oxidizable substrates (Wang *et al.,* 2009).

In recent years, algal polysaccharides have been demonstrated to play an important role as free-radical scavengers and antioxidants for the prevention of oxidative damage in living organisms (Rocha De Souza *et al.,* 2007; Wang *et al.,* 2009). However, no studies have yet been performed about antioxidant activities with the crude extracts from seaweeds from at drift, natural banks and farmed. Thus, the aim of these study is evaluate antioxidant activity of *G. birdiae* from different sources (at drift, natural bank and farmed).

2. Material and methods

2.1 Raw Material

The red seaweed, *Gracilaria birdiae* (at drift, natural bank and farmed) was collected in Rio do Fogo Beach (Rio Grande do Norte, Brazil, 5°16'16.61"S/35°22'54.29"W) by fishermen from the community; the seaweed was taken to the laboratory to cleaned to eliminate residue and epiphytes. After, it was dried at 50º C under ventilation in an oven and they were subsequently crushed. After, this material was homogenized in distilled water at concentrations of 8 mg/mL, agitation for 12 hours and filtered. These solutions were named GbD (extract from *G. birdiae* at drift), GbNB (extract from *G. birdiae* of natural bank) and GbF (extract from *G. birdiae* farmed). At the end, the material was frozen at – 20º C for posterior lyophilization.

2.2 Antioxidant Activity

2.2.1. Determination of Total Antioxidant Capacity

This assay is based on the reduction of Mo (VI) Mo (V) by sulfated polysaccharides and subsequent formation of a phosphate green complex/Mo (V) with acid pH. Tubes containing crude extracts and reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) were incubated at 95 °C for 90 min. After the mixture had cooled to room temperature, the absorbance of each solution was measured at 695 nm against a blank. Total antioxidant capacity was expressed as ascorbic acid equivalent.

2.2.2. Hydroxyl Radical Scavenging Activity Assay

The scavenging assay of the hydroxyl radical was based on the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH$). The results were expressed as inhibition rates. The hydroxyl radicals were generated using 3 mL of sodium phosphate buffer (150 mM, pH 7.4) containing 10 mM $FeSO_4 \cdot 7H_2O$, 10 mM EDTA, 2 mM of sodium salicylate 30% H_2O_2 (200 mL), and different concentrations of crude extract. In the control, sodium phosphate buffer replaced H_2O_2. The solutions were incubated at 37 °C for 1 h, and the presence of the hydroxyl radical was detected through the monitoring of the absorbance at 510 nm. Gallic acid was used as a positive control.

2.2.3. Superoxide Radical Scavenging Activity Assay

This assay was based on the ability of SPs to inhibit the photochemical reduction of tetrazoliumnitroblue (NBT) in the riboflavin-light-NBT system. Every 3 mL of reaction mixture contained 50 mM of phosphate buffer (pH 7.8), 13 mM of methionine, riboflavin 2 mM, EDTA at 100 mM, NBT (75 mM), and 1 mL of the sample solution. After 10 min of illumination with a fluorescent lamp for the production of blue formazan to occur, the samples were read at 560 nm. Identical tubes of the reaction mixture were kept in the dark and served as blanks for the reaction. Gallic acid was used as a positive control.

2.2.4. Ferrous Chelating

Both methods used the ferrozine and $FeCl_2$ complex to determine the antioxidant capacity. In the first method, chitosan at different concentrations (0.01–2 mg/mL) was added to a reaction

mixture containing $FeCl_2$ (0.05 mL, 2 mM) and ferrozine (0.2 mL, 5 mM). The mixture was stirred and incubated for 10 min at room temperature, and the absorbance of the mixture was measured at 562 nm against a blank. EDTA was used as standard. In the second method, chitosan at different concentrations (0.5–2 mg/mL) was mixed with 3.7 mL of methanol and 0.01 mL of 2 mM $FeCl_2$, then 0.2 mL of 5 mM ferrozine were added to initiate the reaction. The mixture was shaken vigorously and kept at room temperature for 10 min. Absorbance was determined at 562 nm against a blank, and EDTA was used as the standard. For both methods, the chelating effect was calculated using the corresponding absorbance (A) in the formula given below, where control is the condition in the absence of chelating agents:

$$\text{Chelating effect (\%)} = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) x100$$

2.2.5. Copper Chelating

The ability to chelate the copper ion from the extracts was determined by the method described by Anton, 1960 (Anton, 1960). Pyrocatechol violet, the reagent used in this assay, has the ability to associate with certain cations, such as aluminum, copper, bismuth and thorium. In the presence of chelating agents, this combination is not formed, resulting in decreased staining. The test is performed in 96-well microplates with a reaction mixture containing different concentrations of samples (0.1–2 mg/mL), pyrocatechol violet (4 mM) and copper II sulfate pentahydrate (50 mg/mL). All wells were homogenized with the aid of a micropipette, and the solution absorbance was measured at 632 nm. The ability of the samples to chelate the copper ion was calculated using the corresponding absorbance (A) in the following formula, where control is the condition in the absence of chelating agents:

$$\text{Copper Chelating (\%)} = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) x100$$

3. Results and discussion

3.1. Antioxidant Activity

Seaweeds inhabit midcoastal areas, especially in harsher environments where they are subjected to repeated immersions and emersions due to tidal fluctuations. As a result, it is exposed twice a day to a variety of environmental stresses, including exposure to ultraviolet radiation, rapid temperature fluctuations, osmotic stress, and desiccation (Costa *et al.*, 2010; Melo *et al.*, 2013). Some of these factors contribute to the generation of free radicals, which in most cases are highly reactive and, therefore, cause damage to the cell structures of algae. However, there are no records of great damage caused by these agents in seaweed, which indicates that there exists a defense mechanism mediated by an efficient antioxidant system constituted of enzymes and probably a myriad of antioxidant molecules. Among these bioactive compounds, crude extract have been study in this work as potent antioxidants.

The term antioxidant refers to compounds that can prevent the formation of biological substances and chemical oxidation damage induced by reactive species. The formation process of these reactive species occurs through a chain reaction involving three stages—initiation, propagation and termination—wherein the antioxidants act through several mechanisms. Thus, different methods are used to evaluate the effect of crude extract in the seaweed by different stages of initiation (total antioxidant capacity), propagation (chelation of copper and iron), and termination (sequestration of the superoxide and hydroxyl radicals).

3.1.1. Total Antioxidant Capacity (TAC)

The TAC test aims to evaluate the ability of a sample to donate electrons, thus neutralizing compounds such as free radicals, like Reactive Oxygen Species (ROS). The results are presented in the form of ascorbic acid equivalents (AAE), or, in other words, mg of ascorbic acid/g of extract (Figure 1).

All extracts from *Gracilaria birdiae* presented activity in the TAC assay, as shown in Figure 1. The GbD and GbF showed a significantly higher TAC ($p < 0.05$) than GbNB, which demonstrated a value of 196.92 mg/g of AAE and 195.02 mg/g of AAE, respectively, while GbNB had a TAC value of 88.58 mg/g of AAE, significantly lower. Thus, the detected values here are extremely interesting, which prompted us to conduct further antioxidant tests to determine the potential antioxidant mechanisms of the crude extracts of *Gracilaria birdiae*.

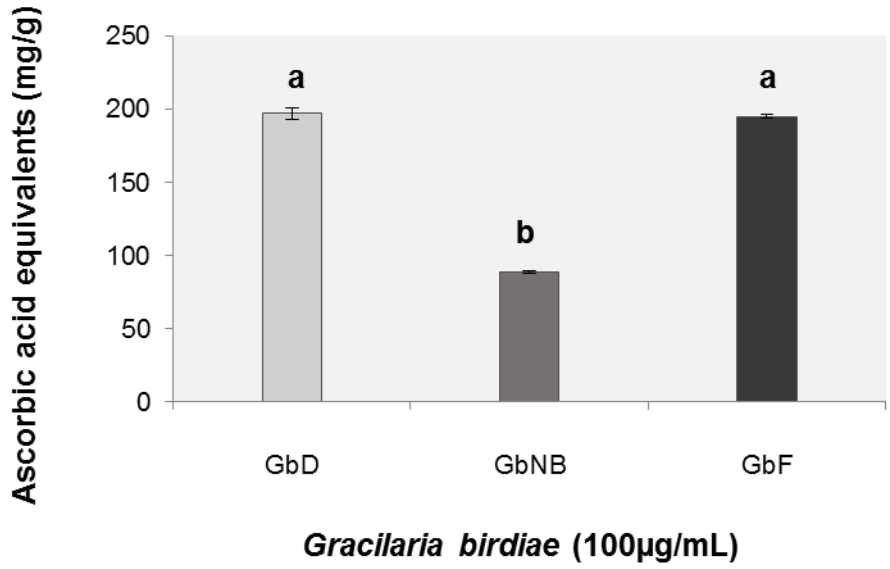


Figure 1. Total antioxidant capacity of crude extracts from the marine red seaweed *Gracilaria birdiae*. The results are expressed as AAE. Each value is the mean ± SD of two determinations: Different letters (a, b) indicate a significant difference ($p < 0.05$) between crude extracts.

3.1.2. Hydroxyl and Superoxide Radical

Hydroxyl radicals and superoxide anions are reactive oxygen species (ROS) implicated in cell damage. The hydroxyl radical is the most reactive of the radicals, making it extremely harmful. Its main source of production *in vivo* is due to the reaction of transition metals with the superoxide ion by the Fenton reaction (Valko *et al.*, 2007; Melo *et al.*, 2013) On the other hand, superoxide anion is considered a primary ROS, capable of generating reactive derivatives by direct interaction with other molecules or by means of processes catalyzed by metals or enzymes (Valko *et al.*, 2007; Melo *et al.*, 2013) being also produced within the mitochondria. Due to the harmful effect in the body, these ROS are associated with numerous diseases, such as strokes, cancer, diabetes, liver, and neuronal lesions (Freinbichler *et al.*, 2008; Melo *et al.*, 2013). No extract concentration of the GbD, GbF and GbNB alga presented hydroxyl scavenging activity and only GbNB (0.05 mg/mL, 40.07%), (0.1 mg/mL, 23.45%), (0.25 mg/mL, 14.90%) and (0.5 mg/mL, 3.98%) and GbF (0.05 mg/mL, 77.03%), (0.1 mg/mL, 58.04%) and (0.25 mg/mL, 22.43%) showed superoxide ion scavenging activity (figure 2). The absence of activity in the test of elimination of hydroxyl radicals is common in sulfated polysaccharides extracted from *Gracilaria birdiae* (Fidelis *et al.*, 2014) and brown algae (Melo *et al.*, 2013), this shows that the elimination of hydroxyl radicals is probably not the main antioxidant mechanism of these polysaccharides. The same could happen with crude extracts from *Gracilaria birdiae*. On the other hands, except GbNB (0.5 mg/mL), the others crude extracts had a high superoxide radical scavenging activity.

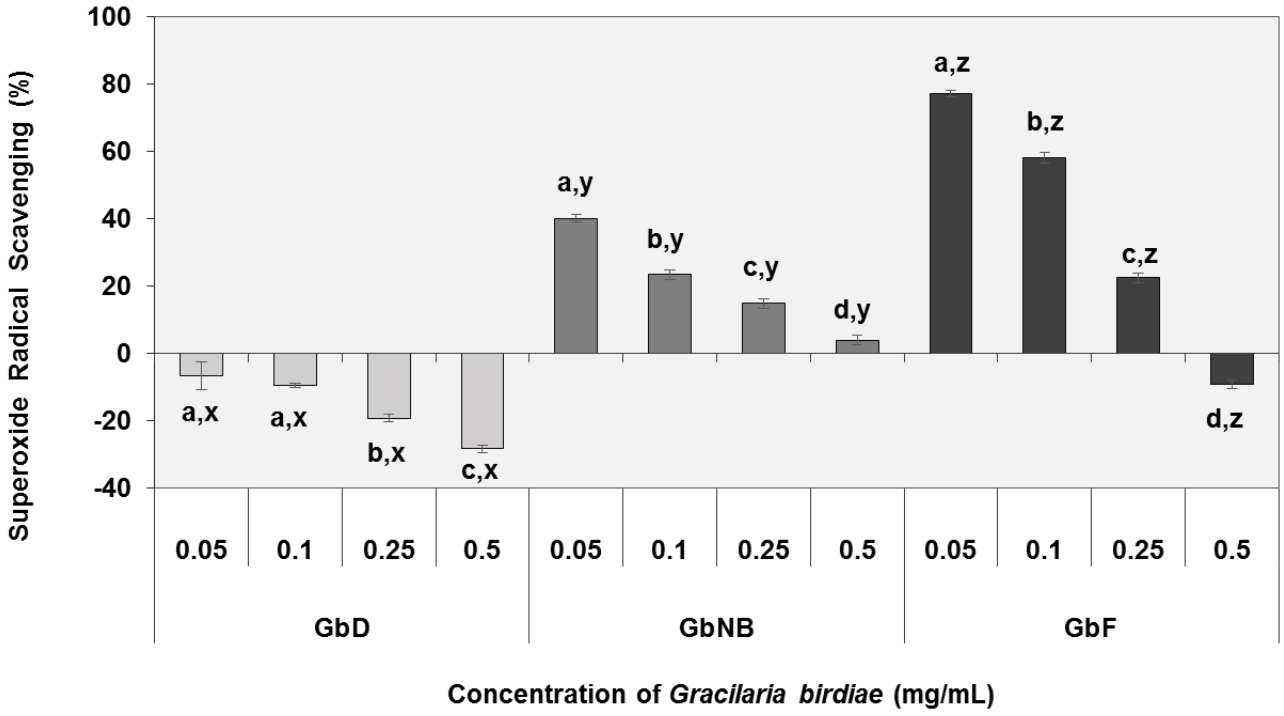


Figure 2. Superoxide radical scavenging extractions from the GbD, GbNB and GbF. Values are shown as mean ± standard deviation (SD) of triplicates. Different superscript letters denote statistical differences by Tukey's test ($P<0.05$).

3.1.3. Iron Chelating Ability

The chelating effect is very important since it inhibits the interaction between lipids and metals by forming insoluble metal complexes with ferrous ions. Furthermore, it is an effective way to eliminate the generation of hydroxyl radicals since it prevents iron from interacting with H_2O_2, thus preventing the decomposition of H_2O_2 and the formation of an even more damaging free radical. The iron-chelating capacity test measures the ability of antioxidants to compete with ferrozine in chelating ferrous ion. Activity is measured as the decrease in absorbance of the red Fe^{2+}/ferrozine complex. The plot of iron chelating capacity as a function of sample concentration is shown in Figura 3. The results revealed that GbD do not presented significative ferrous chelating capacity at 1.0 mg/mL, 1.5 mg/mL and 2.0 mg/mL. It was clear that the effect of the crude extracts

on the chelating capacity was dose dependent upon concentrations. The most active compounds were from GbNb and GbF with 66.80 and 60.89% of ferrous chelating, respectively, at 2 mg/mL.

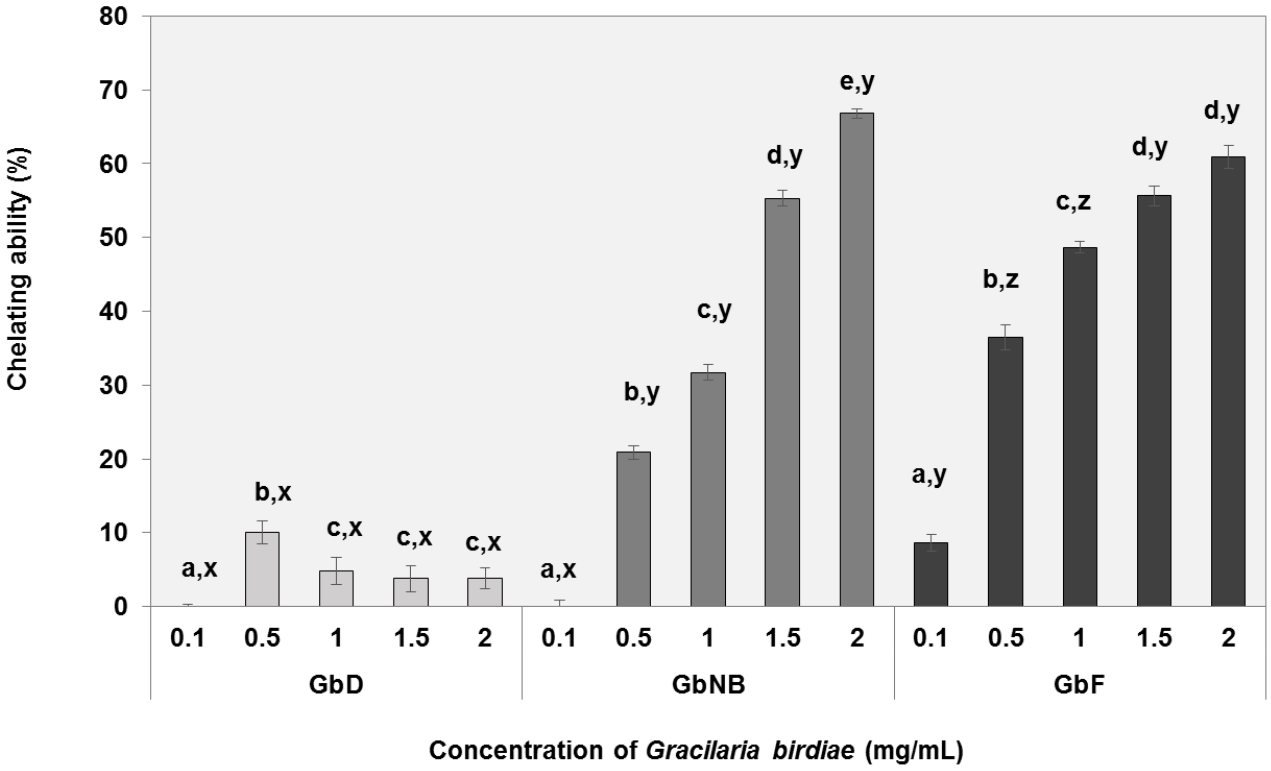


Figure 3. Chelating effect of crude extracts from *Gracilaria birdiae* on ferrous ions. Data are expressed as means standard deviation. Different letters indicates a significant difference between concentrations of individual algal crude extract by one-way Anova followed by Student-Newman-Keuls test ($p < 0.05$).

3.1.4. Copper Chelating Ability

The equilibrium of the concentration of copper ions in biological systems is crucial for the regulation of cellular functions. When an imbalance occurs in its concentration in the human body, this could lead to the development of severe health conditions such as osteoporosis, hypothyroidism, schizophrenia, premenstrual syndrome (Melo *et al.*, 2013). When this imbalance is caused by the increase in the concentration of copper, there is an increase in the production of reactive oxygen species, due in large part to Fenton (Mccord, 1985) and Haber-Weiss (Svingen *et*

al., 1979) reactions. In addition, through the Fenton reaction, the preformed lipid hydroperoxides (LOOH) are decomposed to form alkoxyl radicals (LO), strong oxidizing agents, which can propagate the chain reaction of lipid peroxidation (Melo *et al.*, 2013) or react with other cellular constituents. Consequently, the chelation of Cu^{2+} ions may be crucial for the prevention of the production of reactive species that damage the target biomolecules. Therefore, we have verified the chelating effect of copper ions, displayed by different concentrations of the fractions obtained from crude extracts of *Gracilaria birdiae* (Figure 4).

It can be observed from the data presented in Figure 6 that all crude extracts have copper chelating activity and in all cases the effect is dose dependent. It is worth noting that GbD (2.0 mg/mL) and GbF (2.0 mg/mL) didn't differ significally. However, GbNB (2.0 mg/mL, 76.34%) was more potent than GbD (2.0 mg/mL, 64.68%), for it or ached the plateau of its activity at a concentration lower than that of GBF (2.0 mg/mL, 63.01%). We were unable to identify any other studies that evaluated the effect of crude extract of other species such as copper chelators. Therefore, there is not yet enough data to make further observations on the activity of these crude extracts.

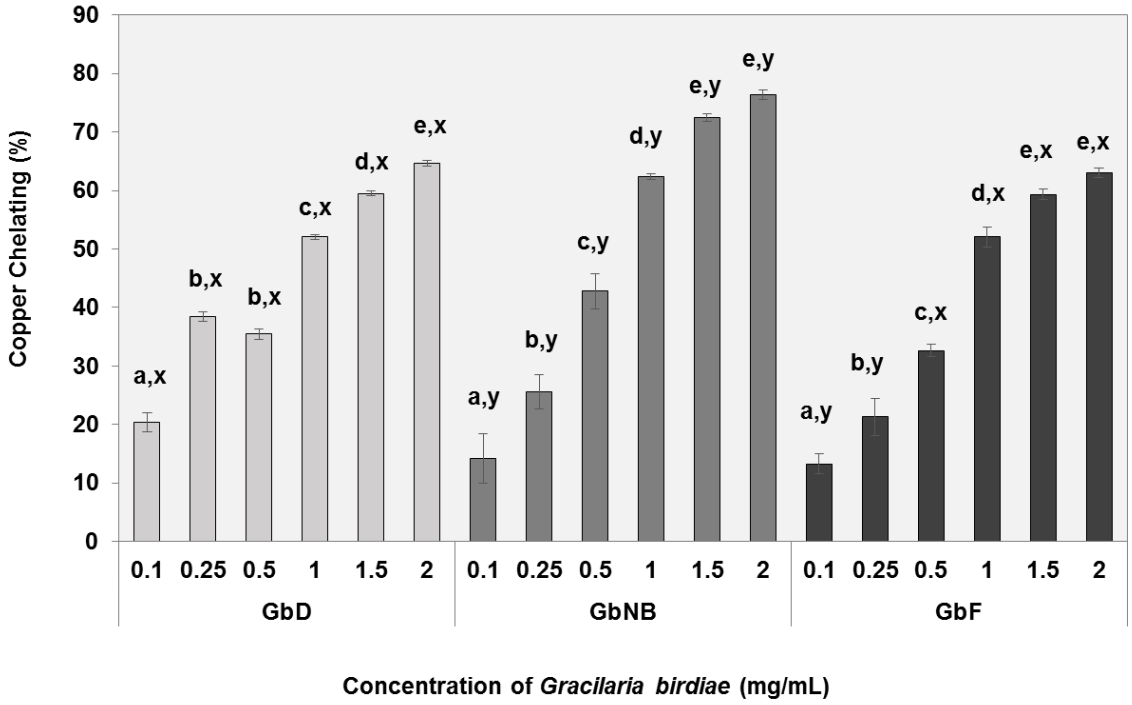


Figure 4. Chelating effect of crude extracts from *Gracilaria birdiae* on copper ions. Data are expressed as means standard deviation. Different letters indicates a significant difference between concentrations of individual algal crude extract by one-way Anova followed by Student-Newman-Keuls test ($p < 0.05$).

4. Conclusions

We demonstrated that crude extracts (GBF, GbNB and GbD) derived from the red alga *Gracilaria birdiae* have an interesting potential biotechnology. It was observed that GbD and GbF samples showed a great total antioxidant capacity in comparison to the GbNB extract. Samples from GbF showed higher superoxide ion scavenging activity. The most active compounds of ferrous chelating capacity were from GbNB and GbF. On the other hand the most active compounds of copper chelating capacity was GbNB. Studies of the bioactivity of algal components contribute to the body of knowledge about marine-derived compounds and their use in the pharmaceutical and chemical industries.

Acknowledgements

Research was supported by CAPES and CNPq, Brazil.

References

ANTON, A. Colorimetric Estimation of Aluminum with Pyrocatechol Violet. **Analytical Chemistry,** v. 32, n. 6, p. 725-726, 1960/05/01 1960. ISSN 0003-2700. Disponível em: < http://dx.doi.org/10.1021/ac60162a052 >.

BECKER, C. F. et al. Conformation of sulfated galactan and sulfated fucan in aqueous solutions: Implications to their anticoagulant activities. **Journal of Molecular Graphics and Modelling,** v. 26, n. 1, p. 391-399, 7// 2007. ISSN 1093-3263. Disponível em: < http://www.sciencedirect.com/science/article/pii/S1093326307000095 >.

COSTA, L. S. et al. Biological activities of sulfated polysaccharides from tropical seaweeds. **Biomedicine & Pharmacotherapy,** v. 64, n. 1, p. 21-28, 1// 2010. ISSN 0753-3322. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0753332209000432 >.

DE S.F-TISCHER, P. C. et al. Chemical structure and antiviral activity of carrageenans from Meristiella gelidium against herpes simplex and dengue virus. **Carbohydrate Polymers,** v. 63, n. 4, p. 459-465, 3/18/ 2006. ISSN 0144-8617. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0144861705004595 >.

FIDELIS, G. P. et al. Proteolysis, NaOH and ultrasound-enhanced extraction of anticoagulant and antioxidant sulfated polysaccharides from the edible seaweed, Gracilaria birdiae. **Molecules,** v. 19, n. 11, p. 18511-26, 2014. ISSN 1420-3049.

FREINBICHLER, W. et al. The detection of hydroxyl radicals in vivo. **J Inorg Biochem,** v. 102, n. 5-6, p. 1329-33, May-Jun 2008. ISSN 0162-0134 (Print)

0162-0134.

MCCORD, J. M. Oxygen-derived free radicals in postischemic tissue injury. **N Engl J Med,** v. 312, n. 3, p. 159-63, Jan 17 1985. ISSN 0028-4793 (Print)

0028-4793.

MELO, K. R. et al. Evaluation of sulfated polysaccharides from the brown seaweed Dictyopteris justii as antioxidant agents and as inhibitors of the formation of calcium oxalate crystals. **Molecules,** v. 18, n. 12, p. 14543-63, 2013. ISSN 1420-3049.

PLASTINO, E. M.; URSI, S.; FUJII, M. T. Color inheritance, pigment characterization, and growth of a rare light green strain of Gracilaria birdiae (Gracilariales, Rhodophyta). **Phycological Research,** v. 52, n. 1, p. 45-52, 2004. ISSN 1440-1835. Disponível em: < http://dx.doi.org/10.1111/j.1440-1835.2003.00324.x >.

QI, H. et al. Antioxidant activity of different molecular weight sulfated polysaccharides from Ulva pertusa Kjellm (Chlorophyta). **Journal of Applied Phycology,** v. 17, n. 6, p. 527-534, 2005// 2005. ISSN 1573-5176. Disponível em: < http://dx.doi.org/10.1007/s10811-005-9003-9 >.

ROCHA DE SOUZA, M. C. et al. Antioxidant activities of sulfated polysaccharides from brown and red seaweeds. **Journal of Applied Phycology**, Dordrecht**,** v. 19, n. 2, p. 153-160, 11/30

11/18/received08/06/accepted 2007. ISSN 0921-8971 1573-5176. Disponível em: < http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2668642/ >.

SOUZA, B. W. S. et al. Chemical characterization and antioxidant activity of sulfated polysaccharide from the red seaweed Gracilaria birdiae. **Food Hydrocolloids,** v. 27, n. 2, p. 287-292, Jun 2012. ISSN 0268-005X. Disponível em: < <Go to ISI>://WOS:000298639600003 >.

SVINGEN, B. A. et al. The mechanism of NADPH-dependent lipid peroxidation. The propagation of lipid peroxidation. **J Biol Chem,** v. 254, n. 13, p. 5892-9, Jul 10 1979. ISSN 0021-9258 (Print) 0021-9258.

TROELL, M. et al. Integrated mariculture: asking the right questions. **Aquaculture,** v. 226, n. 1–4, p. 69-90, 10/31/ 2003. ISSN 0044-8486. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0044848603004691 >.

VALKO, M. et al. Free radicals and antioxidants in normal physiological functions and human disease. **Int J Biochem Cell Biol,** v. 39, n. 1, p. 44-84, 2007. ISSN 1357-2725 (Print)

1357-2725.

WANG, J. et al. Synthesized phosphorylated and aminated derivatives of fucoidan and their potential antioxidant activity in vitro. **Int J Biol Macromol,** v. 44, n. 2, p. 170-4, Mar 1 2009. ISSN 0141-8130.

YANG, Y.-F. et al. Growth of Gracilaria lemaneiformis under different cultivation conditions and its effects on nutrient removal in Chinese coastal waters. **Aquaculture,** v. 254, n. 1–4, p. 248-255, 4/28/ 2006. ISSN 0044-8486. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0044848605006423 >.

YE, H. et al. Purification, antitumor and antioxidant activities in vitro of polysaccharides from the brown seaweed Sargassum pallidum. **Food Chemistry,** v. 111, n. 2, p. 428-432, 11/15/ 2008. ISSN 0308-8146. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0308814608004305 >.

CAPITULO 2

A ser submetido ao jornal

Marine drugs

(ISSN 1660-3397)

www.mdpi.com/journal/marinedrugs

CAPITULO 2: Evaluation of crude extracts from the red seaweed *Gracilaria birdiae* as inhibitors of the formation of Calcium Oxalate Crystals

Werlayne M. Santana[1]; Rafael B. G. Camara[2]; Douglas H. H. Andrade[1]; Jailma A. Lima[2]; Juliett F. X. Silva[3]; Dárlio I. A. Teixeira[4]; Hugo A. O. Rocha[2] e Ranilson S. Bezerra[1*]

[1] Laboratório de Enzimologia (LABENZ), Departamento de Bioquímica, Universidade Federal de Pernambuco (UFPE), Cidade Universitária, 50670-420, Recife-PE, Brasil; E-mails: werlaynemendes@gmail.com (W.M.S.); douglasvicencia@gmail.com (D.H.H.A.); ransoube@uol.com.br (R.S.B.)

[2] Laboratório de Biotecnologia de Polímeros Naturais (BIOPOL), Departamento de Bioquímica, Centro de Biociências, Universidade Federal do Rio Grande do Norte (UFRN), Natal, Rio Grande do Norte 59072-970, Brasil; E-mails: rafael_bgc@yahoo.com.br (R.B.G.C.); hugo@cb.ufrn.br (H.A.O.R.);

[3] Universidade Federal de Alagoas, campus Penedo; E-mail: juliettpesca@yahoo.com.br (J.F.X.S.);

[4] Escola agrícola de Jundiaí, UFRN; E-mail: darlioteixeira@gmail.com (D.I.A.T)

*Corresponding Author:

Ranilson S. Bezerra

Laboratório de Enzimologia (LABENZ), Departamento de Bioquímica e Laboratório de Imunopatologia Keizo Asami (LIKA), Universidade Federal de Pernambuco, Cidade Universitária, 50670-420, Recife-PE, Brazil.

Tel, +55 81 21268540; Fax, +55 81 21268576

e-mail: ransoube@uol.com.br

Evaluation of crude extracts from the red seaweed *Gracilaria birdiae* as inhibitors of the formation of Calcium Oxalate Crystals

Abstract: We investigated the effects of crude extract from the red seaweed *Gracilaria birdiae* (GbD, GbNB and GbF) on calcium oxalate crystallization, and chemical analysis its. To examine the effects of crude extracts on calcium oxalate crystallization, we monitored effect on crystal morphology of calcium oxalate mono, di and tri-hydrate crystals, using sodium citrate as a positive control. We analyzed chemical contents of crude extracts by determining its total sugar, sulfate, protein and total phenolics. The crystal morphology from GbD was 77.2% (COM), 18% (COD) and 4.9% (COT); GbNB was 89.6% (COM), 5.1% (COD) and 5.3% (COT) and GbF 0% (COM), 100% (COD) and 0% (COT). Regarding the use of *Gracilaria birdiae* front of normal and tumor cells she presented an excellent result in 24 h in relation to cell HEK-293 to (GbD, GbNB and GbF) in 48 and 72 hours the best performance was in the lower concentrations (0.1 and 0.25 mg / mL), and the GbNB showed the best results at all times, but the GbF also had an excellent result at all times in the lower concentration (0.1 mg / mL). Regarding the cells 786 GbD presented an excellent antiproliferative compound at 24 and 48 hours. GbNB a compound antiproliferative presented in time 24 and 48 hours and showed no GbF good results. These results suggest that GbF could be a candidate for treating urinary stones because of its ability to inhibit calcium oxalate crystallization.

Keywords: calcium oxalate crystallization; crude extracts; *Gracilaria birdiae*

1. Introduction

Marine algae are valuable sources of structurally diverse bioactive compounds. In recent years, there has been much interest in isolating novel bioactive compounds with beneficial effects on human health from marine resources. Marine red algae of the genus *Gracilaria* are a major agarophyte resource in the world and are cultivated for the phytocolloid industry or for integrated marine culture (Troell *et al.*, 2003). The red marine alga *Gracilaria birdiae* has a great economic impact in Brazil due to agar production (Plastino *et al.*, 2004). Moreover, sulfated polysaccharides from marine algae are known to exhibit many biological and physiological activities including anticoagulant, antiviral, anticancer, anti-inflammatory, and antioxidant (Souza *et al.*, 2012). Zhang et al. 2004 (Zhang *et al.*, 2004) reported that sulfated polysaccharides play important roles as free-radical scavengers and antioxidants, which can prevent oxidative damage in living organisms. Ox and calcium oxalate monohydrate (COM) induce the generation of free radicals, which are major mediators of the pathologic consequences of the formation of kidney stones (Das *et al.*, 2005).

Crystal-forming oxalate salts go through two physico-chemical phases: nucleation and aggregation. The crystal growth, which could be considered a third phase, also occurs. It has been suggested that ROS increase the amount of oxalate crystals since they modulate the process of nucleation, growth and crystal aggregation (Khan, 2013) and it has been confirmed that antioxidants such as vitamin E and ascorbic acid promote the reduction in the size of oxalate crystals and the resulting kidney injuries caused by them (Selvam, 2002). Therefore, sulfated polysaccharides could protect the renal tissue from the aggression caused by oxalate, and by another mechanism they could inhibit the formation of oxalate crystals, as demonstrated by Zhang and colleagues (Zhang *et al.*, 2012). These authors showed that polysaccharides from the Brown seaweed *Sargassum graminifolium* were able to inhibit the crystallization of calcium oxalate *in vitro*.

Oxalate crystals and other types of crystal are the causative agents of urinary lithiasis, or urolithiasis. This disease affects some 10% of the world population and 60% – 90% of the cases are caused primarily by calcium oxalate crystals (Lopez e Hoppe, 2010). The literature also shows that the probability of recurrence of these crystal formations is more than 60%, and despite advances in medical treatments, there are currently no satisfactory drugs for the treatment of urolithiasis (Atmani e Khan, 2000). There search underway for sources of molecules that can provide effective treatment of urolithiasis.

Gracilaria birdiae, red seaweed extensively distributed along the coasts of the Northeastern Brazil, is commonly used to agar extraction for cosmetic and pharmaceutical industry. To fully utilize this rich resource, it is meaningful evaluate new bioactive compounds of *G. birdiae*. The aim of the present study was to investigate the chemical properties of a crude extract from *G. birdiae* farmed, at natural banks and at drift and its effect on the crystallization of calcium oxalate in vitro.

2. Material e methods

2.1 Raw Material

The red seaweed, *Gracilaria birdiae* (farmed, natural bank and at drift) was collected in Rio do Fogo Beach (Rio Grande do Norte, Brazil, 5°16'16.61"S/35°22'54.29"W) by fishermen from the community; the seaweed was taken to the laboratory to cleaned to eliminate residue and epiphytes. After, it was dried at 50º C under ventilation in an oven and they were subsequently crushed. After, this material was homogenized in distilled water at concentrations of 8 mg/mL, agitation for 12 hours and filtered. These solutions were named GbF (extract from *G. birdiae* farmed), GbNB

(extract from *G. birdiae* of natural bank) and GbD (extract from *G. birdiae* at drift). At the end, the material was frozen at – 20º C for posterior lyophilization.

2.2 Chemical Characterization

Total sugars were estimated by the reaction of phenol-H_2SO_4 using L-fucose, as described by Dubois et al., 1956 (Dubois *et al.*, 1956). The sulfate content was determined according to the barium-gelatin method (Dodgson e Price, 1962) by using a standard curve of sodium sulfate. The protein content, in turn, was measured by using the modified Bradford 1976 (Bradford, 1976) method, with bovine serum albumin as standard. The total phenolics content was estimated by the Folin-Ciocalteu method (Taga *et al.*, 1984) to 6.0 ml of distilled water were added to 0.1 mL sample to a final concentration of 2.0 mg / mL and 0.5 ml of Folin-Ciocalteu method, followed by addition of 1.5 mL of Na_2CO_3 (20%). The final volume was adjusted to 10.0 ml. After incubation for 30 minutes at 25° absorbance was measured at 760 nm and total phenolic content was calculated using gallic acid as phenolic pattern.

2.3 Image Analysis Crystal Morphology

The crystals were induced to take shape in the presence or the absence of SP or sodium citrate 0.25 mM. After 30 min, the solutions were centrifuged (5000 × g) and the supernatant was discarded. The crystals were then suspended in 0.5 mL of water and a part of 0.1 mL was put on a histological blade and taken to a microscope. The crystal morphology was analyzed in 10 randomly selected fields at 60 × magnification. Images were captured from different fields. We performed three different experiments.

2.4 Cell Culture

The non-cancerous cell line of kidney cells (HEK-293) and a renal adenocarcinoma cell line (786-0) were purchased at the Federal University of Rio Grande do Norte (Department of biochemistry). HEK-293 cells were grown in culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) and 786-0 cells were grown in culture flasks in RPMI-1640 medium, both supplemented with 10% (v / v) fetal bovine serium.

2.5 MTT Assay

Hek-293 cells were grown in culture flasks in DMEM medium with 10% fetal bovine serium and 786-0 cell were grown in flasks in RPMI-1640 with 10% fetal bovine serium. Cells were plated into 96-well plates at a density of 5×103 cell/well and allowed to attach for overnight at 37 °C and 5% CO_2. In the antiproliferative assay, *Gracilaria birdiae* was added (100; 250; 500 or 1000 µg/mL). After the determinate time (24, 48 or 72 h) incubation, traces of *Gracilaria birdiae* were removed by washing the cells with PBS and fresh medium and 10 µL of 12 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dissolved in PBS was added to determine the effects of the sample on cell proliferation. The cells were then incubated for 4 h at 37 °C and 5% CO_2. To solubilize the reduced of MTT product, isopropanol (100 µL) containing 0.04 N HCl was added to each well and thoroughly mixed using a multichannel pipettor. Within 1 h of HCl-isopropanol addition, the absorbance at 570 nm was read using a Multiskan Ascent Microplate Reader (Thermo Labsystems, Franklin, MA, USA). The percent of cell proliferation was calculated as follows:

$$\text{Cell proliferation (\%)} = \frac{\text{Abs. 570 nm of sample}}{\text{Abs. 570 nm of control}} \text{ x } 100$$

3. Results and Discussion

3.1. Chemical analysis

Chemical analysis of crude extracts is given in Table 1. The red seaweed was washed with water at room temperature and was dried at 50º C under ventilation and subsequently crushed. Several studies showed that yield is associated to temperature. It is not known if the same happens with crude extracts. Maciel et al. (2008) (Maciel *et al.*, 2008) studied the structural characterization of cold extracted fraction of sulfated polysaccharide from *G. birdiae* and showed that the low yield might be due to the low extraction temperature. Melo et al. (2002) (Melo *et al.*, 2002) showed that the temperature is one important factor responsible for the yield of extraction.

Armisen (1995) (Armisen, 1995) showed that in general the yield of extraction of agar from *Gracilaria* species is very variable due to several factors, such as environmental conditions, seasonal variation, physiological factors and extraction methods. As can be seen in Table 1, the extraction sulfate showed a variation of 2.30% to 5.91%, being the highest percentage found in the GbF. When comparing the values recorded in the sulfate content of the crude extract from *G. birdiae* with those described for the sulfated polysaccharides of *G. birdiae* collected in the other region from Northeastern Brazil it, is clear that the percentage of sulfate of the GbF, GbNB, GbD presents a lower value than the polysaccharides of the *G. birdiae* seaweed since the latter's range was from 8.4% (Souza *et al.*, 2012). However, another recent study, conducted by Camara et al., (2011) (Barros Gomes Camara *et al.*, 2011), using the brown seaweed *C. cervicornis*, has shown fucans with sulfate levels around 2.8% and with *Dictyopteris. justii* (Melo *et al.*, 2013) showed sulfate content of the fractions ranged of 3.9% to 7.5%.

The total sugar from crude extracts from *Gracilaria birdiae* ranged from 24.73 % (GbF) to 32.49% (GbD). These values can be considered low when compared with the values found in fractions sulfated polysaccharides of Brown Seaweed *Dictyopteris justii* (59.6% - 80.4%) found by Melo et al. (2013) (Melo *et al.*, 2013) and *Sargassum filipendula* (41.4% - 66.0%) found by Costa et al., (2011) (Costa *et al.*, 2011). In addition, Souza et al., (2012) (Souza *et al.*, 2012) showed percentages of these compounds from 85.6%. Thus, it is clear that the amount of sulfate and total sugar content of seaweed may vary between the same species and according to the species of the studied algae.

As for contamination by proteins, the range was low, ranging from 0.07% to 0.26%. Similar results were found by Melo et al., (2013) (Melo *et al.*, 2013) and Souza et al., (2012) (Souza *et al.*, 2012).

Table 1. Chemical analysis from *Gracilaria birdiae.* Values are shown as mean ± standard deviation (SD) of triplicates. Different italic superscript letters denote statistical differences by Tukey's test ($P<0.05$).

Crude extract	Sulfate (%)	Protein (%)	Total Sugar (%)	phenolics content (%)
GbD	2,30 ± 0,07 ^{a}	0,12 ± 0,01 ^{a}	32,49 ± 4,58 ^{a}	0,53 ± 0,01 ^{a}
GbNB	1,79 ± 0,03 ^{b}	0,07 ± 0,01 ^{b}	26,44 ± 6,85 ^{a,b}	0,30 ± 0,02 ^{b}
GbF	5,91 ± 0,19 ^{c}	0,26 ± 0,01 ^{c}	24,73 ± 2,47 ^{b}	0,78 ± 0,02 ^{c}

3.2 Effect on Crystal Morphology

The crystals of calcium oxalate develops in three different hydrated forms: monohydrated (COM), dihydrated (COD), and trihydrated (COT). COM is the most thermodynamically stable phase, followed by tetragonal COD and then triclinic COT. COM and COD are the major forms found in

most urinary calculi (Yu *et al.*, 2005; Ouyang *et al.*, 2006). The COM form is found in large quantities in kidney stones, while COD is rarer. COT has a large thermodynamic instability, being seldom found within the stones. Figure 1A shows the crystals formed under control conditions. Under these conditions, three types of calcium oxalate crystals are formed, as described in the text of Figure 1. The observation of microscope (Figure 1, Figure 2) slides in ten different fields has demonstrated that 77.2 %; 89.6 % and 0% are of type COM (GbD, GbNB and GbF), respectively; 18 %; 5.1 % and 100% are of type COD (GbD, GbNB and GbF), respectively and 4.9 %; 5.3 % and 0% are of type COT (GbD, GbNB and GbF), respectively. In addition, the crystals formed in the presence of sodium citrate (13.47 µm – COM; 18.36 µm – COD; 9.09 µm – COT) are larger (Figure 1A) than those found than those found in GbD (5.99 µm – COM; 10.53 µm – COD; 5.38 µm – COT) and GbNB (5.15 µm – COM; 9.26 µm – COD; 4.54 µm – COT) and in GbF (0 µm – COM; 10,55 µm – COD; 0 µm – COT).

The change in the morphology of crystals as a result of the crude extract is quite visible. The more rounded geometry of the crystals indicates that they are more amorphous, which is caused by disruption of the crystal structure due to the possible presence of *Gracilaria birdiae* crude extract of which was associated with the crystals. This geometry has a smaller surface area (GbF) compared with COD crystals with sharp edges and points (control group), and the crystals have become smaller which facilitates the removal of these crystals body in urine (Ouyang *et al.*, 2006).

The COD shape, although unstable, is very common in the urine of healthy patients, which indicates that the urine naturally contains molecules that stabilize the COD shape preventing its transformation into a COM geometric shape. This characteristic was also observed with the presence of *Gracilaria birdiae*. Such stabilization has an efficient protective effect against urolithiasis since the crystals have a higher binding capacity to the cells of the renal tubule.

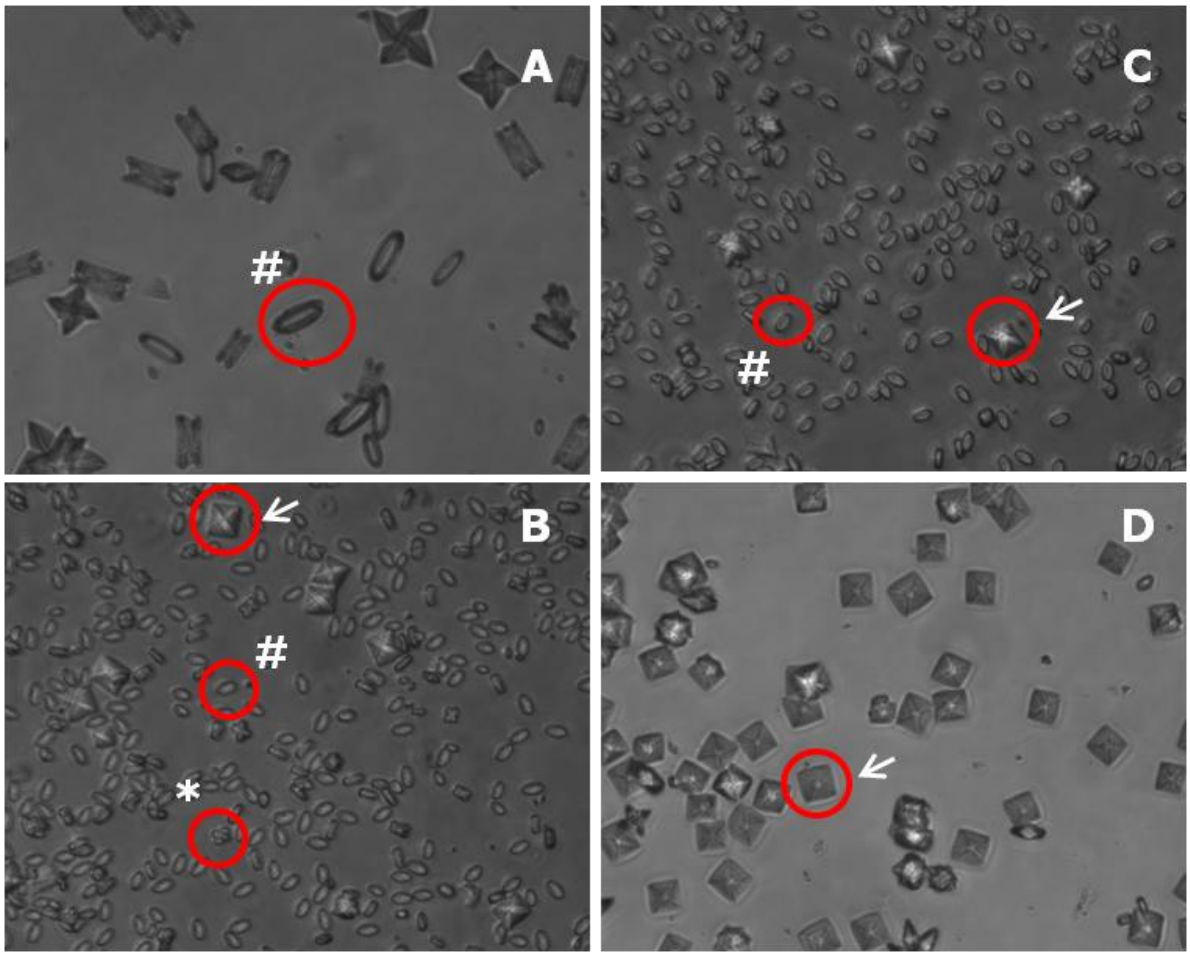


Figure 1. The CaOx crystals, the use of extracts from different sources observed under inversion ted microscope (60×). (A) Control Sodium Citrate (0.25 mM); (B) Formation of crystals with GbD; (C) Formation of crystals of GbNB and (D) formation crystals with the use of GbF. # (COM), arrows (COD), *(COT).

The data shows that the *Gracilaria. birdiae* extract stimulates the formation of crystals in some samples, but inhibits the aggregation, probably prevent large crystals are formed. To confirm this hypothesis, we check the size and morphology of calcium oxalate crystals formed in the presence of the extract of *G. birdiae* (figure 2).

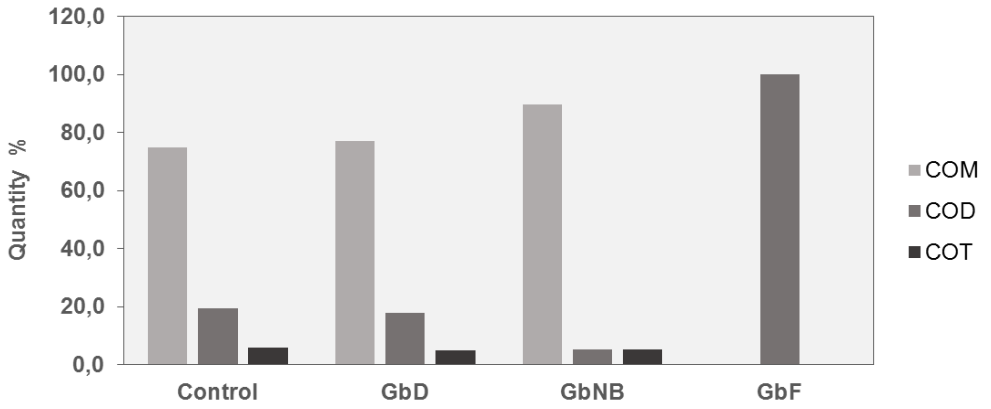


Figure 2. Average size of formed crystals.

3.3. Evaluating cytotoxic of *Gracilaria birdiae* in human renal cell and anti-proliferative activity on tumor cells

How *Gracilaria birdiae* module the formation of calcium oxalate crystals, became interested in checking their proliferative activity against cell lines normal kidney (HEK-293) and anti-proliferative in tumor cells (786-0). The GbD extract in HEK-293 cells, showed good viability at 24 hours incubation (100%) (figure 3), but over time the highest concentrations tends to kill the cell. However, at a concentration of 0.25 mg / mL at 48(figure 4) and 72 hours (figure 5), showed an inhibition of only (20%) in the sample becoming feasible. For 786-0 cells, the best result was in 24 hours incubation. Because it kills up to 55% of that cell.

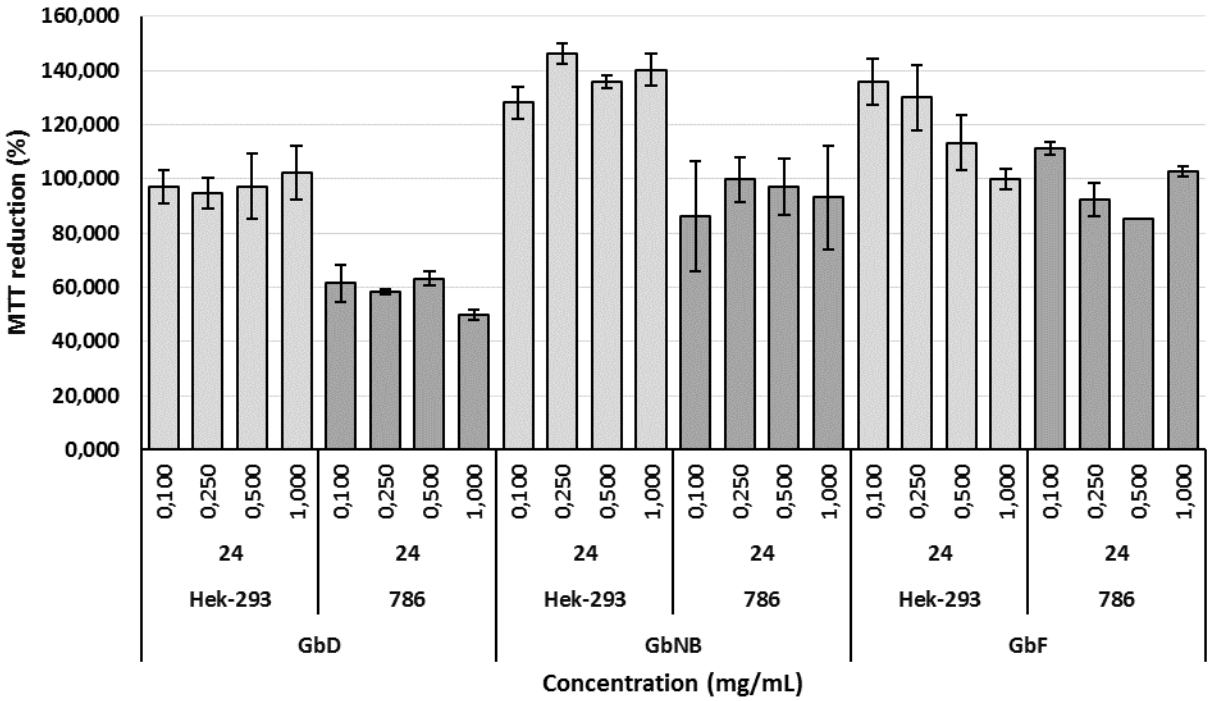


Figure 3. Influence of GbD, GbNB and GbF extracts on inhibition of cell proliferation of HEK-293 and anti-proliferative of 786-0 cells after 24h incubation.

The GbNB extract presented an excellent result for the HEK-293 cells (24, 48 and 72 hours) (figure 3, 4 and 5) and 786-0 cells showed a good results for only 1 mg / mL in 48 hours incubation.

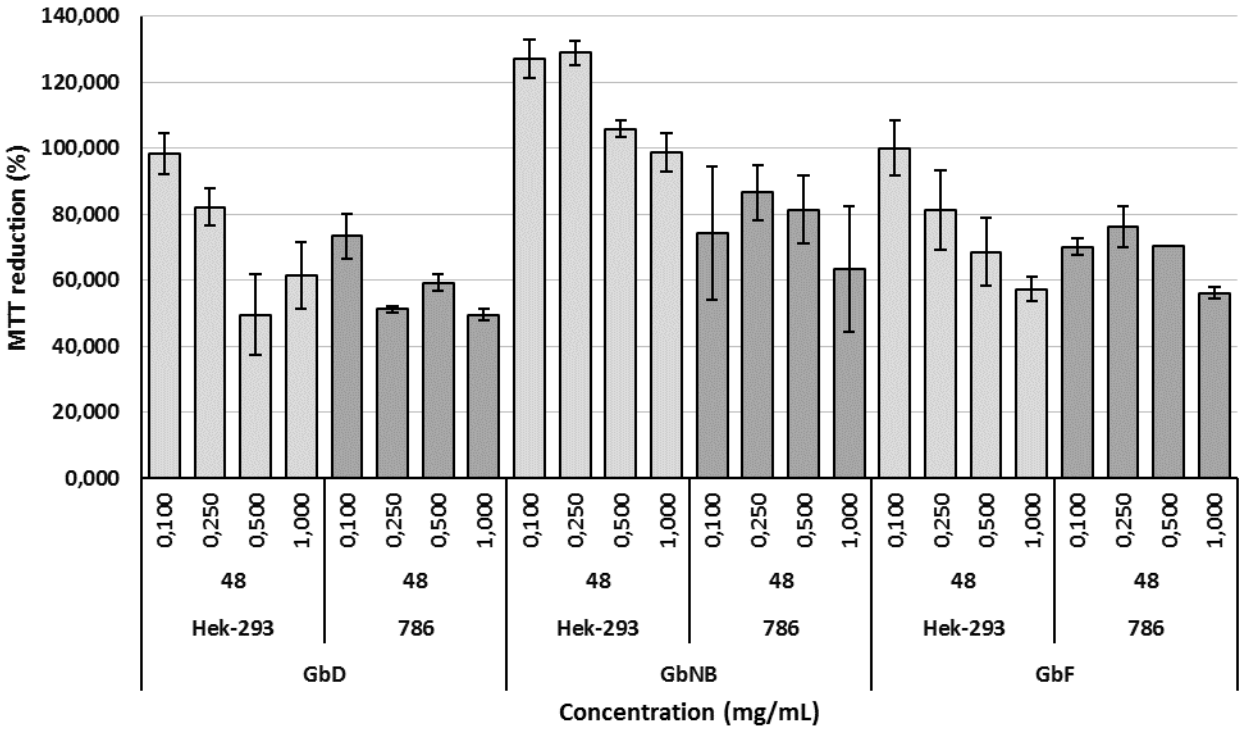


Figure 4. Influence of GbD, GbNB and GbF extracts on inhibition of cell proliferation of HEK-293 and anti-proliferative of 786-0 cells after 48 h incubation.

The GbF (figure 3, 4 and 5) extract showed a good result only in minor concentrations (0.1 to 0.25) at 24, 48 and 72 hours incubation and the cells 786-0 did not inhibit the proliferation of cells, and this negative result. As shown by these results the extract *Gracilaria birdiae* good as to investigate its potential as a potential compound for use in the treatment of urolithiasis. Experiments with animals should be performed in order to confirm this potential.

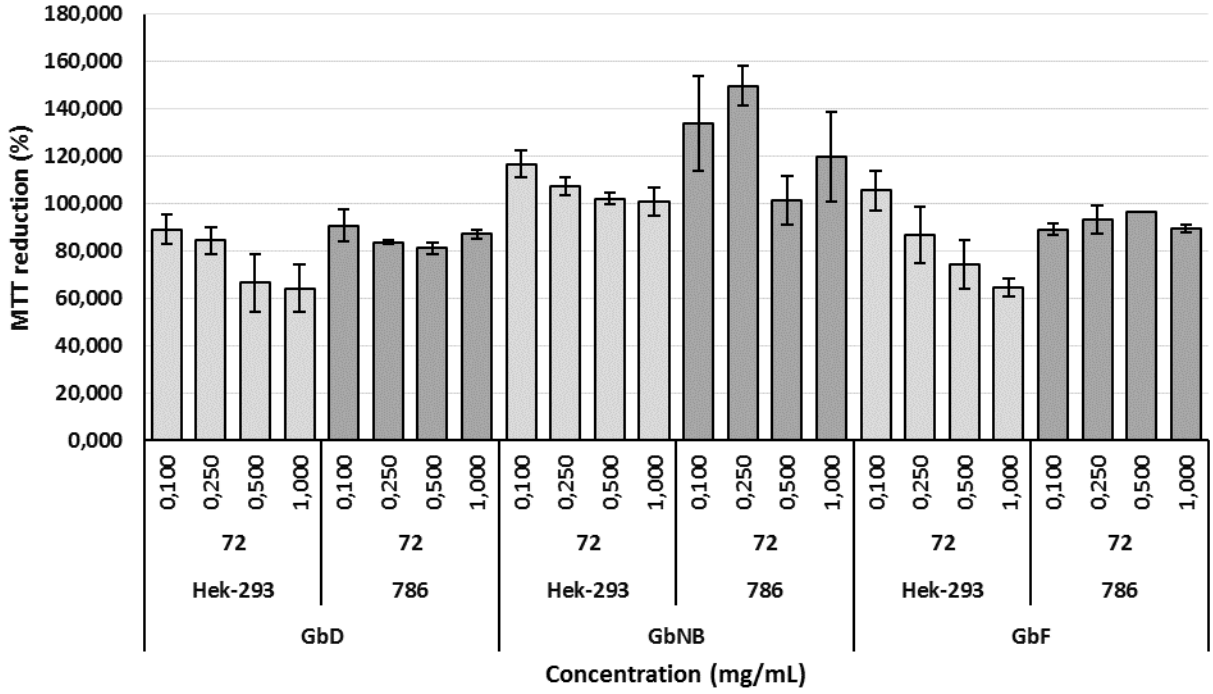


Figure 5. Influence of GbD, GbNB and GbF extracts on inhibition of cell proliferation of HEK-293 and anti-proliferative of 786-0 cells after 72 h incubation.

4. Conclusions

GbF show lower sulfate content than GbD and GbNB, was also the crude extract the better capacity to inhibit the crystallization of calcium oxalate. Regarding the use of *Gracilaria birdiae* front of normal and tumor cells she presented an excellent result in 24 h in relation to cell HEK-293 to (GbD, GbNB and GbF) in 48 and 72 hours the best performance was in the lower concentrations (0.1 and 0.25 mg / mL), and the GbNB showed the best results at all times, but the GbF also had an excellent result at all times in the lower concentration (0.1 mg / mL). Regarding the cells 786 GbD presented an excellent antiproliferative compound at 24 and 48 hours. GbNB a compound antiproliferative presented in time 24 and 48 hours and showed no GbF good results. This bioactive

compounds product is therefore, promising agents for possible application in the treatment of urolithiasis**.** Experiments with animals should be performed in order to confirm this potential.

References

ARMISEN, R. World-wide use and importance of Gracilaria. **Journal of Applied Phycology,** v. 7, n. 3, p. 231-243, 1995// 1995. ISSN 1573-5176. Disponível em: < http://dx.doi.org/10.1007/BF00003998 >.

ATMANI, F.; KHAN, S. R. Effects of an extract from Herniaria hirsuta on calcium oxalate crystallization in vitro. **BJU Int,** v. 85, n. 6, p. 621-5, Apr 2000. ISSN 1464-4096 (Print)

1464-4096.

BARROS GOMES CAMARA, R. et al. Heterofucans from the Brown Seaweed Canistrocarpus cervicornis with Anticoagulant and Antioxidant Activities. **Marine Drugs,** v. 9, n. 1, 2011. ISSN 1660-3397.

BRADFORD, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Analytical Biochemistry,** v. 72, n. 1–2, p. 248-254, 5/7/ 1976. ISSN 0003-2697. Disponível em: < http://www.sciencedirect.com/science/article/pii/0003269776905273 >.

COSTA, L. S. et al. Antioxidant and antiproliferative activities of heterofucans from the seaweed Sargassum filipendula. **Mar Drugs,** v. 9, n. 6, p. 952-66, 2011. ISSN 1660-3397.

DAS, I. et al. In vitro inhibition and dissolution of calcium oxalate by edible plant Trianthema monogyna and pulse Macrotyloma uniflorum extracts. **Journal of Crystal Growth,** v. 273, n. 3–4, p. 546-554, 1/3/ 2005. ISSN 0022-0248. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0022024804011704 >.

DODGSON, K. S.; PRICE, R. G. A note on the determination of the ester sulphate content of sulphated polysaccharides. **Biochemical Journal,** v. 84, n. 1, p. 106-110, 1962. ISSN 0264-6021

1470-8728. Disponível em: < http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1243628/ >.

DUBOIS, M. et al. Colorimetric Method for Determination of Sugars and Related Substances. **Analytical Chemistry,** v. 28, n. 3, p. 350-356, 1956/03/01 1956. ISSN 0003-2700. Disponível em: < http://dx.doi.org/10.1021/ac60111a017 >.

KHAN, S. R. Reactive oxygen species as the molecular modulators of calcium oxalate kidney stone formation: evidence from clinical and experimental investigations. **J Urol,** v. 189, n. 3, p. 803-11, Mar 2013. ISSN 0022-5347.

LOPEZ, M.; HOPPE, B. History, epidemiology and regional diversities of urolithiasis. **Pediatr Nephrol,** v. 25, n. 1, p. 49-59, Jan 2010. ISSN 0931-041x.

MACIEL, J. S. et al. Structural characterization of cold extracted fraction of soluble sulfated polysaccharide from red seaweed Gracilaria birdiae. **Carbohydrate Polymers,** v. 71, n. 4, p. 559-565, 3/7/ 2008. ISSN 0144-8617. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0144861707003463 >.

MELO, K. R. et al. Evaluation of sulfated polysaccharides from the brown seaweed Dictyopteris justii as antioxidant agents and as inhibitors of the formation of calcium oxalate crystals. **Molecules,** v. 18, n. 12, p. 14543-63, 2013. ISSN 1420-3049.

MELO, M. R. S. et al. Isolation and characterization of soluble sulfated polysaccharide from the red seaweed Gracilaria cornea. **Carbohydrate Polymers,** v. 49, n. 4, p. 491-498, 9/1/ 2002. ISSN 0144-8617. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0144861702000061 >.

OUYANG, J.-M.; ZHENG, H.; DENG, S.-P. Simultaneous formation of calcium oxalate (mono-, di-, and trihydrate) induced by potassium tartrate in gelatinous system. **Journal of Crystal Growth,** v. 293, n. 1, p. 118-123, 7/15/ 2006. ISSN 0022-0248. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0022024806004921 >.

PLASTINO, E. M.; URSI, S.; FUJII, M. T. Color inheritance, pigment characterization, and growth of a rare light green strain of Gracilaria birdiae (Gracilariales, Rhodophyta). **Phycological Research,** v. 52, n. 1, p. 45-52, 2004. ISSN 1440-1835. Disponível em: < http://dx.doi.org/10.1111/j.1440-1835.2003.00324.x >.

SELVAM, R. Calcium oxalate stone disease: role of lipid peroxidation and antioxidants. **Urol Res,** v. 30, n. 1, p. 35-47, Mar 2002. ISSN 0300-5623 (Print)

0300-5623.

SOUZA, B. W. S. et al. Chemical characterization and antioxidant activity of sulfated polysaccharide from the red seaweed Gracilaria birdiae. **Food Hydrocolloids,** v. 27, n. 2, p. 287-292, Jun 2012. ISSN 0268-005X. Disponível em: < <Go to ISI>://WOS:000298639600003 >.

TAGA, M. S.; MILLER, E. E.; PRATT, D. E. Chia seeds as a source of natural lipid antioxidants. **Journal of the American Oil Chemists' Society,** v. 61, n. 5, p. 928-931, 1984// 1984. ISSN 1558-9331. Disponível em: < http://dx.doi.org/10.1007/BF02542169 >.

TROELL, M. et al. Integrated mariculture: asking the right questions. **Aquaculture,** v. 226, n. 1–4, p. 69-90, 10/31/ 2003. ISSN 0044-8486. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0044848603004691 >.

YU, J.; TANG, H.; CHENG, B. Influence of PSSS additive and temperature on morphology and phase structures of calcium oxalate. **J Colloid Interface Sci,** v. 288, n. 2, p. 407-11, Aug 15 2005. ISSN 0021-9797 (Print)

0021-9797.

ZHANG, C.-Y. et al. Antioxidant Properties of Polysaccharide from the Brown Seaweed Sargassum graminifolium (Turn.), and Its Effects on Calcium Oxalate Crystallization. **Marine Drugs,** v. 10, n. 1, 2012. ISSN 1660-3397.

ZHANG, Q. et al. The structure of a sulfated galactan from Porphyra haitanensis and its in vivo antioxidant activity. **Carbohydrate Research,** v. 339, n. 1, p. 105-111, 1/2/ 2004. ISSN 0008-6215. Disponível em: < http://www.sciencedirect.com/science/article/pii/S0008621503004701 >.

CAPITULO 3

A ser submetido ao periódico

Molecules

(ISSN 1420-3049)

www.mdpi.com/journal/molecules

CAPITULO 3: Antiproliferative activity of macroalgae *Gracilaria birdiae* against HeLa, A549, B16-F10 and MRC5 cells.

Werlayne M. Santana[1]; Rafael B. G. Camara[2]; Douglas H. H. Andrade[1]; Rafael D. S. Azevedo[1]; Juliett F. X. Silva[3]; Dárlio I. A. Teixeira[4]; Hugo A. O. Rocha[2] e Ranilson S. Bezerra[1*]

[1] Laboratório de Enzimologia (LABENZ), Departamento de Bioquímica, Universidade Federal de Pernambuco (UFPE), Cidade Universitária, 50670-420, Recife-PE, Brasil; E-mails: werlaynemendes@gmail.com (W.M.S.); douglasvicencia@gmail.com (D.H.H.A.); rafaeldavidbio@gmail.com (R.D.S.A.); ransoube@uol.com.br (R.S.B.)

[2] Laboratório de Biotecnologia de Polímeros Naturais (BIOPOL), Departamento de Bioquímica, Centro de Biociências, Universidade Federal do Rio Grande do Norte (UFRN), Natal, Rio Grande do Norte 59072-970, Brasil; E-mails: rafael_bgc@yahoo.com.br (R.B.G.C.); hugo@cb.ufrn.br (H.A.O.R.);

[3] Universidade Federal de Alagoas, campus Penedo; E-mail: juliettpesca@yahoo.com.br (J.F.X.S.);

[4] Escola agrícola de Jundiaí, UFRN; E-mail: darlioteixeira@gmail.com (D.I.A.T)

*Corresponding Author:

Ranilson S. Bezerra

Laboratório de Enzimologia (LABENZ), Departamento de Bioquímica e Laboratório de Imunopatologia Keizo Asami (LIKA), Universidade Federal de Pernambuco, Cidade Universitária, 50670-420, Recife-PE, Brazil.

Tel, +55 81 21268540; Fax, +55 81 21268576

e-mail: ransoube@uol.com.br

Antiproliferative activity of macroalgae *Gracilaria birdiae* against tumor cell lines Hela, A549 and B16-F10 and normal cells MRC5

Abstract: The antiproliferative activity and cytotoxicity of red macroalgae *Gracilaria birdiae* in different farmed sources (GbD), natural bank (GbNB) and at drift (GbF) against tumor cells lines Hela, A549 and B16-F10 and normal cells MRC5 was studied. A crude extract dissolved in water was used, concentrations ranging from 0.1, 0:25, 0.5, 1.0 mg/ml with 24, 48 and 72 hours of incubation using the MTT assay (3- (4,5-dimethylthiazol-2yl) -2,5-diphenyl bromide tetrazolina). The GbD extract inhibited the proliferation of Hela cells, reaching a maximum inhibition (40%) after 48 and 72 hours of incubation, GbNB inhibited 20% of cells after 48 hours incubation and GbF had a 40% inhibition at 1.0 mg / ml after 48 hours incubation. GbD extract the cells A549 after 24 hours of incubation showed an inhibition of 20% at 0.5 and 1.0 mg/ml, and at 48 and 72 h of incubation inhibited 40% (with 0.25, 0.5 and 1.0 mg / ml) in proliferation cells. The GbNB extract did not show inhibition of cell proliferation and GbF showed a 30% inhibition after 24 hours incubation with 0.5 and 1.0 mg/mL, inhibited 50% after 48h incubation with 0.5 and 1.0 mg / ml and 40% after 72h incubation with 1.0mg / ml. The GbD extract inhibited 20% with 0.1, 0.25 and 0.5 mg / ml after 24 and 72 hours of incubation while the concentration (1.0 mg/mL) inhibited 50% by 72 h. GbNB and GbF inhibited 20% after incubation for 72 hours. The GbD extract MRC5 cells had cytotoxicity to cells after incubation used, while GbNB and GbF extract did not inhibit their proliferation after 24 and 48 hours. Therefore, you can affirm that *G. birdae* extracts has antiproliferative activity and this property dependent on seaweed cultivation mode.

Keywords: Cell lines tumor; crude extracts; *Gracilaria birdiae;* antiproliferative.

1. Introduction

Cancer is a generic term for a large group of diseases that can affect any part of the body. Other terms used are malignant tumours and neoplasms. One defining feature of cancer is the rapid creation of abnormal cells that grow beyond their usual boundaries, and which can then invade adjoining parts of the body and spread to other organs, the latter process is referred to as metastasizing. Metastases are the major cause of death from cancer. Cancers figure among the leading causes of morbidity and mortality worldwide, with approximately 14 million new cases and 8.2 million cancer related deaths in 2012 (WHO, 2015).

Due to the increasing incidence of cancer in both developing and developed countries the chemotherapy is still the standard treatment method and its severe side effects cannot be ignored (Moussavou et al., 2014). Especially when it affects normal cells causing serious damage to healthy tissues and organs. This has necessitated the search for alternative anticancer drugs having better efficacy with minimum side-effects (Shaik et al., 2015).

. Various bioactive compounds derived from plants and animals has demonstrated great potential as clinical drugs for inhibition of cell proliferation. (Ali et al., 2001; Casas et al., 2016; Sayari et al., 2016; Zheng et al., 2016). Indeed the evaluation of biological compounds with antiproliferative activity plays an important role in the control and prevention of cancer. Interestingly, seaweed has been a good source of bioactive compounds with antioxidant and antiproliferative properties (Yuan and Walsh, 2006).

Until now, more than 2400 marine bioactive compounds have been isolated from seaweeds of subtropical and tropical populations (Manilal et al., 2009). Findings evidenced that seaweeds possess antiviral, antibacterial, antifungal and antitumor activity (Harada et al., 1997) enabling viable applications for food industry and pharmaceuticals. However, these biological activities might depend on the species and methods of cultivation and collection.

Gracilaria birdiae, is red seaweed extensively distributed along the coasts of the Northeastern Brazil, is commonly used to agar extraction for cosmetic and pharmaceutical industry pelo mundo. *G. birdae* antioxidant, anti-coagulant, anti-inflammatory potential (Brito et al., 2014; Fidelis et al., 2014b; Souza et al., 2012) it has shown promising possibilities for specific therapeutic approaches minimizing the side effects of synthetic chemical for treating cancer and tumors. However, changes in bioactive compounds that have been reported seaweed for environmental and interspecific variations. (Barufi et al., 2015; Ursi et al., 2003). This characteristic may be an interferent in the biological properties recognized for this aquatic organism.

Based on this, this present study aims check for antiproliferative and proliferative activity of the extracts GbD, GbNB and GbF against tumor cell line uterine colon (HeLa), lung adenocarcinoma (A549), skin (B16-F10) and a cell line normal lung (MRC5).

2. Material e methods

2.1 Raw Material

The red seaweed, *Gracilaria birdiae* (farmed, natural bank and at drift) was collected in Rio do Fogo Beach (Rio Grande do Norte, Brazil, 5°16'16.61"S/35°22'54.29"W) by fishermen from the community; the seaweed was taken to the laboratory to cleaned to eliminate residue and epiphytes. After, it was dried at 50º C under ventilation in an oven and they were subsequently crushed. After, this material was homogenized in distilled water at concentrations of 8 mg/mL, agitation for 12 hours and filtered. These solutions were named GbF (extract from *G. birdiae* farmed), GbNB (extract from *G. birdiae* of natural bank) and GbD (extract from *G. birdiae* at drift). At the end, the material was frozen at – 20º C for posterior lyophilization.

Cell Culture

The non-cancerous cell line of kidney cells (HEK-293) and lung (MRC5) and a tumor cell lines of renal adenocarcinoma (786-0) and lung (A549) were purchased from the Federal University of Rio do Norte (Department of biochemistry). HEK-293 cells, MRC5 and A549 were grown in culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) and 786-0 cells were grown in culture flasks in RPMI-1640 medium, both supplemented with 10% (v/v) fetal bovine serum.

MTT Assay

MRC-5, HEK-293 and A549 cells were grown in culture flasks in DMEM medium with 10% fetal bovine serum and 786-0 cell were grown in flasks in RPMI-1640 with 10% fetal bovine serum. Cells were plated into 96-well plates at a density of 5×103 cell/well and allowed to attach for overnight at 37 °C and 5% CO_2. In the antiproliferative assay, *Gracilaria birdiae* extracts was added (100; 250; 500 or 1000 mg/mL). After the determinate time (24, 48 or 72 h) incubation, traces of *Gracilaria birdiae* were removed by washing the cells with PBS and fresh medium and 10 µL of 12 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dissolved in PBS was added to determine the effects of the sample on cell proliferation. The cells were then incubated for 4 h at 37 °C and 5% CO_2. To solubilize the reduced of MTT product, isopropanol (100 µL) containing 0.04 N HCl was added to each well and thoroughly mixed using a multichannel pipettor. Within 1 h of HCl-isopropanol addition, the absorbance at 570 nm was read using a Multiskan Ascent Microplate Reader (Thermo Labsystems, Franklin, MA, USA). The percent of cell proliferation was calculated as follows:

$$\text{Cell proliferation (\%)} = \frac{\text{Abs. 570 nm of sample}}{\text{Abs. 570 nm of control}} \text{ x } 100$$

3. Results and discussion

Hela

We evaluated the effect of the extracts GbD, GbNB and GbF on the proliferation of tumor cells and normal **(Figure 1)**. The cells used were (HeLa, A549, MRC5 and B16-F10*).* No is observed a dose-dependent effect for *G. birdae* extracts, however, all the extracts showed antiproliferative activity during the time intervals or concentrations used.

Inhibition of GbD within the first 24 shows the antiproliferative efficacy of this extract process. After GbD extract inhibited the proliferation of Hela cells, reaching a maximum inhibition (40%) after 48 and 72 hours of incubation, GbNB inhibited 20% of cells after 48 hours incubation and GbF had a 40% inhibition at 1.0 mg / ml after 48 hours incubation. This inhibition to HeLa cells can be related to biological activity of sulfated polysaccharides, as already shown for various seaweed.

The inhibitory activity recorded in this study was higher than the activities of algae *C. cupresoides, C. sertularioides* described by (Costa et al., 2010) at concentration of 2.0 mg/mL. Extracts of 1.0 mg/ml of edible seaweed *L. setchellii, M. integrifolia, N. leutkeana* has antiproliferative activity to lower GBD, GbNB and GBF (Yuan and Walsh, 2006).

Thus, it is possible to assume that *GbD, GbNB and GbF* has the ability to inhibit cancers. What associated with potential anti-inflammatory effects (Coura et al., 2015), anticoagulants and antioxidants (Fidelis et al., 2014a) demonstrate that *G. birdiae* can combine multiple positives in response to conditions. Finally, it is possible to observe that GbNB extracts and GBF after 72 hours favored the proliferation of HeLa cells. This result as well as demonstrating the ineffectiveness these extracts, alert to the opposite effect to hypothesize.

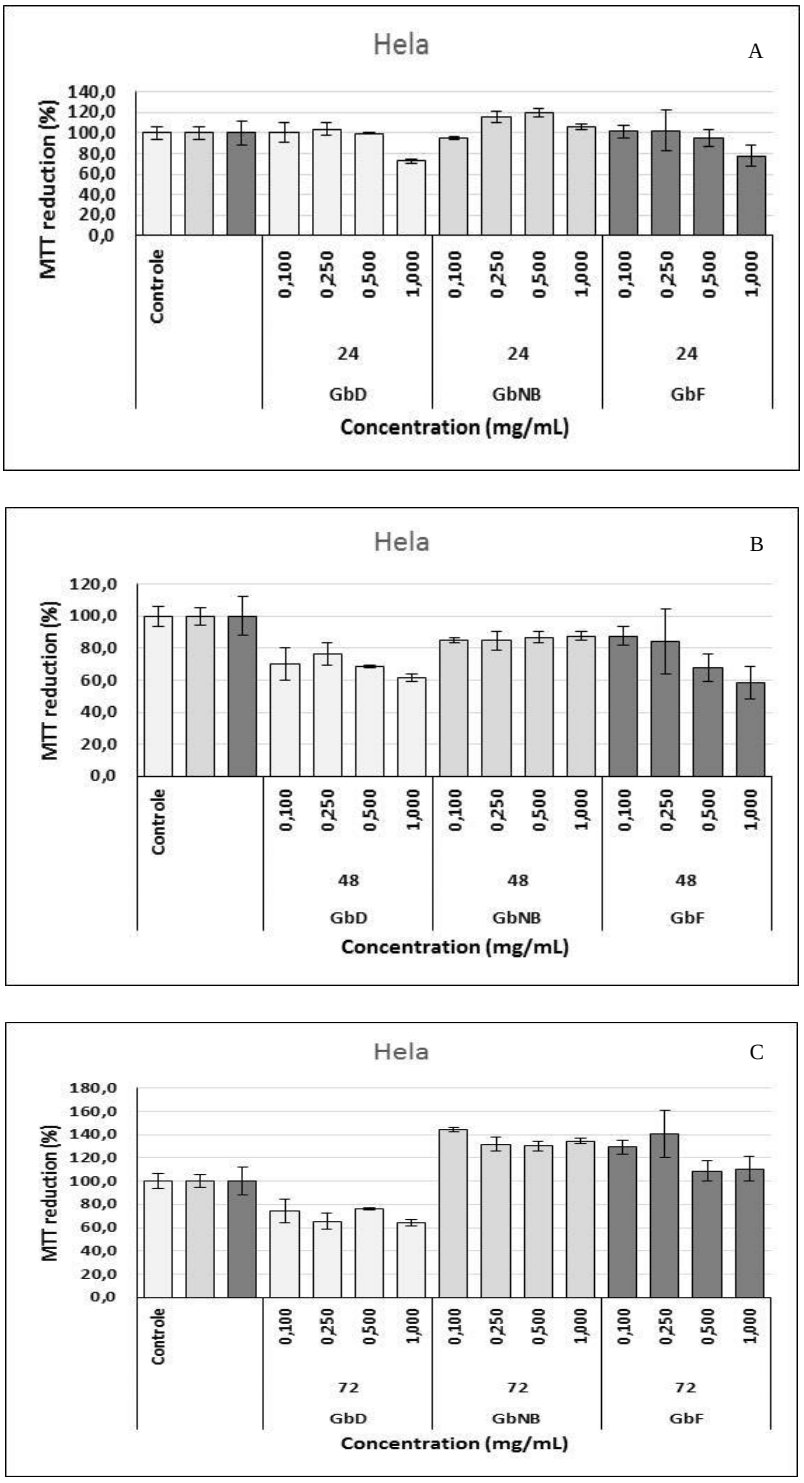


Figure 1. Influence (%) of GbD, GbNB and GbF extracts on inhibition of proliferative of HeLa cells after 24 (A), 48 (B) e 72h (C) incubation.

Cell A549

Antiproliferative activity of seaweed against A549 cells has enabled the identification of biomolecules with high potential anticancer **(Figure 2)**. GbD extract the cells A549 after 24 hours of incubation showed an inhibition of 20% at 0.5 and 1.0 mg/ml, and at 48 and 72 h of incubation inhibited 40% (with 0.25, 0.5 and 1.0 mg/ml) in proliferation cells, demonstrating dose and time dependent effect for GbD. The GbNB extract did not show inhibition of cell proliferation and GbF showed a 30% inhibition after 24 hours incubation with 0.5 and 1.0 mg/mL, inhibited 50% after 48h incubation with 0.5 and 1.0 mg / ml and 40% after 72h incubation with 1.0mg/ml.

Green, brown and red seaweed has the potential antiproliferative against A549 cells (Murugan and Iyer, 2013). Several biological components of these algae has antiproliferative activity against cancer cells. The IC_{50} registered for GbF against A549 is also observed for other species of the genus, such as *Gracilaria edulis* (Sakthivel et al., 2016). Beyond *G. edulis*, antiproliferative activity of biological components of seaweed against A549 has been evaluated for brown seaweed *Cystoseira sedoides, Sargassum plagiophyllum*, *Turbinaria ornata*, *Kappaphycus alvarezii*, *Acanthophora spicifera* and *Gracilaria corticata* (Mhadhebi et al., 2011; Murugan and Iyer, 2014; Sakthivel et al., 2016; Suresh et al., 2013). All satisfactory results using small concentrations of the biological components.

Is argued that main components responsible for inhibitory activity present in *G. edulis* is phytol, the acetate fraction to *C. sedoides* and sulfated polysaccharides to *Sargassum plagiophyllum*. Emphasis components that have little or no effect on normal cells which maximizes the potential of an alternative therapy against tumors.

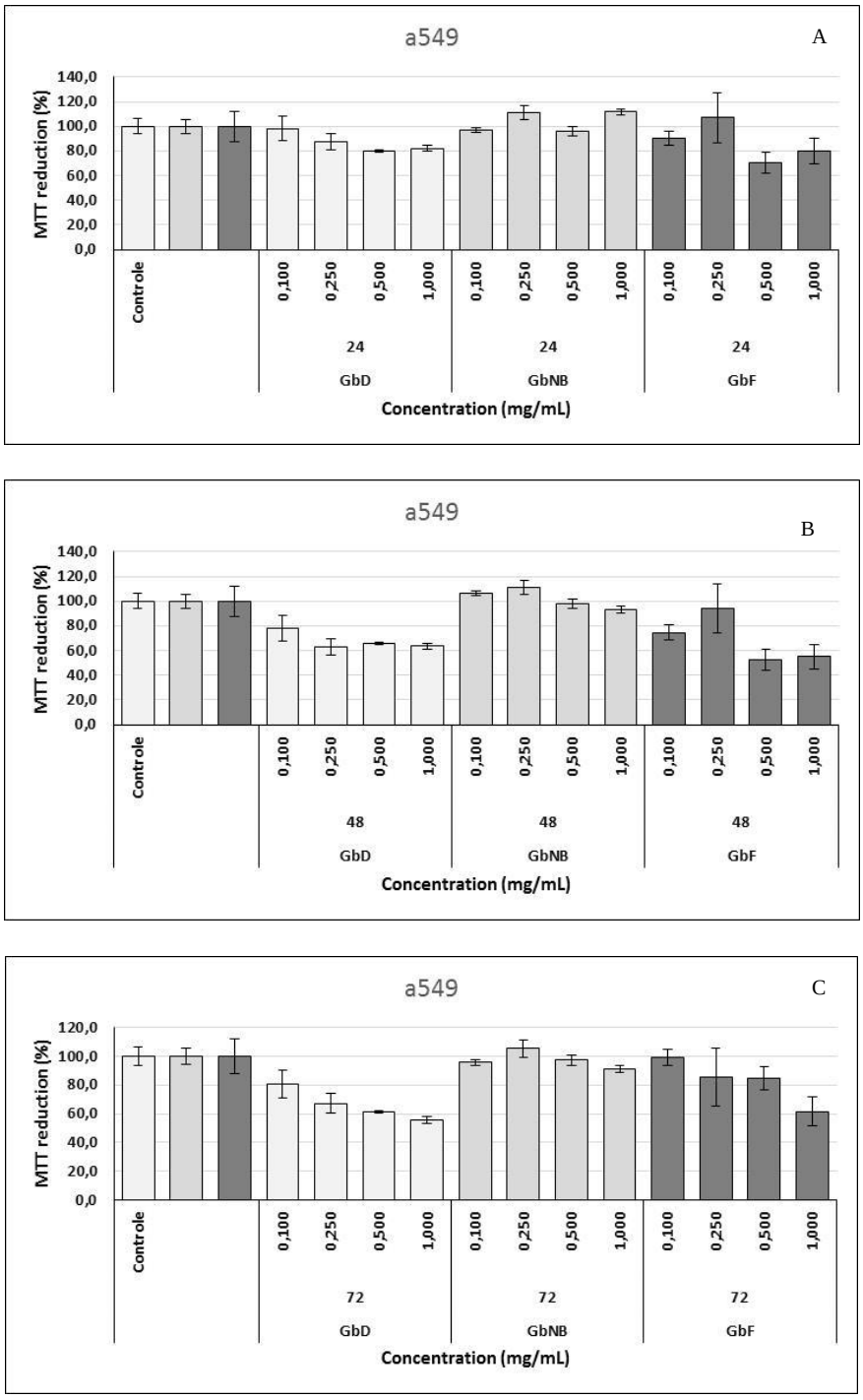


Figure 2. Influence of GbD, GbNB and GbF extracts on inhibition of proliferative of A549 cell after 24 (A), 48 (B) e 72h (C) incubation.

Cell B16-F10

Antiproliferative effect to melanoma B16-F10 it was evaluated for the same extracts of *G. birdae* (GbD, GbNB and GbF) **(Figure 3)**. The GbD extract inhibited 20% with 0.1, 0.25 and 0.5 mg / ml after 24 and 72 hours of incubation while the concentration (1.0 mg/mL) inhibited 50% by 72 h. What demonstrates a time-dependency ratio for the extract GbD. GbNB and GbF inhibited 20% after incubation for 72 hours.

Antiproliferative effect of B16-F10 cells it has been extensively studied using several synthetic drugs such as paclitaxel, pentoxifylline and theophylline (Gude et al., 2001; Lentini et al., 2010). However, the use of bioactive natural compounds have had similar effects minimizamdo side effects on normal cells. Positive effects are observed for plant extracts, with IC_{50} para 250µg/mL against B16-F10 until 300µg/mL for HeLa and A2780 cells (Diaconeasa et al., 2015). The bioavailability for obtaining the GbD is an advantage compared obtaining of plant extracts.

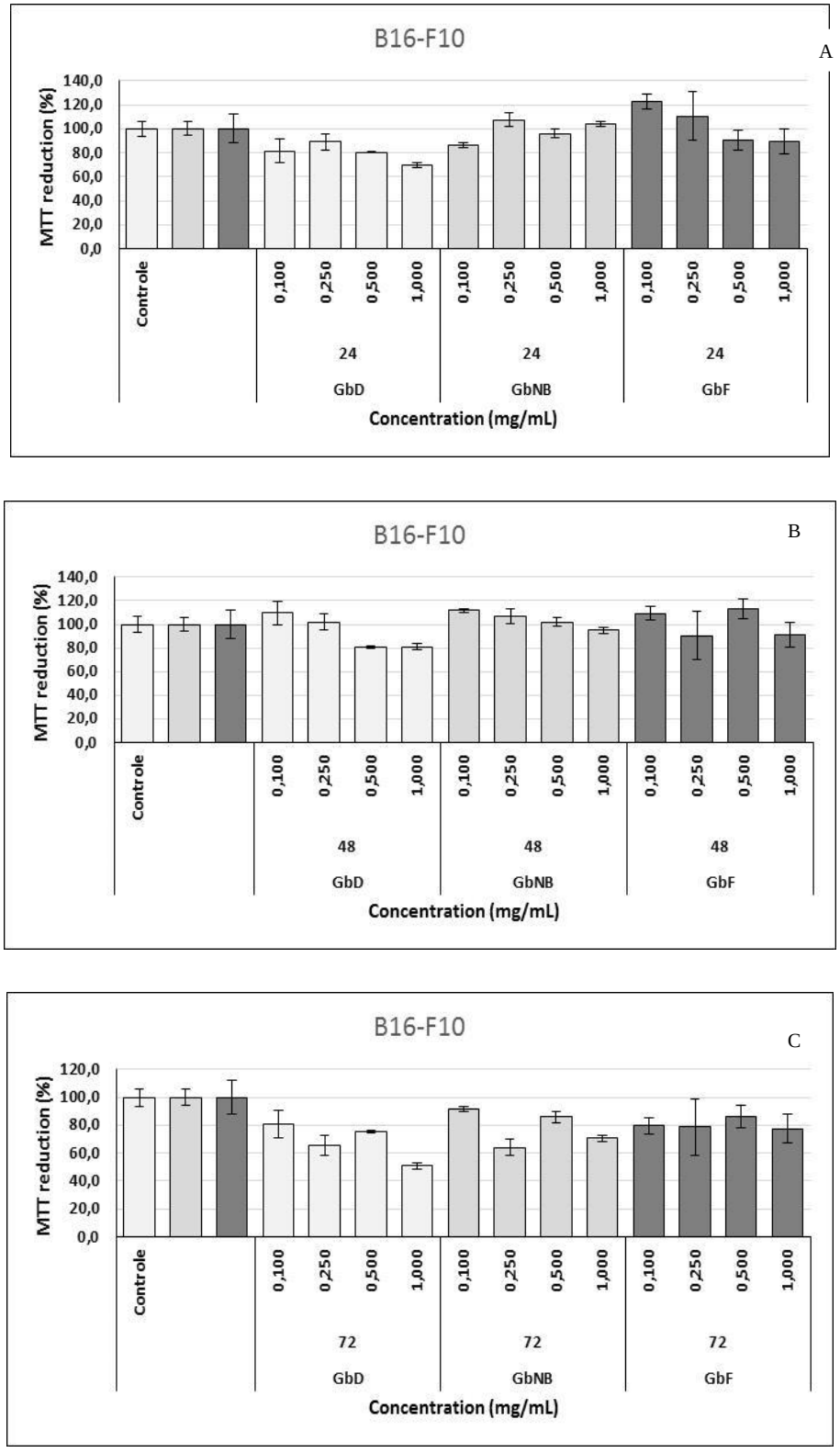
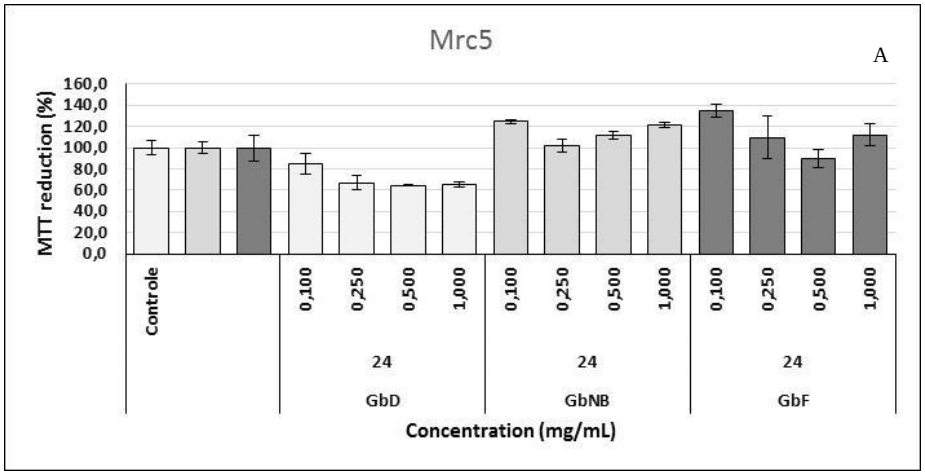
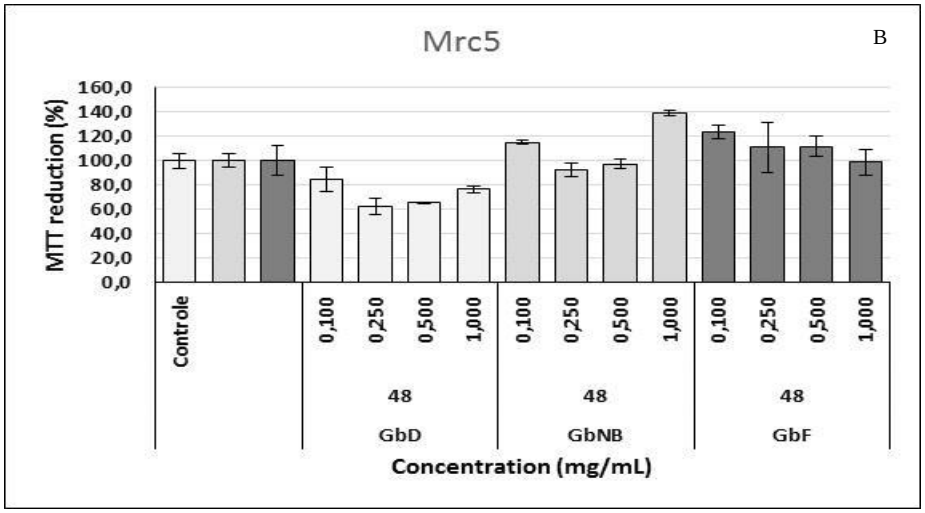


Figure 3. Influence of GbD, GbNB and GbF extracts on inhibition of proliferative of B-16-F10 cell after 24 (A), 48 (B) and 72 (C) hours of incubation.

Cell MRC-5

The GbD extract MRC-5 cells had cytotoxicity to cells after incubation used, while GbNB and GbF extract did not inhibit their proliferation after 24 and 48 hours **(Figure 4)**. This result show possibilits to GbNB and GbF use for proliferation inhibiton with minimal damage to normal cells. 0.5mg/mL of GbD extratct concentration show bioactivity lower than 30% MRC-5 cell line. (Figure 4 – C).





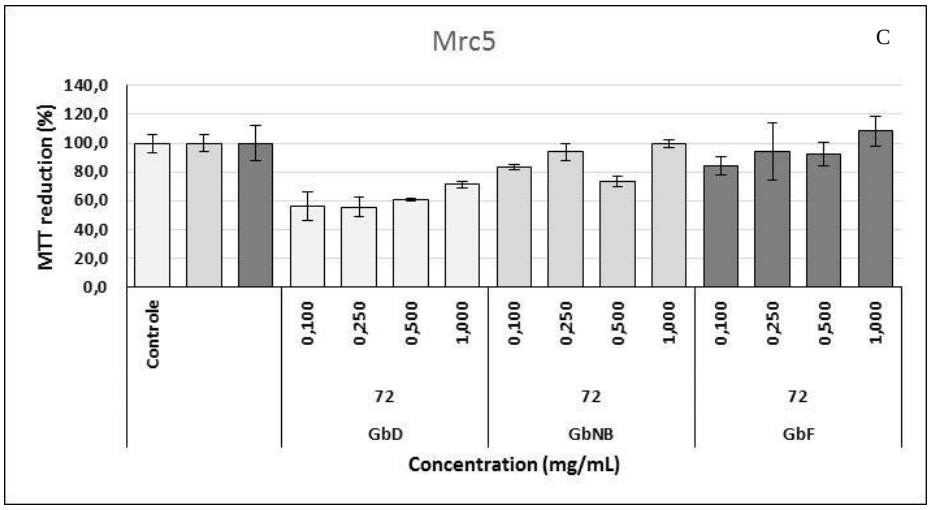


Figure 4. Influence of GbD, GbNB and GbF extracts on cytotoxic of cell proliferation of Mrc5 after 24, 48 e 72h incubation.

CONCLUSION

The GbD, GbNB and GbF extract showed a favorable outcome for the samples adenocarcinomas, and the GbD extract showed lower cytotoxicity on the growth of normal cells. More detailed study should be performed *in vivo* to identify, isolate and assess the specific compounds which are responsible for the cytotoxic and antiproliferative activity for confirmation that potential. Falar sobre as diferenças entre as algas relacionadas as metodologias de coleta, sobre como isso pode Furthermore, it is possible to assume that different sampling methodology can mask results and damage of identity alternative therapies and / or compounds with a high probability to minimize side effects of current chemotherapeutics.

References

World Health Organization. 2015. Cancer. Available online: http://www.who.int/mediacentre/factsheets/fs297/en (accessed on 27 march 2016).

Ali, M. S., A. H. Banskota, Y. Tezuka, I. Saiki, and S. Kadota, 2001, Antiproliferative activity of diarylheptanoids from the seeds of Alpinia blepharocalyx: Biological & Pharmaceutical Bulletin, v. 24, p. 525-528.

Barufi, J. B., F. L. Figueroa, and E. M. Plastino, 2015, Effects of light quality on reproduction, growth and pigment content of Gracilaria birdiae (Rhodophyta: Gracilariales): Scientia Marina, v. 79, p. 15-24.

Brito, T. V., J. Neto, R. S. Prudencio, J. A. Batista, J. S. C. Junior, R. O. Silva, A. X. Franco, K. S. Aragao, P. M. G. Soares, M. Souza, L. S. Chaves, A. L. P. Freitas, J. V. R. Medeiros, and A. L. R. Barbosa, 2014, Sulfated-polysaccharide fraction extracted from red algae Gracilaria birdiae ameliorates trinitrobenzenesulfonic acid-induced colitis in rats: Journal of Pharmacy and Pharmacology, v. 66, p. 1161-1170.

Casas, M. P., V. Rodriguez-Hermida, P. Perez-Larran, E. Conde, M. T. Liveri, D. Ribeiro, E. Fernandes, and H. Dominguez, 2016, In vitro bioactive properties of phlorotannins recovered from hydrothermal treatment of Sargassum muticum: Separation and Purification Technology, v. 167, p. 117-126.

Costa, L. S., G. P. Fidelis, S. L. Cordeiro, R. M. Oliveira, D. A. Sabry, R. B. G. Câmara, L. T. D. B. Nobre, M. S. S. P. Costa, J. Almeida-Lima, E. H. C. Farias, E. L. Leite, and H. A. O. Rocha, 2010, Biological activities of sulfated polysaccharides from tropical seaweeds: Biomedicine & Pharmacotherapy, v. 64, p. 21-28.

Coura, C. O., R. B. Souza, J. A. G. Rodrigues, E. D. O. Vanderlei, I. W. F. de Arajo, N. A. Ribeiro, A. F. Frota, K. A. Ribeiro, H. V. Chaves, K. M. A. Pereira, R. M. S. da Cunha, M. M. Bezerra, and N. M. B. Benevides, 2015, Mechanisms Involved in the Anti-Inflammatory Action of a Polysulfated Fraction from Gracilaria cornea in Rats: Plos One, v. 10, p. 18.

Diaconeasa, Z., L. Leopold, D. Rugina, H. Ayvaz, and C. Socaciu, 2015, Antiproliferative and antioxidant properties of anthocyanin rich extracts from blueberry and blackcurrant juice: Int J Mol Sci, v. 16, p. 2352-65.

Fidelis, G. P., R. B. Camara, M. F. Queiroz, M. S. Santos Pereira Costa, P. C. Santos, H. A. Rocha, and L. S. Costa, 2014a, Proteolysis, NaOH and ultrasound-enhanced extraction of anticoagulant and antioxidant sulfated polysaccharides from the edible seaweed, Gracilaria birdiae: Molecules, v. 19, p. 18511-26.

Fidelis, G. P., R. B. G. Camara, M. F. Queiroz, M. Costa, P. C. Santos, H. A. O. Rocha, and L. S. Costa, 2014b, Proteolysis, NaOH and Ultrasound-Enhanced Extraction of Anticoagulant and Antioxidant Sulfated Polysaccharides from the Edible Seaweed, Gracilaria birdiae: Molecules, v. 19, p. 18511-18526.

Gude, R. P., M. M. Binda, A. L. Boquete, and R. D. Bonfil, 2001, Inhibition of endothelial cell proliferation and tumor-induced angiogenesis by pentoxifylline: Journal of Cancer Research and Clinical Oncology, v. 127, p. 625-630.

Harada, H., T. Noro, and Y. Kamei, 1997, Selective antitumor activity in vitro from marine algae from Japan coasts: Biol Pharm Bull, v. 20, p. 541-6.

Lentini, A., C. Tabolacci, P. Mattioli, B. Provenzano, and S. Beninati, 2010, Antitumor Activity of Theophylline in Combination with Paclitaxel: A Preclinical Study on Melanoma Experimental Lung Metastasis: Cancer Biotherapy and Radiopharmaceuticals, v. 25, p. 497-503.

Mhadhebi, L., A. Laroche-Clary, J. Robert, and A. Bouraoui, 2011, Antioxidant, anti-inflammatory, and antiproliferative activities of organic fractions from the Mediterranean brown seaweed Cystoseira sedoides: Canadian Journal of Physiology and Pharmacology, v. 89, p. 911-921.

Moussavou, G., D. H. Kwak, B. W. Obiang-Obonou, C. A. Maranguy, S. D. Dinzouna-Boutamba, D. H. Lee, O. G. Pissibanganga, K. Ko, J. I. Seo, and Y. K. Choo, 2014, Anticancer effects of different seaweeds on human colon and breast cancers: Mar Drugs, v. 12, p. 4898-911.

Murugan, K., and V. V. Iyer, 2013, Differential growth inhibition of cancer cell lines and antioxidant activity of extracts of red, brown, and green marine algae: In Vitro Cellular & Developmental Biology-Animal, v. 49, p. 324-334.

Murugan, K., and V. V. Iyer, 2014, ANTIOXIDANT AND ANTIPROLIFERATIVE ACTIVITIES OF EXTRACTS OF SELECTED RED AND BROWN SEAWEEDS FROM THE MANDAPAM COAST OF TAMIL NADU: Journal of Food Biochemistry, v. 38, p. 92-101.

Sakthivel, R., S. Muniasamy, G. Archunan, and K. P. Devi, 2016, Gracilaria edulis exhibit antiproliferative activity against human lung adenocarcinoma cell line A549 without causing adverse toxic effect in vitro and in vivo: Food & Function, v. 7, p. 1155-1165.

Sayari, N., A. Sila, B. E. Abdelmalek, R. Ben Abdallah, S. Ellouz-Chaabouni, A. Bougatef, and R. Balti, 2016, Chitin and chitosan from the Norway lobster by-products: Antimicrobial and anti-proliferative activities: International Journal of Biological Macromolecules, v. 87, p. 163-171.

Souza, B. W. S., M. A. Cerqueira, A. I. Bourbon, A. C. Pinheiro, J. T. Martins, J. A. Teixeira, M. A. Coimbra, and A. A. Vicente, 2012, Chemical characterization and antioxidant activity of sulfated polysaccharide from the red seaweed Gracilaria birdiae: Food Hydrocolloids, v. 27, p. 287-292.

Suresh, V., N. Senthilkumar, R. Thangam, M. Rajkumar, C. Anbazhagan, R. Rengasamy, P. Gunasekaran, S. Kannan, and P. Palani, 2013, Separation, purification and preliminary characterization of sulfated polysaccharides from Sargassum plagiophyllum and its in vitro anticancer and antioxidant activity: Process Biochemistry, v. 48, p. 364-373.

Ursi, S., M. Pedersen, E. Plastino, and P. Snoeijs, 2003, Intraspecific variation of photosynthesis, respiration and photoprotective carotenoids in Gracilaria birdiae (Gracilariales : Rhodophyta): Marine Biology, v. 142, p. 997-1007.

Yuan, Y. V., and N. A. Walsh, 2006, Antioxidant and antiproliferative activities of extracts from a variety of edible seaweeds: Food and Chemical Toxicology, v. 44, p. 1144-1150.

Zheng, L. Y., X. L. Zhao, P. Zhang, C. D. Chen, S. J. Liu, R. Q. Huang, M. Q. Zhong, C. J. Wei, and Y. L. Zhang, 2016, Hemocyanin from Shrimp Litopenaeus vannamei Has Antiproliferative Effect against HeLa Cell In Vitro: Plos One, v. 11, p. 15.

4. CONSIDERAÇÕES FINAIS

O potencial farmacológico da macroalga vermelha *Gracilaria birdiae* foi identificado através da avaliação química do composto do extrato bruto que apresentou diferentes mecanismos antioxidantes *in vitro*.

O extrato foi considerado eficiente a partir do momento que foi observada a inibição da agregação de cristais de oxalato de cálcio, além da diminuição na quantidade e morfologia dos mesmos. Além disso, o extrato não apresentou citotoxicidade para células renais em algumas concentrações e tempso de incubação, além de inibir sua proliferação em células tumorais.

O efeito da atividade antiproliferativa para linhagens de células tumorais observado durante os estudos estimula o interesse na investigação do potencial farmacológico desse composto.

Outro ponto importante que pode ser levado em consideração é a possibilidade de geração de renda para comunidades extrativistas que poderão investir no cultivo de algas, para atender uma demanda de mercado.

Contudo, faz-se necessário, estudos *in vivo* para melhor avaliação das propriedades do extrato bruto dessas diferentes fontes da *Gracilaria birdiae*.

REFERÊNCIAS BIBLIOGRÁFICAS

AGUILAR-ROSAS, R.; Marcos-Ramirez, R.; Lobo-Niembro, J. M. & Zertuche Gonzalez, J. A. 1993. Seasonal variation of reproductive and vegetative phases of Gracilaria pacifica Abbott, in Estero de Punta Banda, Baja California, Mexico. Ciencias Marinas 19: 219-228.

ALMEIDA, C. L. F., Falcão, H. S., Lima, G. R. M., Montenegro, C. A., Lira, N. S., Athayde-Filho, P. F., Rodrigues, L. C., Souza, M. F. V., Barbosa-Filho, J. M., Batista, L. M. Bioactivities from marine algae of the genus Gracilaria. International Journal of Molecular Sciences, v. 12, p. 4540 – 4573, 2011.

BARNHAM, K. J.; MASTERS, C. L.; BUSH, A. I. Neurodegenerative diseases and oxidative stress. Nature Reviews: Drug Discovery, [S.l.], v. 3, p. 205-214, 2004.

BARUFI, J.B. 2004. Efeitos da luz na reprodução, no crescimento e no conteúdo pigmentar de Gracilaria birdiae (Gracilariales, Rhodophyta). Dissertação. Univ. S. Paulo. São Paulo. 112p.

BELLORIN, A. M., Oliveira, M. C., Oliveira, E. C. Phylogenyand systematics of the marine algal family Graciariaceae (Gracilariales, Rhodophyta) based on small subunit rDNA and its sequences on Atlantic and Pacific species. Journal of Phycology, v. 38, p. 551 – 563, 2002.

BEZERRA, A. F., Marinho-Soriano, E. Cultivation of the red seaweed Gracilaira birdiae (Gracilariales, Rhodophyta) in tropical waters of northeast brazil. Biomass and Bioenergy, v. 34, p. 1813 – 1817, 2010.

BRAY F, Jemal A, Grey N, Ferlay J, Forman D. Global cancer transitions according to the Human Development Index (2008–2030): a populationbased study. The Lancet Oncology 2012; 13(8):790–801.Rang et al., 200

CACHAT F, Barbey F, Daudon M. Medical treatment of urinary lithiasis. Rev Med Suisse Romande 2004 ;124 (8):461-4.

CARDOZO KHM, Marques LG, Carvalho MV, Carignan MO, Pinto E, Marinho-Soriano E, Colepicolo P (2011) Analyses of photoprotective compounds in red algae from the Brazilian coast. Rev Bras Farmacogn 21:202–208.

CARVALHO, L.R. & ROQUE, N.F. 2000. Halogenated and/or sulphated phenols from marine macroalgae. Química Nova 23:757-764.

CHAOYUAN. W. 1990. Training Manual On Gracilaria Culture And Seaweed processing In China, Zhanjiang Fisheries College, Regional Seafarming Resources Atlas. FAO/UNDP Region al Seafarming Development and Demonstration Project (RAS/86/024), January 1990. (Atlas series No. 1). 83 pp.

COE, Fredric L.; PARKS, Joan H.; FAVUS, Murray J. Diet and calcium: the end of an era. Annals of internal medicine, v. 126, n. 7, p. 553-555, 1997.

COSTA, V.L. 2005. Diversidade intraespecífica em gametófitos de Gracilaria birdiae (Gracilariales, Rhodophyta): efeitos fisiológicos da concentração de nitrato no meio de cultura. Tese. Univ. S. Paulo. São Paulo. 100p

CUZZOCREA, S.; RILEY, D. P.; CAPUTI, A. P.; SALVEMINI, D. Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. Pharmacological reviews, 53(1), 135-159. 2001

DE CASABIANCA M. L., Marinho-Soriano E. & LAUGIER T. 1997. Growth of Gracilaria bursapastoris in a mediterranean lagoon: Thau, France. Botanica Marina, 40: 29-37.

DEGANELLO, Sergio. Interaction between nephrocalcin and calcium oxalate monohydrate: a structural study. Calcified tissue international, v. 48, n. 6, p. 421428, 1991.

DEVASAGAYAM, T. et al. Free radicals and antioxidants in human health: current status and future prospects. Japi, v. 52, p. 794-804, 2004.

EKNOYAN, Garabed. History of urolithiasis. Clinical reviews in bone and mineral metabolism, v. 2, n. 3, p. 177-185, 2004.

ESPINOZA-AVALOS J., 2005. Fenología de macroalgas marinas. Hidrobiologica 15: 109-122.

FANG, Yun-Zhong; YANG, Sheng; WU, Guoyao. Free radicals, antioxidants, and nutrition. Nutrition, v. 18, n. 10, p. 872-879, 2002.

FAO. The State of World Fisheries and Aquaculture 2014. Rome. 223 pp., 2014.

FERNANDES, D. R. P., V. P. de Oliveira, and Y. Y. Valentin, 2014, Seaweed biotechnology in Brazil: six decades of studies on natural products and their antibiotic and other biological activities: Journal of Applied Phycology, v. 26, p. 1923-1937.

FERREIRA, A. L. A.; MATSUBARA, L. S. Radicais livres: conceitos, doenças relacionadas, sistema de defesa e estresse oxidativo. Revista da Assossiação Médica Brasileila, v. 43, n. 1, p. 61-68, 1997

FERREIRA, L.B. 2008. Diversidade intraespecífica em Gracilaria domingensis (Gracilariales, Rhodophyta): estudos fisiológicos na interpretação do polimorfismo de cor. Tese. Univ. S. Paulo. São Paulo. 200p.

FONG-NGERN, Kedsarin et al. Large-scale identification of calcium oxalate monohydrate crystal-binding proteins on apical membrane of distal renal tubular epithelial cells. Journal of Proteome Research, v. 10, n. 10, p. 4463-4477, 2011.

GIULIETTI, A. M.; HARLEY, R. M.; DE QUEIROZ, L. P.; WANDERLEY, M. G. L.; VAN DEN BERG, C. Biodiversidade e conservação das plantas no Brasil. Megadiversidade, v. 1, n. 1, p. 52-61, 2005.

GOMES, J. et al. Urolitíase e cólica renal. Perspectiva terapêutica em Urologia. Acta medica portuguesa, v. 15, n. 5, p. 369-80, 2002.

GRASES, F. et al. Effects of phytic acid on renal stone formation in rats.Scandinavian journal of urology and nephrology, v. 32, n. 4, p. 261-265, 1998.

GRESSLER, V., YOKOYA, N. S., FUJII, M. T., COLEPICOLO, P., MANCINI-FILHO, J., TORRES, R. P., PINTO, E. Lipid, fatty acid, protein, amino acid and ash contents in four Brazilian red algae species. Food Chemistry, v. 120, p. 585–590, 2010.

GUIMARÃES, M. 2000. Aspectos fisiológicos de Gracilaria domingensis (Gracilairales, Rhodophyta): subsídios para a compreensão da manutenção do polimorfismo pigmentar. Tese. Univ. S. Paulo. São Paulo. 88p.

GUIRY, M. D. Gracilariaceae. In: GUIRY, M. D.; GUIRY, G. M. AlgaeBase. World-wide eletronic publicatin, National University of Ireland, Galway, 2011.

HALLIWELL, Barry. Reactive oxygen species and the central nervous system. In: Free Radicals in the Brain. Springer Berlin Heidelberg, 1992. p. 21-40.

HALLIWELL B. Antioxidants in human health and desease. Annu Rev Nutr. 1996; 16; 3350.

HOLDT, S. L.; KRAAN, S. Bioactive compounds in seaweed: functional food applications and legislation. Journal of Applied Phycology, v. 23, p. 543-597, 2011.

ITO, N.; FUKUSHIMA, S.; TSUDA, H. Carcinogenicity and modification of the carcinogenic response by BHA, BHT, and other antioxidants. Critical Reviews in Toxicology, [S.l.], v. 15, n. 2, p. 109-150, 1985

KANETO, H.; KATAKAMI, N.; MATSUHISA, M.; MATSUOKA, T. Role of reactive oxygen species in the progression of type 2 diabetes and atherosclerosis. Mediators of Inflammation, [S.l.], p. 1-11, 2010.

KNOLL, Thomas; PEARLE, Margaret Sue (ORGs.). Clinical management of urolithiasis. EUA: Springer, 2013 p43-50.

KNOLL, Thomas. Epidemiology, pathogenesis, and pathophysiology of urolithiasis. European Urology Supplements, v. 9, n. 12, p. 802-806, 2010.

LÉPEZ, I., WERLINGER, C., KLEMPAU, A., SOBARZO, G. Acuicultura: cultivo y producción de organismos acuáticos. Werlinger, C. (ed.). Biologia Marina y Oceanografia: Conceptos y Processos. Concepción,. Universidad de Concepción. Trama Impressores S.A., Chile. Tomo II, p. 559 - 585, 2004.

LÓPEZ, Michelle; HOPPE, Bernd. History, epidemiology and regional diversities of urolithiasis. Pediatric Nephrology, v. 25, n. 1, p. 49-59, 2010.

MARINHO-SORIANO, E. Cultivo experimental de *Gracilaria* no Rio Grande do Norte. pp. 115-124. In: Anais da X Reunião Brasileira de Ficologia. Salvador 2004. Rio de Janeiro, Museu Nacional. Série Livros 10, 2005.

MCHUGH, D.J., 2001. Prospects for Seaweed Production in Developing Countries. FAO Fisheries Circular nº 968, FIIU/C968, 28 pp.

MIRANDA, G. E. C. Avaliação do impacto da explotação (simulada) da alga agarófita *Gracilaria caudata* J. Agardh no litoral da Paraíba. Dissertação de Mestrado. Universidade de São Paulo. São Paulo. 107p. 2000.

MOE, Orson W. Kidney stones: pathophysiology and medical management.The lancet, v. 367, n. 9507, p. 333-344, 2006.

NORDBERG, J.; Arnér, S.J. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. Free Radical Biology & Medicine, v. 31, p. 1287-1312, 2001.

OLIVEIRA, E. C.; Horta, P. A.; Amancio, C. E.; Sant'Anna, C. L. Algas e angiospermas marinhas bênticas do litoral brasileiro: diversidade, explotação e conservação. Disponível em http://www.plantas_marinhasbrasil.pdf. Acesso em 02/11/2010.

OLIVEIRA, E.C., Alveal, K., Anderson, R. Mariculture of ágar-producing gracilarioid red algae. Reviews in Fisheries Science 8: 345-378, 2000.

OLIVEIRA, E. C. The Growth of the seaweed industry and the Latin-American potential. In: Proceedings of Aquicultura Brasil 98. Recife, p. 435 – 436, 1998.

OLIVEIRA, E. C. & PLASTINO, E. M. Gracilariaceae. In: AKATSUKA, I. (ed).Biology of economic seaweeds. SPB Academic Publishing. Netherlands. 185 – 226 pp., 1994.

PEARL, M.S, Calhoun, E.A, Curhan, G.C et al.Urologic diseases of America project: Urolithiasis. J Urol. 2005; Mar;173(3):848-57.

PIETTA, P.G. Flavonoids as antioxidants. Journal of Natural Products, v. 63, p. 1035-1042, 2000.

PLASTINO, E. M., and E. C. Oliveira, 2002, Gracilaria birdiae (Gracilariales, Rhodophyta), a new species from the tropical South American Atlantic with a terete frond and deep spermatangial conceptacles: Phycologia, v. 41, p. 389-396.

PLASTINO, E.M. 2004. Diversidade intraespecifica em algas gracilarióides. Tese de Livre-Docência. Univ. S. Paulo. São Paulo. 52 p.

PERERA, J. G.; MESQUITA, J. X.; ARAGÃO, K. S.; FRANCO, A. X.; SOUZA, M. H. L. P.; BRITO, T. V.; DIAS, J. M.; SILVA, R. O.; MEDEIROS, J. V. R.; OLIVEIRA, J. S.; ABREU, C. M. W.S.; DE PAULA, R. C. M.; BARBOSA, A. L. R.; FREITAS, A. L. P. Polysaccharides isolated from Digenea simplex inhibit inflammatoryand nociceptive responses. Carbohydrate Polymers, v. 108, p. 17–25, 2014.

PRAJAPATI, V. D.; MAHERIYA, P. M.; JANI, G. K.; SOLANKI, H. K. Carrageenan: A natural seaweed polysaccharide and its applications. Carbohydrate polymers, v. 105, p. 97112, 2014.

QI, H. et al. Antioxidant activity of different sulfate content derivatives of polysaccharide extracted from Ulva pertusa (Chlorophyta) in vitro. International Journal of Biological Macromolecules, [S.l.], v. 37, n. 4, p. 195-199, 2005a.

RANG HP, Dale MM, Ritter JM, Moore PK. Farmacologia. 5ed. Rio de Janeiro: Elsevier 2004, p. 789-809.

RIELLA MC, Martins C. Nutrição e o Rim. Rio de Janeiro: Guanabara Koogan, 2001.

RINAUDO, M. Main properties and current applications of some polysaccharides as biomaterials. Polymer International, v. 57, p. 397-430, 2008.

ROCHA, H. A. O.; FARIAS, E. H. C.; BEZERRA, L. C. L. M.; ALBUQUERQUE, I. R. L.; MEDEIROS, V. P.; QUEIROZ, K. C. S.; LEITE, E. L. Polissacarídeos sulfatados de algas marinhas com atividade anticoagulante. Infarma, v. 16, n. 1-2, p. 82-87, 2004.

ROCHA F. D, Pereira R. C., Kaplan M. A. C., Teixeira V. L. Produtos naturais de algas marinhas e seu potencial antioxidante. Rv Bras Farmacogn. 17(4): 631-9, 2007.

RODRIGUES, J. A. G. Atividade anticoagulante de galactanas sulfatadas de algas marinhas vermelhas do gênero *Halymenia* e seu efeito imunoestimulante no camarão marinho *Litopenaeus vannamei*. Tese de Doutorado. UFC, 2006, 77p.

ROTHENBERG ML, Carbone DP, Johnson DH. Improving the evaluation of new cancer treatments: challenges and opportunities. Nat Rev Cancer 2003; 3(4):303-9

RUSSEL, K. C. Nucleation mechanisms in aqueous solutions with comments on urine In: FINLAYSON, B.; HENCH, L. L.; SMITH, L.H. Smith, Urolithiasis: Physical Aspects: Proceedings of a Conference Washington: National Academy of Siences, v. 17, p. 129-143, 1972.

SELVAM, Ramasamy. Calcium oxalate stone disease: role of lipid peroxidation and antioxidants. Urological research, v. 30, n. 1, p. 35-47, 2002.

SIES, H.; STAHL, W. Vitamins E and C, b-carotene, and other carotenoids as antioxidants. American Journal of Clinical Nutrition, Bethesda, v.62,n.6,p.1315-1321,Nov. 1995.

SIMIC, M. G. Mechanisms of inhibition of free-radical processes in mutagenesis and carcinogenesis. Mutagenesis Research, v. 202, n. 2, p. 377-386, 1988.

SOUZA, B. W. S., Cerqueira, M. A., Martins, J. T., Quintas, M. A. C., Ferreira, A. C. S., Teixeira, J. A., Vicente, A. A. Antioxidant potential of two red seaweeds from the Brazilian coasts. Journal of Agricultural and Food Chemistry, v. 59, n. 10, p. 5589 -5594, 2011.

TEIXEIRA, D.I.A.; AMARAL, N.M.; SILVA, J.S. e MASIH NETO, T., 2009. Cultivo de Macroalgas Marinhas no Nordeste do Brasil: uma perspectiva sócio-ambiental para as comunidades tradicionais. Anais do XXIII Congresso Latinoamericando de Ciencias del Mar e VIII Congresso de Ciencias del Mar, MarCuba, Havana.

TEIXEIRA, D.I.A. & Masih Neto, T. Cultivo de Gracilaria em Flecheiras e Guajiru. Anais da X Reunião da Sociedade Brasileira de Ficologia. Salvador, 2004.

TEIXEIRA, D. I. A., Bezerra, C. A. B., Masih Neto, T. Cultivo Experimental da Alga Marinha Gracilaria corneanas praias de Flecheiras e Guajiru-Ceará-Brasil Visando Recuperação de Estoques Naturais. Anais do Congresso Brasileiro de Meio Ambiente, 2000.

TISELIUS HG. (2003). Epidemiology and medical management of stone disease. BJU International; 91 (8): 758–767.

TONON, A., Oliveira, M. C., Soriano, E. M., Colepicolo, P. Absroption of metals and characterization of chemical elements present in three species of Gracilaria (Gracilariaceae) Greville: a genus of economical importance. Revista Brasileira de Farmacognosia, v. 21, n. 2, p. 355 – 360, 2011.

URSI S. & PLASTINO E.M. 200l. Crescimento in vitro de linhagens de coloraçao vermelha e verde clara de Gracilaria sp. (Gracilariales, Rhodophyta) em dois meios de cultura: analise de diferentes estadios reprodutivos. Revista Brasileira de Botiinica 24, supplement:587-594.

URSI, S. 2005. Diversidade intraespecífica de Gracilaria birdiae (Gracilariales, Rhodophyta): crescimento, fotossíntese, pigmentos, polissacarídeos e genes da ficoeritrina de linhagens selvagens e variantes. Tese. Univ. S. Paulo. São Paulo. 121p.

VALKO, M.; RHODES, C.J.; MONCOL, J.; IZAKOVIC, M.M.; MAZUR, M. Free radicals,

metals and antioxidants in oxidative stress-induced cancer. Chemicobiological interactions, 160(1), 1-40. 2006

VALKO, M. et al., Free radicals and antioxidant in normal physiological functions and human disease. The International Journal of Biochemistry & Cell Biological, v. 39, p. 44-84, 2007.

VIDOTTI, E. C.; ROLLEMBERG, M. C. E. Algas: da economia nos ambientes aquáticos à bioremediação e à química analítica. Quim. Nova, v. 27, n. 1, p. 139-145, 2004.

Werlinger. C. et al, 2004. Acuicultura: cultivo y produccíon de organismos acuáticos In: Biología Marina y Oceanografía: Conceptos y Procesos. Nacional del libero y la Lectura. Universidad de Concepción. Chile. Trama Impresores. v. II, p. 559 -594.

WHO. Global Status Report on Non-Communicable Diseases 2010. Disponível em: http://www.who.int/nmh/publications/ncd_report2010/en/.>Accesso em: Feb 05, 2014.

YU, S.-L., et al. Oxalate impairs aminophospholipid translocase activity in renal epithelial cells via oxidative stress: implications for calcium oxalate urolithiasis. The Journal of urology, 186, 3, p1114-1120, set./ 2011.

ZHANG, X.C. & Van Der Meer, J.P. 1987. A study on heterosis in diploid gametophytes of the marine red alga Gracilaria tikvahiae. Botanica marina 30:309-314.

ANEXOS

Molecules — Instructions for Authors

Shortcuts

- Manuscript Submission Overview
- Preparation of a Manuscript
- Qualification for Authorship
- Research Ethics Guidelines
- Correct Identification of Components of Natural Products
- Potential Conflicts of Interest
- Editorial Procedure and Peer-Review
- Suggestion of Reviewers
- English Corrections
- Publication Ethics Statement
- Supplementary Materials and Data Deposit
- Guidelines for Deposition of Sequences and of Expression Data

Submission Checklist

Please

1) read the Aims & Scope to gain an overview and assess if your manuscript is suitable for this journal;

2) use the Microsoft Word template or LaTeX template to prepare your manuscript;

3) make sure you have appropriately considered issues about publication ethics, research ethics, copyright, authorship, figure formats, and references format;

4) ensure that all authors have approved the content of the submitted manuscript.

Manuscript Submission Overview

Types of Publications

Molecules has no restrictions on the length of manuscripts, provided that the text is concise and comprehensive. Full experimental details must be provided so that the results can be reproduced by other groups. *Molecules* encourages authors to publish all experimental controls and full datasets as supplementary files (please read the guidelines about Supplementary Materials carefully and references to unpublished data).

The different types of articles published in *Molecules* are indicated in the first section of the Aims & Scope. The main types are:

- *Articles:* research manuscripts report new evidence or new conclusions which have neither been published before nor are under consideration for publication in another journal. MDPI considers all original research manuscripts provided that the work reports scientifically sound experiments and provides a substantial amount of new information. We strongly recommend authors not to unnecessarily divide their work into several related manuscripts.
- *Short communications* of preliminary, but significant, results will also be considered.
- *Reviews:* review manuscripts provide concise and precise updates on the latest progress made in a given area of research.

- *Conference Papers:* Expanded and high quality conference papers are also considered in *Molecules* if they fulfill the following requirements: (1) the paper should be expanded to the size of a research article; (2) the conference paper should be cited and noted on the first page of the paper; (3) if the authors do not hold the copyright to the published conference paper, authors should seek the appropriate permission from the copyright holder; (4) authors are asked to disclose that it is conference paper in their cover letter and include a statement on what has been changed compared to the original conference paper.

Submission Process

Manuscripts for *Molecules* should be submitted online at susy.mdpi.com. The submitting author, who is generally the corresponding author, is responsible for the manuscript during the submission and peer-review process. The submitting authors must ensure that all co-authors have been included in the author list (read the criteria to qualify for authorship) and that they all have read and approved the submitted version of the manuscript. To submit your manuscript, register and log in to this website. Once you are registered, click here to go to the submission form for *Molecules*. All co-authors can see the manuscript details in the submission system, if they register and log in using the e-mail address provided during manuscript submission.

Accepted File Formats

Authors must use the Microsoft Word template or LaTeX template to prepare their manuscript. Using the template file will substantially shorten the time to complete copy-editing and publication of accepted manuscripts. Accepted file formats are:

- *Microsoft Word:* Manuscripts prepared in Microsoft Word must be converted into a single file before submission. When preparing manuscripts in Microsoft Word, the *Molecules* Microsoft Word template file must be used. Please insert your graphics (schemes, figures, *etc.*) in the main text after the paragraph of its first citation.
- *LaTeX:* Manuscripts prepared in LaTeX must be collated into one ZIP folder (include all source files and images, so that the Editorial Office can recompile the submitted PDF). When preparing manuscripts in LaTeX, please use the *Molecules* LaTeX template files. You can now also use the online application writeLaTeX to submit articles directly to *Molecules*. The MDPI LaTeX template file should be selected from the writeLaTeX template gallery.

Cover Letter

A cover letter must be included with each manuscript submission. It should be concise and explain why the content of your paper is significant, placing your findings in the context of existing work and why it fits the scope of the journal. Please confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal. Any prior submissions of the manuscript to MDPI journals must be acknowledged. The names of proposed and excluded reviewers should be provided in the submission system, not in the cover letter.

Note for Authors Funded by the National Institutes of Health (NIH)

The editors of this journal are able to deposit papers to the NIH Manuscript Submission System (NIHMS, http://nihms.nih.gov/) on your behalf. If you are funded by NIH, please request this service from our editors after acceptance of your paper.

[Return to top]

Preparation of a Manuscript

General Considerations

- **Research manuscripts** should comprise:
    - Front matter: Title, Author list, Affiliations, Abstract, Keywords
    - Research manuscript sections: Introduction, Results, Discussion, Conclusions (optional), Materials and Methods, Supplementary Materials
    - Back matter: Acknowledgments, Author Contributions, Conflict of Interests, References.
- **Review manuscripts** should comprise the front matter, literature review sections and the back matter. The template file can also be used to prepare the front and back matter of your review manuscript. It is not necessary to follow the remaining structure.
- **Abstract Graphic:** Authors are encouraged to provide a graphical abstract to display on the website alongside the textual abstract. It should be a self-explanatory snapshot of your article giving a view on its rationale, study design, and/or conclusions. The graphic should not exceed 550 pixels. When prepared in Adobe Photoshop or Microsoft PowerPoint, the frame should be 5–15 cm in width and height. The text should be kept to a minimum and the font size comprised between 10 pt and 14 pt to ensure readability. The graphic should be provided as a JPG, TIFF, PNG or GIF file.
- **"Data not shown"** should be avoided in research manuscripts. We encourage our authors to publish all observations related to the submitted manuscript as Supplementary Materials. **"Unpublished data"** intended for publication in a different manuscript, *i.e.*, in a manuscript that is either planned, **"in preparation"** or that have been **"submitted"** but not yet accepted, should be cited in the text and a reference should be added in the References section. **"Personal Communications"** should also be cited in the text and reference added in the References section. (see also the MDPI reference list and citations style guide).
- **Abbreviations** should be defined in parentheses the first time they appear in the abstract, main text and in figure captions.
- **SI Units** (International System of Units) should be used for this journal. Imperial, US customary and other units should be converted to SI units whenever possible before submission of a manuscript to the journal.
- **Accession numbers** of RNA, DNA and protein sequences used in the manuscript should be provided in the Materials and Methods section. Please also read the Guidelines for Deposition of Sequences and of Expression Data
- **Equations:** If you are using Word, please use either the Microsoft Equation Editor or the MathType add-on in your paper. Equations should be editable by the editorial office and not appear in a picture format.
- **Chemical Structures and Reaction Schemes:** Chemical structures and reaction schemes should be drawn using an appropriate software package designed for this purpose. As a guideline, these should be drawn to a scale such that all the details and text are clearly legible when placed in the manuscript (*i.e.*, text should be no smaller that 8-9 pt.). To facilitate editing we recommend the use of any of the software packages widely available for this purpose: MDL® Isis/Draw, ACD/ChemSketch®, CS ChemDraw®, ChemWindow®, *etc*.. Free versions of some of these products are available for personal or academic use from the respective publishers. If another less common structure drawing software is used, authors should ensure the figures are saved in a file format compatible with of one of these products.
- **Physical and Spectroscopic Data:** Physical and spectroscopic data as well as tables for NMR data should be prepared according to the ACS's *Preparation and Submission of Manuscripts* standard (page 4).
- **Experimental Data:** To allow for correct abstracting of the manuscripts all compounds should be mentioned by correct chemical name, followed by any numerals used to refer to them in the paper. The use of the IUPAC nomenclature conventions is preferred, although alternate naming systems (for example CAS rules) may be used provided that a single consistent naming system is used throughout a manuscript. For authors perhaps unfamiliar with chemical nomenclature in English we recommend the use of compound naming software such as AutoNom. Full experimental details must be provided, or, in the case of many compounds prepared by a similar method, a representative typical procedure should be given. The general style used in the Journal of Organic Chemistry is preferred. Complete characterization data must be given for all new compounds. For papers mentioning large numbers of compounds a tabular format is acceptable. For known compounds appropriate literature references must be given.
- **X-Ray Crystallographic Data:** to avoid publication of extensive compilations of crystallographic data and facilitate the refereeing of manuscripts, *Molecules* asks authors to **deposit the crystallographic data prior to the submission of the manuscript**.
    - **COD:** Preferably, the data should be deposited with the Crystallography Open Database (COD). Please deposit as "pre-publication data" at http://www.crystallography.net/initiate_deposition.php prior to the submission of the manuscript. COD numbers for structures will be displayed immediately after the data is validated and deposited, and should be included in the manuscript, along with the following text: "COD ..... contains the supplementary crystallographic data for this paper. These data

can be obtained free of charge via http://www.crystallography.net/search.html". This text may be included in the experimental section or as a suitably referenced endnote.

- **CCDC:** Alternatively, the data can be deposited to the Cambridge Crystallographic Data Centre (CCDC). For instructions on doing this, see: http://www.ccdc.cam.ac.uk/conts/depositing.html. The deposition numbers are usually provided by the CCDC within three working days and should be included in the manuscript, along with the following text: "CCDC ..... contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html (or from the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; Fax: +44 1223 336033; E-mail: deposit@ccdc.cam.ac.uk)". This text may be included in the experimental section or as a suitably referenced endnote.

- **Supplementary Materials and Research Data:** To maintain the transparency and reproducibility of research results, authors are encouraged to make their experimental and research data openly available either by depositing into data repositories or by publishing the data and files as "Supplementary Materials". Large datasets and files should be deposited in specialized data repositories. Small datasets, spreadsheets, images, video sequences, conference slides, software source code, *etc.* can be uploaded as "Supplementary Files" during the manuscript submission process. The supplementary files will also be made available to the referees during the peer-review process and be published online alongside the manuscript. Please read the information about Supplementary Materials and Data Deposit for additional guidelines.

[Return to top]

Front Matter

These sections should appear in all manuscript types

- **Title:** The title of your manuscript should be concise, specific and relevant. When gene or protein names are included, the abbreviated name rather than full name should be used.
- **Authors List and Affiliations:** Authors' full first and last names must be provided. The initials of any middle names can be added. The PubMed/MEDLINE standard format is used for affiliations: complete address information including city, zip code, state/province, country, and all email addresses. At least one author should be designated as corresponding author, and his or her email address and other details should be included at the end of the affiliation section. Please read the criteria to qualify for authorship.
- **Abstract:** The abstract should be a total of about 200 words maximum. The abstract should be a single paragraph and should follow the style of structured abstracts, but without headings: 1) Background: Place the question addressed in a broad context and highlight the purpose of the study; 2) Methods: Describe briefly the main methods or treatments applied; 3) Results: Summarize the article's main findings; and 4) Conclusion: Indicate the main conclusions or interpretations. The abstract should be an objective representation of the article: it must not contain results which are not presented and substantiated in the main text and should not exaggerate the main conclusions.
- **Keywords:** Three to ten pertinent keywords need to be added after the abstract. We recommend that the keywords are specific to the article, yet reasonably common within the subject discipline.

Research Manuscript Sections

- **Introduction:** The introduction should briefly place the study in a broad context and highlight why it is important. It should define the purpose of the work and its significance. The current state of the research field should be reviewed carefully and key publications should be cited. Please highlight controversial and diverging hypotheses when necessary. Finally, briefly mention the main aim of the work and highlight the main conclusions. As far as possible, please keep the introduction comprehensible to scientists outside your particular field of research.
- **Results:** This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation as well as the experimental conclusions that can be drawn.
- **Discussion:** This section may be divided by subheadings. Authors should discuss the results and how they can be interpreted in perspective of previous studies and of the working hypotheses. The findings and their

implications should be discussed in the broadest context possible. Future research directions may also be highlighted.

- **Conclusions:** This section is not mandatory, but can be added to the manuscript if the discussion is unusually long or complex.
- **Materials and Methods:** This section should be divided by subheadings. Materials and Methods should be described with sufficient details to allow others to replicate and build on published results. Please note that publication of your manuscript implicates that you must make all materials, data, and protocols associated with the publication available to readers. Please disclose at the submission stage any restrictions on the availability of materials or information. New methods and protocols should be described in detail while well-established methods can be briefly described and appropriately cited.
    - Research manuscripts reporting large datasets that are deposited in a publicly available database should specify where the data have been deposited and provide the relevant accession numbers. If the accession numbers have not yet been obtained at the time of submission, please state that they will be provided during review. They must be provided prior to publication.
    - Research manuscripts using human or animal subjects, tissues, field samples or cell lines must include research ethics statements. Please read the Ethical Research Guidelines.
- **Supplementary Materials:** This section should be included when supplementary information is published online alongside the manuscript. Please indicate the name and title of each supplementary file as follows **Figure S1:** title, **Table S1:** title, *etc.*

[Return to top]

Back Matter

- **Acknowledgments:** All sources of funding of the study should be disclosed. Please clearly indicate grants that you have received in support of your research work. Clearly state if you received funds for covering the costs to publish in open access. Note that some funders will not refund article processing charges (APC) if the funder and grant number are not clearly identified in the paper. Funding information can be entered separately into the submission system by the authors during submission of their manuscript. Such funding information, if available, will be deposited to FundRef if the manuscript is finally published.
- **Author Contributions:** For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "X.X. and Y.Y. conceived and designed the experiments; X.X. performed the experiments; X.X. and Y.Y. analyzed the data; W.W. contributed reagents/materials/analysis tools; Y.Y. wrote the paper. **Authorship must be limited to those who have contributed substantially to the work reported. Please read the section concerning the criteria to qualify for authorship carefully.**
- **Conflicts of Interest:** Authors must identify and declare any personal circumstances or interest that may be perceived as inappropriately influencing the representation or interpretation of reported research results. If there is no conflict of interest, please state "The authors declare no conflict of interest." Any role of the funding sponsors in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript, or in the decision to publish the results must be declared in this section. If there is no role, please state "The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results".
- **References:** References must be numbered in order of appearance in the text (including tables and legends) and listed individually at the end of the manuscript. We recommend preparing the references with a bibliography software package, such as EndNote, ReferenceManager or Zotero to avoid typing mistakes and duplicated references.

  Citations and References in Supplementary files are permitted provided that they also appear in the main text and in the reference list.

    - In the text, reference numbers should be placed in square brackets [ ], and placed before the punctuation; for example [1], [1–3] or [1,3]. For embedded citations in the text with pagination, use both parentheses and brackets to indicate the reference number and page numbers; for example [5] (p. 10). or [6] (pp. 101–105).

- The Reference list should include the full title as recommended by the ACS style guide. The style file for endnote, MDPI.ens, can be found at http://endnote.com/downloads/style/mdpi
- References should be described as follows depending on the type of work:
  - Journal Articles:
    1. Author 1, A.B.; Author 2, C.D. Title of the article. *Abbreviated Journal Name* **Year**, *Volume*, page range, DOI or other identifier. Available online: URL (accessed on Day Month Year).
  - Books and Book Chapters:
    2. Author 1, A.; Author 2, B. *Book Title*, 3rd ed.; Publisher: Publisher Location, Country, Year; pp. 154–196.
    3. Author 1, A.; Author 2, B. Title of the chapter. In *Book Title*, 2nd ed.; Editor 1, A.; Editor 2, B., Eds.; Publisher: Publisher Location, Country, Year; Volume 3, pp. 154–196.
  - Unpublished work, submitted work, personal communication:
    4. Author 1, A.B.; Author 2, C. Title of Unpublished Work. status (unpublished; manuscript in preparation).
    5.Author 1, A.B.; Author 2, C. Title of Unpublished Work. *Abbreviated Journal Name* stage of publication (under review; accepted; in press).
    6.Author 1, A.B. (University, City, State, Country); Author 2, C. (Institute, City, State, Country). Personal communication, Year.
  - Conference Proceedings:
    7. Author 1, A.B.; Author 2, C.D.; Author 3, E.F. Title of Presentation. In *Title of the Collected Work* (if available), Proceedings of the Name of the Conference, Location of Conference, Country, Date of Conference; Editor 1, Editor 2, Eds. (if available); Publisher: City, Country, Year (if available); Abstract Number (optional), Pagination (optional).
  - Thesis:
    8. Author 1, A.B. Title of Thesis. Level of Thesis, Degree-Granting University, Location of University, Date of Completion.
  - Websites:
    9.Title of Site. Available online: URL (accessed on Day Month Year).
    Unlike published works, websites may change over time or disappear, so we encourage you create an archive of the cited website using a service such as WebCite. Archived websites should be cited using the link provided as follows:
    10. Title of Site. URL (archived on Day Month Year).

See the Reference List and Citations Guide for more detailed information.

Preparing Figures, Schemes and Tables

- All figure files should be separately uploaded during submission.
- Figures and schemes must be provided at a sufficiently high resolution (minimum 1000 pixels width/height, or a resolution of 300 dpi or higher). All Figure file formats are accepted. However, TIFF, JPEG, EPS and PDF files are preferred.
- *Molecules* can publish multimedia files in articles or as supplementary materials. Please get in touch with the Editorial office for further information.
- All Figures, Schemes and Tables should also be inserted into the main text close to their first citation and must be numbered following their number of appearance (Figure 1, Scheme I, Figure 2, Scheme II, Table 1, *etc.*).
- All Figures, Schemes and Tables should have a short explanatory title and a caption placed above it.
- All table columns should have an explanatory heading. To facilitate the copy-editing of larger tables, smaller fonts may be used, but in no less than 8 pt. in size. Authors should use the Table option of Microsoft Word to create tables.
- For multi-panel figures, the file must contain all data in one file. For tips on creating multi-panel figures, please read the helpful advice provided by L2 Molecule.
- Authors are encouraged to prepare figures and schemes in color (RGB at 8-bit per channel). Full color graphics will be published free of charge.

[Return to top]

Qualification for Authorship

Authorship must include and be strictly limited to researchers who have substantially contributed to the reported work. To qualify for authorship, a researcher should have made a substantial contribution to the design of the study, or to the production, analysis or interpretation of the results. Authors should also have been involved in the preparation and have approved the submitted manuscript. Those who contributed to the work but do not qualify for authorship should be listed in the acknowledgments. According to the Committee on Publication Ethics (COPE) standard, to which this journal adheres, "all authors should agree to be listed and should approve the submitted and accepted versions of the publication. Any change to the author list should be approved by all authors including any who have been removed from the list. The corresponding author should act as a point of contact between the editor and the other authors and should keep co-authors informed and involve them in major decisions about the publication (e.g. answering reviewers' comments)." [1]

1. Wager, E.; Kleinert, S. Responsible research publication: international standards for authors. A position statement developed at the 2nd World Conference on Research Integrity, Singapore, July 22-24, 2010. In Promoting Research Integrity in a Global Environment; Mayer, T., Steneck, N., eds.; Imperial College Press / World Scientific Publishing: Singapore; Chapter 50, pp. 309-16.

[Return to top]

Research Ethics Guidelines

1. Research Involving Animals

The editors will require that the benefits potentially derived from any research causing harm to animals are significant in relation to any suffering endured by animals, and that procedures followed are unlikely to cause offense to the majority of readers. Authors should particularly ensure that their research complies with the commonly-accepted '3Rs':

- Replacement of animals by alternatives wherever possible,
- Reduction in number of animals used, and
- Refinement of experimental conditions and procedures to minimize the harm to animals.

Any experimental work must be conducted in accordance with relevant national legislation on the use of animals for research. Authors should follow the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines (http://www.nc3rs.org.uk/page.asp?id=1357) for reporting experiments using live animals. Authors may use the ARRIVE guidelines as a checklist (www.nc3rs.org.uk/ARRIVEchecklist).

An approval from an ethics committee must be obtained before undertaking the research. The project identification code, date of approval and name of the ethics committee or institutional review board should be cited in the Methods section.

Editors reserve the rights to reject any submission that does not meet these requirements.

An example of Ethical Statements:

The animal protocols used in this work were evaluated and approved by the Animal Use and Ethic Committee (CEUA) of the Institute Pasteur Montevideo (Protocol 2009_1_3284). They are in accordance with FELASA guidelines and the National law for Laboratory Animal Experimentation (Law no. 18.611).

2. Research Involving Human Subjects

When reporting on research that involves human subjects, human material, human tissues or human data, authors must declare that the investigations were carried out following the rules of the Declaration of Helsinki of 1975 (http://www.wma.net/en/30publications/10policies/b3/), revised in 2008. According to point 23 of this declaration, an

approval from an ethics committee should have been obtained before undertaking the research. As a minimum, a statement including the project identification code, date of approval and name of the ethics committee or institutional review board should be cited in the Methods Section of the article. Data relating to individual participants must be described in detail, but private information identifying participants need not be included unless the identifiable materials are of relevance to the research (for example, photographs of participants' faces that show a particular symptom). A written informed consent for publication must be obtained from participating patients in this case.

Editors reserve the rights to reject any submission that does not meet these requirements.

Example of Ethical Statements:

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of XXX (Project identification code).

3. Research Involving Cell Lines

Methods sections for submissions reporting on research with cell lines should state the origin of any cell lines. For established cell lines the provenance should be stated and references must also be given to either a published paper or to a commercial source. If previously unpublished *de novo* cell lines were used, including those gifted from another laboratory, details of institutional review board or ethics committee approval must be given, and confirmation of written informed consent must be provided if the line is of human origin.

An example of Ethical Statements:

The HCT116 cell line was obtained from XXXX. The $MLH1^{+}$ cell line was provided by XXXXX, Ltd. The DLD-1 cell line was obtained from Dr. XXXX. The DR-GFP and SA-GFP reporter plasmids were obtained from Dr. XXX and the Rad51K133A expression vector was obtained from Dr. XXXX.

4. Research Involving Plants

Experimental research on plants (either cultivated or wild) including collection of plant material, must comply with institutional, national, or international guidelines. We recommend that authors comply with the Convention on Biological Diversity and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

For each submitted manuscript supporting genetic information and origin must be provided. For research manuscripts involving rare and non-model plants (other than, e.g., *Arabidopsis thaliana*, *Nicotiana benthamiana, Oriza sativa*, or many other typical model plants), voucher specimens must be deposited in an accessible herbarium or museum. Vouchers may be requested for review by future investigators to verify the identity of the material used in the study (especially if taxonomic rearrangements occur in the future). They should include details of the populations sampled on the site of collection (GPS coordinates), date of collection, and document the part(s) used in the study where appropriate. For rare, threatened or endangered species this can be waived but it is necessary for the author to describe this in the cover letter.

Editors reserve the rights to reject any submission that does not meet these requirements.

An example of Ethical Statements:

Torenia fournieri plants were used in this study. White-flowered Crown White (CrW) and violet-flowered Crown Violet (CrV) cultivars selected from 'Crown Mix' (XXX Company, City, Country) were kindly provided by Dr. XXX (XXX Institute, City, Country).

Arabidopis mutant lines (SALKxxxx, SAILxxxx,…) were kindly provided by Dr. XXX , institute, city, country).

[Return to top]

Correct Identification of Components of Natural Products

The correct identification of the various components of extracts from natural sources is of key importance, and as publishers we are keenly aware of our responsibility to the scientific community in this area. Consequently, for papers on this topic, we have adopted the recommendations of the Working Group on Methods of Analysis of the International Organization of the Flavour Industry (IOFI), as published in *Flavour Fragr. J.* **2006**, *21*, 185. These recommendations may be summarized as follows:

Any identification of a natural compound must pass scrutiny by the latest forms of available analytical techniques. This implies that its identity must be confirmed by at least two different methods, for example, comparison of chromatographic and spectroscopic data (including mass, IR and NMR spectra) with those of an authentic sample, either isolated or synthesized. For papers claiming the first discovery of a given compound from a natural source, the authors must provide full data obtained by their own measurements of both the unknown and an authentic sample, whose source must be fully documented. Authors should also consider very carefully potential sources of artifacts and contaminants resulting from any extraction procedure or sample handling.

[Return to top]

Potential Conflicts of Interest

It is the authors' responsibility to identify and declare any personal circumstances or interests that may be perceived as inappropriately influencing the representation or interpretation of clinical research. If there is no conflict, please state here "The authors declare no conflict of interest." This should be conveyed in a separate "Conflicts of Interest" section preceding the "References" sections at the end of the manuscript.

[Return to top]

Editorial Procedure and Peer-Review

Initial Checks

All submitted manuscripts received by the Editorial Office will be checked by a professional in-house *Managing Editor* to determine whether it is properly prepared and whether the manuscript follows the ethical policies of the journal, including those for human and animal experimentation. Manuscripts that do not fit the journal or are not in line with our ethical policy may be rejected before peer-review. Manuscripts that are not properly prepared will be returned to the authors for revision and resubmission. The *Managing Editor* will consult the journals' *Editor-in-Chief*, the *Guest Editor* or an *Editorial Board member* to determine whether the manuscript fits the scope of the journal and whether it is scientifically sound. No judgment on the significance or potential impact of the work will be made at this stage.

Peer-Review

Once a manuscript passes the initial checks, it will be assigned to at least two independent experts for peer-review. A single-blind peer-review process is applied, where authors' names are revealed to reviewers. In-house assistant editors generally invite experts recommended by the *Editor-in-Chief* or identified by literature searches. These experts may also include Editorial Board members and Guest Editors of the journal. Potential reviewers suggested by the authors may also be considered. Reviewers should not have published with any of the co-authors during the past five years and should not currently work or collaborate with one of the institutes of the co-authors of the submitted manuscript.

Editorial Decision and Revision

Based on the comments and advices of the peer-reviewers, an external editor – usually the *Editor-in-Chief* or a *Guest Editor* – will make a decision to accept, reject, or to ask authors to revise the manuscript.

For *Minor Revisions* the authors will have one week to resubmit their revised manuscript. For *Major Revisions* the authors will have two weeks to resubmit their revised manuscript. However, authors should contact the editorial office if extended revision time is anticipated.

Author Appeals

Authors may appeal a rejection by sending an e-mail to the Editorial Office of the journal. The appeal must provide a detailed justification, including point-by-point responses to the reviewers' and/or Editor's comments. The *Managing Editor* of the journal will forward the manuscript and relating information (including the identities of the referees) to an Editorial Board member who was not involved in the initial decision-making process. If no appropriate Editorial Board member is available, the editor will identify a suitable external scientist. The Editorial Board member will be asked to give an advisory recommendation on the manuscript and may recommend acceptance, further peer-review, or uphold the original rejection decision. A reject decision at this stage will be final and cannot be revoked.

Production and Publication

Once accepted, the manuscript will undergo professional copy-editing, English editing, proofreading by the authors, final corrections, pagination, and, publication on the www.mdpi.com website.

[Return to top]

Suggestion of Reviewers

During the submission process, authors have the possibility to suggest potential reviewers with the appropriate expertise to review the manuscript. The editors will not necessarily approach these referees. Please provide detailed contact information (address, homepage, phone, e-mail address). The proposed referees should neither be current collaborators of the co-authors nor have published with any of the co-authors of the manuscript within the last five years. Proposed reviewers should be from different institutions to the authors. You may identify appropriate Editorial Board members of the journal as potential reviewers. You may also suggest reviewers from among the authors that you frequently cite in your paper.

[Return to top]

English Corrections

This journal is published in English. To facilitate proper peer-reviewing of your manuscript, it is essential that it is submitted in grammatically correct English. If you are not a native English speaker, we strongly recommend that you have your manuscript professionally edited before submission or read by a native English-speaking colleague. Professional editing will mean that reviewers and future readers are better able to read and assess the content of your manuscript. For additional information see the English Editing Guidelines for Authors.

[Return to top]

Publication Ethics Statement

Molecules is a member of the Committee on Publication Ethics (COPE). We fully adhere to its Code of Conduct and to its Best Practice Guidelines.

The editors of this journal take the responsibility to enforce a rigorous peer-review process together with strict ethical policies and standards to ensure to add high quality scientific works to the field of scholarly publication. Unfortunately, cases of plagiarism, data falsification, image manipulation, inappropriate authorship credit, and the like, do arise. The editors of *Molecules* take such publishing ethics issues very seriously and are trained to proceed in such cases with a zero tolerance policy.

Authors wishing to publish their papers in *Molecules* are asked to abide to the following rules:

- Any facts that might be perceived as a possible conflict of interest of the author(s) must be disclosed in the paper prior to submission.
- Authors should accurately present their research findings and include an objective discussion of the significance of their findings.
- Data and methods used in the research need to be presented in sufficient detail in the paper, so that other researchers can replicate the work.
- Raw data should preferably be publicly deposited by the authors before submission of their manuscript. Authors need to at least have the raw data readily available for presentation to the referees and the editors of the journal, if requested. Authors need to ensure appropriate measures are taken so that raw data is retained in full for a reasonable time after publication.
- Simultaneous submission of manuscripts to more than one journal is not tolerated.
- Republishing content that is not novel is not tolerated (for example, an English translation of a paper that is already published in another language will not be accepted).
- If errors and inaccuracies are found by the authors after publication of their paper, they need to be promptly communicated to the editors of this journal so that appropriate actions can be taken. Please refer to our policy regarding publication of publishing addenda and corrections.
- Your manuscript should not contain any information that has already been published. If you include already published figures or images, please obtain the necessary permission from the copyright holder to publish under the CC-BY license.
- Plagiarism, data fabrication and image manipulation are not tolerated.
    - **Plagiarism is not acceptable** in *Molecules* submissions.

      Plagiarism includes copying text, ideas, images, or data from another source, even from your own publications, without giving any credit to the original source.

      Reuse of text that is copied from another source must be between quotes and the original source must be cited. If a study's design or the manuscript's structure or language has been inspired by previous works, these works must be explicitly cited.

      If plagiarism is detected during the peer review process, the manuscript may be rejected. If plagiarism is detected after publication, we may publish a correction or retract the paper.

    - **Image files must not be manipulated or adjusted in any way** that could lead to misinterpretation of the information provided by the original image.

      Irregular manipulation includes: 1) introduction, enhancement, moving, or removing features from the original image; 2) grouping of images that should obviously be presented separately (e.g., from different parts of the same gel, or from different gels); or 3) modifying the contrast, brightness or color balance to obscure, eliminate or enhance some information.

      If irregular image manipulation is identified and confirmed during the peer review process, we may reject the manuscript. If irregular image manipulation is identified and confirmed after publication, we may correct or retract the paper.

  Our in-house editors will investigate any allegations of publication misconduct and may contact the authors' institutions or funders if necessary. If evidence of misconduct is found, appropriate action will be taken to

correct or retract the publication. Authors are expected to comply with the best ethical publication practices when publishing with MDPI.

[Return to top]

Supplementary Materials and Data Deposit

In order to maintain the integrity, transparency and reproducibility of research records, and to retain important chemical and structural information, authors are strongly encouraged to make their experimental and research data openly available either by depositing into data repositories or by publishing the data and files as supplementary information in this journal. Additional data and files can be uploaded as "Supplementary Files" during the manuscript submission process. The supplementary files will also be available to the referees as part of the peer-review process, although referees are not specifically asked to review these files. Accepted file formats include (but are not limited to):

- spectral data (NMR, IR, Raman, ESR, etc.) in JCAMP (JDX) format
- 3D coordinate structures (in PDB, MOL, XYZ or other common format)
- crystallographic information (in CIF format)
- data tables and spreadsheets (text files, MS Excel, OpenOffice, CSV, XML, *etc.*)
- text documents (text files, PDF, MS Word, OpenOffice, *etc.*; text documents will usually be converted to PDF files for publication)
- images (JPEG, PNG, GIF, TIFF, BMP, *etc.*)
- videos (AVI, MPG, QuickTime, *etc.*)
- executables (EXE, Java, *etc.*)
- software source code

Citations and References in Supplementary files are permitted provided that they also appear in the main text and in the reference list.

Large data sets and files should be deposited to specialized service providers (such as Figshare) or institutional/subject repositories, preferably those that use the DataCite mechanism. For a list of specialized repositories for the deposit of scientific and experimental data, please consult databib.org or re3data.org. The data repository name, link to the data set (URL) and accession number, doi or handle number of the data set must be provided in the paper. The journal *Data* (ISSN 2306-5729) also accepts submissions of data set papers, and the publication of small data sets along with the paper, and/or software source codes is encouraged.

Guidelines for Deposition of Sequences and of Expression Data

New sequence information must be deposited to the appropriate database prior to submission of the manuscript. Accession numbers provided by the database should be included in the submitted manuscript. Manuscripts will not be published until the accession number is provided.

- **New nucleic acid sequences** must be deposited in one of the following databases: GenBank, EMBL, or DDBJ. Sequences should be submitted to only one database.
- **New high throughput sequencing (HTS) datasets**(RNA-seq, ChIP-Seq, degradome analysis, …) must be deposited either in the GEO database or in the NCBI's Sequence Read Archive.
- **New microarray data** must be deposited either in GEO or ArrayExpress databases. The "Minimal Information About a Microarray Experiment" (MIAME) guidelines published by the Microarray Gene Expression Data Society must be followed.
- **New protein sequences** obtained by protein sequencing must be submitted to UniProt (submission tool SPIN). All sequence names and the accession numbers provided by the databases should be provided in the Materials and Methods section of the article.

ANEXO 2:

Marine Drugs — Instructions for Authors

Shortcuts

- Manuscript Submission Overview
- Preparation of a Manuscript
- Qualification for Authorship
- Correct Identification of Components of Natural Products
- Research Ethics Guidelines
- Potential Conflicts of Interest
- Editorial Procedure and Peer-Review
- Suggestion of Reviewers
- English Corrections
- Publication Ethics Statement
- Supplementary Materials and Data Deposit
- Guidelines for Deposition of Sequences and of Expression Data

Submission Checklist

Please

1) read the Aims & Scope to gain an overview and assess if your manuscript is suitable for this journal;

2) use the Microsoft Word template or LaTeX template to prepare your manuscript;

3) make sure you have appropriately considered issues about publication ethics, research ethics, copyright, authorship, figure formats, and references format;

4) ensure that all authors have approved the content of the submitted manuscript.

Manuscript Submission Overview

Types of Publications

Marine Drugs has no restrictions on the length of manuscripts, provided that the text is concise and comprehensive. Full experimental details must be provided so that the results can be reproduced by other groups. *Marine Drugs* encourages authors to publish all experimental controls and full datasets as supplementary files (please read the guidelines about Supplementary Materials carefully and references to unpublished data).

The different types of articles published in *Marine Drugs* are indicated in the first section of the Aims & Scope. The main types are:

- *Articles:* research manuscripts report new evidence or new conclusions which have neither been published before nor are under consideration for publication in another journal. MDPI considers all original research manuscripts provided that the work reports scientifically sound experiments and provides a substantial amount of new information. We strongly recommend authors not to unnecessarily divide their work into several related manuscripts.
- *Short communications* of preliminary, but significant, results will also be considered.
- *Reviews:* review manuscripts provide concise and precise updates on the latest progress made in a given area of research.
- *Conference Papers:* Expanded and high quality conference papers are also considered in *Marine Drugs* if they fulfill the following requirements: (1) the paper should be expanded to the size of a research article; (2) the

conference paper should be cited and noted on the first page of the paper; (3) if the authors do not hold the copyright to the published conference paper, authors should seek the appropriate permission from the copyright holder; (4) authors are asked to disclose that it is conference paper in their cover letter and include a statement on what has been changed compared to the original conference paper.

Submission Process

Manuscripts for *Marine Drugs* should be submitted online at susy.mdpi.com. The submitting author, who is generally the corresponding author, is responsible for the manuscript during the submission and peer-review process. The submitting authors must ensure that all co-authors have been included in the author list (read the criteria to qualify for authorship) and that they all have read and approved the submitted version of the manuscript. To submit your manuscript, register and log in to this website. Once you are registered, click here to go to the submission form for *Marine Drugs*. All co-authors can see the manuscript details in the submission system, if they register and log in using the e-mail address provided during manuscript submission.

Accepted File Formats

Authors must use the Microsoft Word template or LaTeX template to prepare their manuscript. Using the template file will substantially shorten the time to complete copy-editing and publication of accepted manuscripts. Accepted file formats are:

- *Microsoft Word:* Manuscripts prepared in Microsoft Word must be converted into a single file before submission. When preparing manuscripts in Microsoft Word, the *Marine Drugs* Microsoft Word template file must be used. Please insert your graphics (schemes, figures, *etc.*) in the main text after the paragraph of its first citation.
- *LaTeX:* Manuscripts prepared in LaTeX must be collated into one ZIP folder (include all source files and images, so that the Editorial Office can recompile the submitted PDF). When preparing manuscripts in LaTeX, please use the *Marine Drugs* LaTeX template files. You can now also use the online application writeLaTeX to submit articles directly to *Marine Drugs*. The MDPI LaTeX template file should be selected from the writeLaTeX template gallery.

Cover Letter

A cover letter must be included with each manuscript submission. It should be concise and explain why the content of your paper is significant, placing your findings in the context of existing work and why it fits the scope of the journal. Please confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal. Any prior submissions of the manuscript to MDPI journals must be acknowledged. The names of proposed and excluded reviewers should be provided in the submission system, not in the cover letter.

Note for Authors Funded by the National Institutes of Health (NIH)

This journal automatically deposits papers to PubMed Central after publication of an issue. Authors do not need to separately submit their papers through the NIH Manuscript Submission System (NIHMS, http://nihms.nih.gov/).

[Return to top]

Preparation of a Manuscript

General Considerations

- **Research manuscripts** should comprise:
  - Front matter: Title, Author list, Affiliations, Abstract, Keywords

    - Research manuscript sections: Introduction, Results, Discussion, Conclusions (optional), Materials and Methods, Supplementary Materials
    - Back matter: Acknowledgments, Author Contributions, Conflict of Interests, References.
- **Review manuscripts** should comprise the front matter, literature review sections and the back matter. The template file can also be used to prepare the front and back matter of your review manuscript. It is not necessary to follow the remaining structure.
- **Abstract Graphic:** Authors are encouraged to provide a graphical abstract to display on the website alongside the textual abstract. It should be a self-explanatory snapshot of your article giving a view on its rationale, study design, and/or conclusions. The graphic should not exceed 550 pixels. When prepared in Adobe Photoshop or Microsoft PowerPoint, the frame should be 5–15 cm in width and height. The text should be kept to a minimum and the font size comprised between 10 pt and 14 pt to ensure readability. The graphic should be provided as a JPG, TIFF, PNG or GIF file.
- **"Data not shown"** should be avoided in research manuscripts. We encourage our authors to publish all observations related to the submitted manuscript as Supplementary Materials. **"Unpublished data"** intended for publication in a different manuscript, *i.e.*, in a manuscript that is either planned, **"in preparation"** or that have been **"submitted"** but not yet accepted, should be cited in the text and a reference should be added in the References section. **"Personal Communications"** should also be cited in the text and reference added in the References section. (see also the MDPI reference list and citations style guide).
- **Abbreviations** should be defined in parentheses the first time they appear in the abstract, main text and in figure captions.
- **SI Units** (International System of Units) should be used for this journal. Imperial, US customary and other units should be converted to SI units whenever possible before submission of a manuscript to the journal.
- **Accession numbers** of RNA, DNA and protein sequences used in the manuscript should be provided in the Materials and Methods section. Please also read the Guidelines for Deposition of Sequences and of Expression Data
- **Equations:** If you are using Word, please use either the Microsoft Equation Editor or the MathType add-on in your paper. Equations should be editable by the editorial office and not appear in a picture format.
- **Chemical Structures and Reaction Schemes:** Chemical structures and reaction schemes should be drawn using an appropriate software package designed for this purpose. As a guideline, these should be drawn to a scale such that all the details and text are clearly legible when placed in the manuscript (*i.e.*, text should be no smaller that 8-9 pt.). To facilitate editing we recommend the use of any of the software packages widely available for this purpose: MDL® Isis/Draw, ACD/ChemSketch®, CS ChemDraw®, ChemWindow®, *etc.*. Free versions of some of these products are available for personal or academic use from the respective publishers. If another less common structure drawing software is used, authors should ensure the figures are saved in a file format compatible with of one of these products.
- **Physical and Spectroscopic Data:** Physical and spectroscopic data as well as tables for NMR data should be prepared according to the ACS's *Preparation and Submission of Manuscripts* standard (page 4).
- **Experimental Data:** To allow for correct abstracting of the manuscripts all compounds should be mentioned by correct chemical name, followed by any numerals used to refer to them in the paper. The use of the IUPAC nomenclature conventions is preferred, although alternate naming systems (for example CAS rules) may be used provided that a single consistent naming system is used throughout a manuscript. For authors perhaps unfamiliar with chemical nomenclature in English we recommend the use of compound naming software such as AutoNom. Full experimental details must be provided, or, in the case of many compounds prepared by a similar method, a representative typical procedure should be given. The general style used in the Journal of Organic Chemistry is preferred. Complete characterization data must be given for all new compounds. For papers mentioning large numbers of compounds a tabular format is acceptable. For known compounds appropriate literature references must be given.
- **Supplementary Materials and Research Data:** To maintain the transparency and reproducibility of research results, authors are encouraged to make their experimental and research data openly available either by depositing into data repositories or by publishing the data and files as "Supplementary Materials". Large datasets and files should be deposited in specialized data repositories. Small datasets, spreadsheets, images, video sequences, conference slides, software source code, *etc.* can be uploaded as "Supplementary Files" during the manuscript submission process. The supplementary files will also be made available to the referees during the peer-review process and be published online alongside the manuscript. Please read the information about Supplementary Materials and Data Deposit for additional guidelines.

[Return to top]

Front Matter

These sections should appear in all manuscript types

- **Title:** The title of your manuscript should be concise, specific and relevant. When gene or protein names are included, the abbreviated name rather than full name should be used.
- **Authors List and Affiliations:** Authors' full first and last names must be provided. The initials of any middle names can be added. The PubMed/MEDLINE standard format is used for affiliations: complete address information including city, zip code, state/province, country, and all email addresses. At least one author should be designated as corresponding author, and his or her email address and other details should be included at the end of the affiliation section. Please read the criteria to qualify for authorship.
- **Abstract:** The abstract should be a total of about 200 words maximum. The abstract should be a single paragraph and should follow the style of structured abstracts, but without headings: 1) Background: Place the question addressed in a broad context and highlight the purpose of the study; 2) Methods: Describe briefly the main methods or treatments applied; 3) Results: Summarize the article's main findings; and 4) Conclusion: Indicate the main conclusions or interpretations. The abstract should be an objective representation of the article: it must not contain results which are not presented and substantiated in the main text and should not exaggerate the main conclusions.
- **Keywords:** Three to ten pertinent keywords need to be added after the abstract. We recommend that the keywords are specific to the article, yet reasonably common within the subject discipline.

Research Manuscript Sections

- **Introduction:** The introduction should briefly place the study in a broad context and highlight why it is important. It should define the purpose of the work and its significance. The current state of the research field should be reviewed carefully and key publications should be cited. Please highlight controversial and diverging hypotheses when necessary. Finally, briefly mention the main aim of the work and highlight the main conclusions. As far as possible, please keep the introduction comprehensible to scientists outside your particular field of research.
- **Results:** This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation as well as the experimental conclusions that can be drawn.
- **Discussion:** This section may be divided by subheadings. Authors should discuss the results and how they can be interpreted in perspective of previous studies and of the working hypotheses. The findings and their implications should be discussed in the broadest context possible. Future research directions may also be highlighted.
- **Conclusions:** This section is not mandatory, but can be added to the manuscript if the discussion is unusually long or complex.
- **Materials and Methods:** This section should be divided by subheadings. Materials and Methods should be described with sufficient details to allow others to replicate and build on published results. Please note that publication of your manuscript implicates that you must make all materials, data, and protocols associated with the publication available to readers. Please disclose at the submission stage any restrictions on the availability of materials or information. New methods and protocols should be described in detail while well-established methods can be briefly described and appropriately cited.
    - Research manuscripts reporting large datasets that are deposited in a publicly available database should specify where the data have been deposited and provide the relevant accession numbers. If the accession numbers have not yet been obtained at the time of submission, please state that they will be provided during review. They must be provided prior to publication.
    - Research manuscripts using human or animal subjects, tissues, field samples or cell lines must include research ethics statements. Please read the Ethical Research Guidelines.
- **Supplementary Materials:** This section should be included when supplementary information is published online alongside the manuscript. Please indicate the name and title of each supplementary file as follows **Figure S1:** title, **Table S1:** title, *etc.*

[Return to top]

Back Matter

- **Acknowledgments:** All sources of funding of the study should be disclosed. Please clearly indicate grants that you have received in support of your research work. Clearly state if you received funds for covering the costs to publish in open access. Note that some funders will not refund article processing charges (APC) if the funder and grant number are not clearly identified in the paper. Funding information can be entered separately into the submission system by the authors during submission of their manuscript. Such funding information, if available, will be deposited to FundRef if the manuscript is finally published.
- **Author Contributions:** For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "X.X. and Y.Y. conceived and designed the experiments; X.X. performed the experiments; X.X. and Y.Y. analyzed the data; W.W. contributed reagents/materials/analysis tools; Y.Y. wrote the paper. **Authorship must be limited to those who have contributed substantially to the work reported. Please read the section concerning the criteria to qualify for authorship carefully.**
- **Conflicts of Interest:** Authors must identify and declare any personal circumstances or interest that may be perceived as inappropriately influencing the representation or interpretation of reported research results. If there is no conflict of interest, please state "The authors declare no conflict of interest." Any role of the funding sponsors in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript, or in the decision to publish the results must be declared in this section. If there is no role, please state "The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results".
- **References:** References must be numbered in order of appearance in the text (including tables and legends) and listed individually at the end of the manuscript. We recommend preparing the references with a bibliography software package, such as EndNote, ReferenceManager or Zotero to avoid typing mistakes and duplicated references.

  Citations and References in Supplementary files are permitted provided that they also appear in the main text and in the reference list.

  - In the text, reference numbers should be placed in square brackets [ ], and placed before the punctuation; for example [1], [1–3] or [1,3]. For embedded citations in the text with pagination, use both parentheses and brackets to indicate the reference number and page numbers; for example [5] (p. 10). or [6] (pp. 101–105).
  - The Reference list should include the full title as recommended by the ACS style guide. The style file for endnote, MDPI.ens, can be found at http://endnote.com/downloads/style/mdpi
  - References should be described as follows depending on the type of work:
    - Journal Articles:
      1. Author 1, A.B.; Author 2, C.D. Title of the article. *Abbreviated Journal Name* **Year**, *Volume*, page range, DOI or other identifier. Available online: URL (accessed on Day Month Year).
    - Books and Book Chapters:
      2. Author 1, A.; Author 2, B. *Book Title*, 3rd ed.; Publisher: Publisher Location, Country, Year; pp. 154–196.
      3. Author 1, A.; Author 2, B. Title of the chapter. In *Book Title*, 2nd ed.; Editor 1, A.; Editor 2, B., Eds.; Publisher: Publisher Location, Country, Year; Volume 3, pp. 154–196.
    - Unpublished work, submitted work, personal communication:
      4. Author 1, A.B.; Author 2, C. Title of Unpublished Work. status (unpublished; manuscript in preparation).
      5.Author 1, A.B.; Author 2, C. Title of Unpublished Work. *Abbreviated Journal Name* stage of publication (under review; accepted; in press).
      6.Author 1, A.B. (University, City, State, Country); Author 2, C. (Institute, City, State, Country). Personal communication, Year.
    - Conference Proceedings:
      7. Author 1, A.B.; Author 2, C.D.; Author 3, E.F. Title of Presentation. In *Title of the Collected Work* (if available), Proceedings of the Name of the Conference, Location of

Conference, Country, Date of Conference; Editor 1, Editor 2, Eds. (if available); Publisher: City, Country, Year (if available); Abstract Number (optional), Pagination (optional).

- Thesis:
  8. Author 1, A.B. Title of Thesis. Level of Thesis, Degree-Granting University, Location of University, Date of Completion.
- Websites:
  9.Title of Site. Available online: URL (accessed on Day Month Year).
  Unlike published works, websites may change over time or disappear, so we encourage you create an archive of the cited website using a service such as WebCite. Archived websites should be cited using the link provided as follows:
  10. Title of Site. URL (archived on Day Month Year).

See the Reference List and Citations Guide for more detailed information.

Preparing Figures, Schemes and Tables

- All figure files should be separately uploaded during submission.
- Figures and schemes must be provided at a sufficiently high resolution (minimum 1000 pixels width/height, or a resolution of 300 dpi or higher). All Figure file formats are accepted. However, TIFF, JPEG, EPS and PDF files are preferred.
- *Marine Drugs* can publish multimedia files in articles or as supplementary materials. Please get in touch with the Editorial office for further information.
- All Figures, Schemes and Tables should also be inserted into the main text close to their first citation and must be numbered following their number of appearance (Figure 1, Scheme I, Figure 2, Scheme II, Table 1, *etc.*).
- All Figures, Schemes and Tables should have a short explanatory title and a caption placed above it.
- All table columns should have an explanatory heading. To facilitate the copy-editing of larger tables, smaller fonts may be used, but in no less than 8 pt. in size. Authors should use the Table option of Microsoft Word to create tables.
- For multi-panel figures, the file must contain all data in one file. For tips on creating multi-panel figures, please read the helpful advice provided by L2 Molecule.
- Authors are encouraged to prepare figures and schemes in color (RGB at 8-bit per channel). Full color graphics will be published free of charge.

[Return to top]

Qualification for Authorship

Authorship must include and be strictly limited to researchers who have substantially contributed to the reported work. To qualify for authorship, a researcher should have made a substantial contribution to the design of the study, or to the production, analysis or interpretation of the results. Authors should also have been involved in the preparation and have approved the submitted manuscript. Those who contributed to the work but do not qualify for authorship should be listed in the acknowledgments. According to the Committee on Publication Ethics (COPE) standard, to which this journal adheres, "all authors should agree to be listed and should approve the submitted and accepted versions of the publication. Any change to the author list should be approved by all authors including any who have been removed from the list. The corresponding author should act as a point of contact between the editor and the other authors and should keep co-authors informed and involve them in major decisions about the publication (e.g. answering reviewers' comments)." [1]

1. Wager, E.; Kleinert, S. Responsible research publication: international standards for authors. A position statement developed at the 2nd World Conference on Research Integrity, Singapore, July 22-24, 2010. In Promoting Research Integrity in a Global Environment; Mayer, T., Steneck, N., eds.; Imperial College Press / World Scientific Publishing: Singapore; Chapter 50, pp. 309-16.

[Return to top]

Correct Identification of Components of Natural Products

The correct identification of the various components of extracts from natural sources is of key importance, and as publishers we are keenly aware of our responsibility to the scientific community in this area. Consequently, for papers on this topic, we have adopted the recommendations of the Working Group on Methods of Analysis of the International Organization of the Flavour Industry (IOFI), as published in *Flavour Fragr. J.* **2006**, *21*, 185. These recommendations may be summarized as follows:

Any identification of a natural compound must pass scrutiny by the latest forms of available analytical techniques. This implies that its identity must be confirmed by at least two different methods, for example, comparison of chromatographic and spectroscopic data (including mass, IR and NMR spectra) with those of an authentic sample, either isolated or synthesized. For papers claiming the first discovery of a given compound from a natural source, the authors must provide full data obtained by their own measurements of both the unknown and an authentic sample, whose source must be fully documented. Authors should also consider very carefully potential sources of artifacts and contaminants resulting from any extraction procedure or sample handling.

[Return to top]

Research Ethics Guidelines

1. Research Involving Animals

The editors will require that the benefits potentially derived from any research causing harm to animals are significant in relation to any suffering endured by animals, and that procedures followed are unlikely to cause offense to the majority of readers. Authors should particularly ensure that their research complies with the commonly-accepted '3Rs':

- Replacement of animals by alternatives wherever possible,
- Reduction in number of animals used, and
- Refinement of experimental conditions and procedures to minimize the harm to animals.

Any experimental work must be conducted in accordance with relevant national legislation on the use of animals for research. Authors should follow the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines (http://www.nc3rs.org.uk/page.asp?id=1357) for reporting experiments using live animals. Authors may use the ARRIVE guidelines as a checklist (www.nc3rs.org.uk/ARRIVEchecklist).

An approval from an ethics committee must be obtained before undertaking the research. The project identification code, date of approval and name of the ethics committee or institutional review board should be cited in the Methods section.

Editors reserve the rights to reject any submission that does not meet these requirements.

An example of Ethical Statements:

The animal protocols used in this work were evaluated and approved by the Animal Use and Ethic Committee (CEUA) of the Institute Pasteur Montevideo (Protocol 2009_1_3284). They are in accordance with FELASA guidelines and the National law for Laboratory Animal Experimentation (Law no. 18.611).

2. Research Involving Human Subject

When reporting on research that involves human subjects, human material, human tissues or human data, authors must declare that the investigations were carried out following the rules of the Declaration of Helsinki of 1975 (http://www.wma.net/en/30publications/10policies/b3/), revised in 2008. According to point 23 of this declaration, an approval from an ethics committee should have been obtained before undertaking the research. As a minimum, a statement including the project identification code, date of approval and name of the ethics committee or institutional review board should be cited in the Methods Section of the article. Data relating to individual participants must be described in detail, but private information identifying participants need not be included unless the identifiable materials

are of relevance to the research (for example, photographs of participants' faces that show a particular symptom). A written informed consent for publication must be obtained from participating patients in this case.

Editors reserve the rights to reject any submission that does not meet these requirements.

Example of Ethical Statements:

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of XXX (Project identification code).

3. Research Involving Cell Line

Methods sections for submissions reporting on research with cell lines should state the origin of any cell lines. For established cell lines the provenance should be stated and references must also be given to either a published paper or to a commercial source. If previously unpublished *de novo* cell lines were used, including those gifted from another laboratory, details of institutional review board or ethics committee approval must be given, and confirmation of written informed consent must be provided if the line is of human origin.

An example of Ethical Statements:

The HCT116 cell line was obtained from XXXX. The $MLH1^{+}$ cell line was provided by XXXXX, Ltd. The DLD-1 cell line was obtained from Dr. XXXX. The DR-GFP and SA-GFP reporter plasmids were obtained from Dr. XXX and the Rad51K133A expression vector was obtained from Dr. XXXX.

[Return to top]

Potential Conflicts of Interest

It is the authors' responsibility to identify and declare any personal circumstances or interests that may be perceived as inappropriately influencing the representation or interpretation of clinical research. If there is no conflict, please state "The authors declare no conflict of interest". This should be conveyed in a separate "Conflicts of Interest" section preceding the "References" sections at the end of the manuscript. Financial support for the study must be fully disclosed in the "Acknowledgments" section.

[Return to top]

Editorial Procedure and Peer-Review

Initial Checks

All submitted manuscripts received by the Editorial Office will be checked by a professional in-house *Managing Editor* to determine whether it is properly prepared and whether the manuscript follows the ethical policies of the journal, including those for human and animal experimentation. Manuscripts that do not fit the journal or are not in line with our ethical policy may be rejected before peer-review. Manuscripts that are not properly prepared will be returned to the authors for revision and resubmission. The *Managing Editor* will consult the journals' *Editor-in-Chief*, the *Guest Editor* or an *Editorial Board member* to determine whether the manuscript fits the scope of the journal and whether it is scientifically sound. No judgment on the significance or potential impact of the work will be made at this stage.

Peer-Review

Once a manuscript passes the initial checks, it will be assigned to at least two independent experts for peer-review. A single-blind peer-review process is applied, where authors' names are revealed to reviewers. In-house assistant editors

generally invite experts recommended by the *Editor-in-Chief* or identified by literature searches. These experts may also include Editorial Board members and Guest Editors of the journal. Potential reviewers suggested by the authors may also be considered. Reviewers should not have published with any of the co-authors during the past five years and should not currently work or collaborate with one of the institutes of the co-authors of the submitted manuscript.

Editorial Decision and Revision

Based on the comments and advices of the peer-reviewers, an external editor – usually the *Editor-in-Chief* or a *Guest Editor* – will make a decision to accept, reject, or to ask authors to revise the manuscript.

For *Minor Revisions* the authors will have one week to resubmit their revised manuscript. For *Major Revisions* the authors will have two weeks to resubmit their revised manuscript. However, authors should contact the editorial office if extended revision time is anticipated.

Author Appeals

Authors may appeal a rejection by sending an e-mail to the Editorial Office of the journal. The appeal must provide a detailed justification, including point-by-point responses to the reviewers' and/or Editor's comments. The *Managing Editor* of the journal will forward the manuscript and relating information (including the identities of the referees) to an Editorial Board member who was not involved in the initial decision-making process. If no appropriate Editorial Board member is available, the editor will identify a suitable external scientist. The Editorial Board member will be asked to give an advisory recommendation on the manuscript and may recommend acceptance, further peer-review, or uphold the original rejection decision. A reject decision at this stage will be final and cannot be revoked.

Production and Publication

Once accepted, the manuscript will undergo professional copy-editing, English editing, proofreading by the authors, final corrections, pagination, and, publication on the www.mdpi.com website.

[Return to top]

Suggestion of Reviewers

During the submission process, authors have the possibility to suggest potential reviewers with the appropriate expertise to review the manuscript. The editors will not necessarily approach these referees. Please provide detailed contact information (address, homepage, phone, e-mail address). The proposed referees should neither be current collaborators of the co-authors nor have published with any of the co-authors of the manuscript within the last five years. Proposed reviewers should be from different institutions to the authors. You may identify appropriate Editorial Board members of the journal as potential reviewers. You may also suggest reviewers from among the authors that you frequently cite in your paper.

[Return to top]

English Corrections

This journal is published in English. To facilitate proper peer-reviewing of your manuscript, it is essential that it is submitted in grammatically correct English. If you are not a native English speaker, we strongly recommend that you have your manuscript professionally edited before submission or read by a native English-speaking colleague. Professional editing will mean that reviewers and future readers are better able to read and assess the content of your manuscript. For additional information see the English Editing Guidelines for Authors.

[Return to top]

Publication Ethics Statement

Marine Drugs is a member of the Committee on Publication Ethics (COPE). We fully adhere to its Code of Conduct and to its Best Practice Guidelines.

The editors of this journal take the responsibility to enforce a rigorous peer-review process together with strict ethical policies and standards to ensure to add high quality scientific works to the field of scholarly publication. Unfortunately, cases of plagiarism, data falsification, image manipulation, inappropriate authorship credit, and the like, do arise. The editors of *Marine Drugs* take such publishing ethics issues very seriously and are trained to proceed in such cases with a zero tolerance policy.

Authors wishing to publish their papers in *Marine Drugs* are asked to abide to the following rules:

- Any facts that might be perceived as a possible conflict of interest of the author(s) must be disclosed in the paper prior to submission.
- Authors should accurately present their research findings and include an objective discussion of the significance of their findings.
- Data and methods used in the research need to be presented in sufficient detail in the paper, so that other researchers can replicate the work.
- Raw data should preferably be publicly deposited by the authors before submission of their manuscript. Authors need to at least have the raw data readily available for presentation to the referees and the editors of the journal, if requested. Authors need to ensure appropriate measures are taken so that raw data is retained in full for a reasonable time after publication.
- Simultaneous submission of manuscripts to more than one journal is not tolerated.
- Republishing content that is not novel is not tolerated (for example, an English translation of a paper that is already published in another language will not be accepted).
- If errors and inaccuracies are found by the authors after publication of their paper, they need to be promptly communicated to the editors of this journal so that appropriate actions can be taken. Please refer to our policy regarding publication of publishing addenda and corrections.
- Your manuscript should not contain any information that has already been published. If you include already published figures or images, please obtain the necessary permission from the copyright holder to publish under the CC-BY license.
- Plagiarism, data fabrication and image manipulation are not tolerated.
    - **Plagiarism is not acceptable** in *Marine Drugs* submissions.

      Plagiarism includes copying text, ideas, images, or data from another source, even from your own publications, without giving any credit to the original source.

      Reuse of text that is copied from another source must be between quotes and the original source must be cited. If a study's design or the manuscript's structure or language has been inspired by previous works, these works must be explicitly cited.

      If plagiarism is detected during the peer review process, the manuscript may be rejected. If plagiarism is detected after publication, we may publish a correction or retract the paper.

    - **Image files must not be manipulated or adjusted in any way** that could lead to misinterpretation of the information provided by the original image.

      Irregular manipulation includes: 1) introduction, enhancement, moving, or removing features from the original image; 2) grouping of images that should obviously be presented separately (e.g., from different parts of the same gel, or from different gels); or 3) modifying the contrast, brightness or color balance to obscure, eliminate or enhance some information.

If irregular image manipulation is identified and confirmed during the peer review process, we may reject the manuscript. If irregular image manipulation is identified and confirmed after publication, we may correct or retract the paper.

Our in-house editors will investigate any allegations of publication misconduct and may contact the authors' institutions or funders if necessary. If evidence of misconduct is found, appropriate action will be taken to correct or retract the publication. Authors are expected to comply with the best ethical publication practices when publishing with MDPI.

[Return to top]

Supplementary Materials and Data Deposit

In order to maintain the integrity, transparency and reproducibility of research records, and to retain important chemical and structural information, authors are strongly encouraged to make their experimental and research data openly available either by depositing into data repositories or by publishing the data and files as supplementary information in this journal. Additional data and files can be uploaded as "Supplementary Files" during the manuscript submission process. The supplementary files will also be available to the referees as part of the peer-review process, although referees are not specifically asked to review these files. Accepted file formats include (but are not limited to):

- spectral data (NMR, IR, Raman, ESR, etc.) in JCAMP (JDX) format
- 3D coordinate structures (in PDB, MOL, XYZ or other common format)
- crystallographic information (in CIF format)
- data tables and spreadsheets (text files, MS Excel, OpenOffice, CSV, XML, *etc.*)
- text documents (text files, PDF, MS Word, OpenOffice, *etc.*; text documents will usually be converted to PDF files for publication)
- images (JPEG, PNG, GIF, TIFF, BMP, *etc.*)
- videos (AVI, MPG, QuickTime, *etc.*)
- executables (EXE, Java, *etc.*)
- software source code

Citations and References in Supplementary files are permitted provided that they also appear in the main text and in the reference list.

Large data sets and files should be deposited to specialized service providers (such as Figshare) or institutional/subject repositories, preferably those that use the DataCite mechanism. For a list of specialized repositories for the deposit of scientific and experimental data, please consult databib.org or re3data.org. The data repository name, link to the data set (URL) and accession number, doi or handle number of the data set must be provided in the paper. The journal *Data* (ISSN 2306-5729) also accepts submissions of data set papers, and the publication of small data sets along with the paper, and/or software source codes is encouraged.

Guidelines for Deposition of Sequences and of Expression Data

New sequence information must be deposited to the appropriate database prior to submission of the manuscript. Accession numbers provided by the database should be included in the submitted manuscript. Manuscripts will not be published until the accession number is provided.

- **New nucleic acid sequences** must be deposited in one of the following databases: GenBank, EMBL, or DDBJ. Sequences should be submitted to only one database.
- **New high throughput sequencing (HTS) datasets**(RNA-seq, ChIP-Seq, degradome analysis, …) must be deposited either in the GEO database or in the NCBI's Sequence Read Archive.
- **New microarray data** must be deposited either in GEO or ArrayExpress databases. The "Minimal Information About a Microarray Experiment" (MIAME) guidelines published by the Microarray Gene Expression Data Society must be followed.

- **New protein sequences** obtained by protein sequencing must be submitted to UniProt (submission tool SPIN). All sequence names and the accession numbers provided by the databases should be provided in the Materials and Methods
    - section of the article.