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A esponja marinha *Polymastia janeirensis* como fonte de novos fármacos contra o câncer

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“ Imagination is more important than knowledge.

Knowledge is limited,

imagination encircles the world.”

(Albert Einstein)

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RESUMO

Nos últimos anos, o ambiente marinho tem sido pesquisado para uma variedade de compostos com diferentes atividades biológicas. Entre todos os organismos marinhos, as esponjas representam uma das fontes mais promissoras de novas drogas contra o câncer. Embora o litoral brasileiro seja o segundo mais extenso depois da Austrália, existem poucos estudos com esponjas marinhas coletadas no Brasil objetivando a descoberta de novos fármacos. Nesse trabalho, nós investigamos os efeitos de diferentes extratos da esponja marinha *Polymastia janeirensis* na viabilidade de uma linhagem de glioma humano (U138MG). Além disso, avaliamos a seletividade do efeito, caracterizamos os mecanismos envolvidos no processo de morte celular e isolamos as frações ativas responsáveis pelas atividades encontradas. De uma maneira geral, um efeito antiproliferativo foi observado quando as células forma expostas aos extratos (aquoso e orgânico) da esponja. Entretanto, as doses mais altas (50 e 100 µg/ml) foram extremamente citotóxicas, inibindo mais de 80% a proliferação e a viabilidade celular. Além disso, uma morte celular por necrose foi observada com essas doses, enquanto uma morte celular por apoptose foi observada com a dose mais baixa (10 µg/ml). Nós também demonstramos que a rota apoptótica ativada em resposta aos extratos era a via intrínseca, e que a produção de radicais livres estava, pelo menos em parte, relacionada ao efeito bifásico (apoptose ou necrose) encontrado. Os resultados aqui apresentados demonstram a existência de uma seletividade do efeito citotóxico, uma vez que os extratos não induziram morte em culturas de astrócitos na dose em que foi observada morte por apoptose nos gliomas. Ademais, uma potente atividade apoptótica foi observada com o composto ativo isolado da esponja marinha alvo desse estudo. Os nossos resultados sugerem que a esponja marinha alvo desse estudo pode ser considerada uma boa fonte para o desenvolvimento de novas drogas contra o câncer.

ABSTRACT

Over the last years, the marine environment has been screened for a variety of compounds with different biological activities. Among all marine organisms, sponges represent one of the most promising source of new drug leads for cancer. Although Brazil has the second most extensive coastline after Australia, there are just few reports in which the authors have screened Brazilian sponge extracts for biological activities. In this work, we examine the anti-proliferative effects of crude extracts of the marine sponge *Polymastia janeirensis* in the U138MG human glioma cell line. Moreover, we investigate the effects on selective cytotoxicity in the glioma cells in comparison with a normal, untransformed cell culture, as well as examine the apoptotic pathway activated in response to treatments with extracts of *P. janeirensis*. Yet, we performed a bioassay-guided fractionation to found the active fractions in the extracts. Exposure of glioma cells to treatments resulted in cell number decrease with both aqueous and organic extracts. Moreover, sponge extracts reduced glioma cell viability. However, higher doses (50 e 100 µg/ml) induced a stronger cytotoxic effect when compared to the lower dose tested (10 µg/ml), inhibiting more than 80% of cellular growth and viability. Our results showed that sponge extracts caused necrosis in the glioma cell line at higher doses, while a high percentage of apoptotic glioma cells were observed at 10 µg/ml. We also demonstrated that marine sponge extracts induces oxidative cell death through a receptor-independent pathway under our assays condictions, and ROS productions may be related to the biphasic effect (apoptosis or necrosis) observed. Furthermore, our results suggested a selective cytotoxic effect on glioma cell line compared to a normal cell culture, since the effect on viability found in glioma cells was not observed in astrocyte cultures with the lower dose (10 µg/ml). Yet, a strong apoptotic activity was observed with the active fraction isolated from *P. janeirensis*. Our results suggest that this marine sponge may be considered a good source for new antiglioma metabolites.

LISTA DE ABREVIATURAS

U138MG - linhagem de glioma humano U138MG

TMZ - Temozolomida

Z-VAD - inibidor de caspases

Z-LEHD - inibidor específico de caspase-9

Z-IETD - inibidor específico de caspase-8

HPLC - high performance liquid chromatography

PDA detector - detector de arranjo de fotodiodos

ROS - espécies reativas de oxigênio

DCFH - diclorohidrofluoresceína

PI - iodeto de propídeo

PARP - poly-ADP-ribose polymerase

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APRESENTAÇÃO

Essa Tese sugere que a esponja marinha *Polymastia janeirensis* pode ser considerada uma boa fonte para o desenvolvimento de novas drogas contra o câncer. Os "Materiais e Métodos" e os "Resultados" estão apresentados na forma de artigos científicos. O primeiro artigo descreve um efeito citotóxico seletivo de diferentes extratos da esponja marinha em uma linhagem de glioma humano (U138MG), e foi aceito para publicação no periódico *Investigational New Drugs*, um dos mais importantes na área do desenvolvimento de novas drogas contra o câncer. O segundo artigo descreve o mecanismo de morte ativado em resposta aos extratos, bem como o envolvimento de espécies reativas de oxigênio nos efeitos encontrados. Esse artigo foi submetido para publicação no periódico *Toxicology in Vitro*. O terceiro trabalho descreve o isolamento da fração ativa responsável pela atividade antiproliferativa encontrada nos extratos, tendo sido esse submetido para publicação, como "Rapid Communication", no periódico *The Biological Bulletin*.

A "Discussão" acerca dos resultados obtidos nessa Tese contém uma ampla interpretação dos mesmos, além de importantes considerações no que se refere aos resultados encontrados na literatura específica.

As "Referências Bibliográficas" se referem àquelas apresentadas na "Introdução" e na "Discussão" dessa Tese. Por fim, são apresentadas as conclusões e as perspectivas geradas com esse trabalho.

I - INTRODUÇÃO

I. INTRODUÇÃO

I. 1 Produtos naturais de origem marinha

I. 1. 1 Um breve histórico

Nas últimas décadas, a bioquímica ecológica dos organismos marinhos tem emergido como uma interessante área a ser pesquisada. Os oceanos são fontes de um grande grupo de produtos naturais, e a descrição de novos compostos, muitas vezes únicos em suas estruturas, tem superado todas as expectativas (Faulkner, 2000). Esses potentes compostos marinhos são utilizados como defesa química contra predadores e parasitas, incluindo bactérias e fungos, mas também são conhecidos por modularem diversos processos biológicos (Pawlik et al., 1993; Proksch et al., 1994; Ebel et al., 1997).

Os primeiros estudos com compostos de origem marinha remetem ao início do século XX, com o isolamento de alguns esteróides de esponjas e de outros animais, de carotenóides presentes em diversos invertebrados marinhos, além de alguns estudos com toxinas encontradas em peixes da família Tetraodontideae (Doré, 1990; Henze, 1909; Lederer, 1938; Tahara, 1910). Todavia, foi somente a partir de 1950 que a atenção voltada à fauna marinha teve seu início. Com um trabalho pioneiro realizado por Bergman e seus colaboradores, foram isolados diversos arabino-nucleosídeos da esponja *Cryptotethya crypta* (Bergman e Feeney, 1950). Essa importante descoberta levou à síntese de análogos sintéticos como o Ara C e o Ara A (Kijjoa & Sawangwong, 2004). O Ara-C, atualmente vendido pela Pharmacia & Upjohn Company sob o nome de Cytosar-U ®, é um composto utilizado na terapia contra o câncer (Newman & Cragg, 2004). Já o composto Ara A, vendido sob o nome de Vidarabine ® ou Vidarabin Thilo ®, possui atividade antiviral (Kijjoa & Sawangwong, 2004). Além disso, outros

nucleosídeos foram obtidos a partir desses estudos, como o acyclovir e o AZT (Munro et al., 1993; Newman et al., 2000; Pettit et al., 2001).

I. 1. 2 Esponjas marinhas como fontes de novos fármacos

As esponjas (Filo Porifera) constituem um importante grupo no Reino Animal, sendo um modelo essencial na transição entre os organismos unicelulares e pluricelulares. Esses animais surgiram há mais de 550 milhões de anos, no período Pré-Cambriano, Era Paleozóica. No Cambriano Inferior já existiam representantes de todos os grupos encontrados recentemente (Bergquist, 1978) e aproximadamente 7.000 espécies das 15.000 estimadas, já foram descritas pelos pesquisadores (Hooper and Lévi, 1994). Aproximadamente 99% das espécies de esponjas ocorrem no ambiente marinho, e somente 1% das espécies encontra-se em ambientes de água doce.

O nome “Porifera” é justificado pela presença de pequenas aberturas na superfície, chamadas poros. Cada poro é formado por um porócito, uma célula em forma de anel que se estende da superfície externa até a cavidade central denominada átrio ou espongiocele. Internamente, a parede do corpo é revestida pelos coanócitos, que são células flageladas típicas dos poríferos. Os coanócitos promovem a filtração da água capturando microorganismos e partículas alimentares nela presentes. Após a filtração, a água é expelida para o meio externo através de uma abertura maior chamada ósculo. A parede corporal é relativamente simples, com a superfície externa revestida de células achatadas conhecidas como pinacócitos, que constituem a pinacoderme. Os pinacócitos secretam um material que fixa a esponja ao substrato. Por baixo da pinacoderme está o mesohilo, que consiste em uma matriz protéica gelatinosa contendo o material esquelético e células amebóides. O mesohilo é equivalente ao tecido conjuntivo dos outros metazoários. O esqueleto é relativamente complexo e proporciona uma estrutura

de sustentação para as células vivas do animal. A maioria das esponjas tem esqueleto formado por fibras de espongina juntamente com estruturas chamadas espículas, parecidas com pequenas agulhas cristalinas de sílica ou de carbonato de cálcio. O esqueleto que sustenta as esponjas é constituído por uma rede de espículas rígidas, fibras flexíveis e sedimentos externos, como areia. A combinação das dimensões, do tipo e da distribuição das espículas, bem como sua relação com o esqueleto fibroso, é a principal ferramenta utilizada para identificar esponjas.

As esponjas são organismos filtradores capazes de ingerir partículas de tamanho entre 5-50 μm através de células do mesohilo e da pinacoderme, e micropartículas de 0,3 a 1 μm pelas câmaras de coanócitos (Simpson, 1984). Um espécime da pequena esponja silicosa de 1 kg, *Geodia cydonium*, filtra 24.000 litros de água por dia (Vogel, 1977). Sésseis quando adultos, sua distribuição está condicionada à duração de seu curto período larval livre natante, em geral de poucas horas.

Devido a sua condição séssil e filtradora, as esponjas marinhas desenvolveram, ao longo da evolução, um efetivo sistema contra o estresse ambiental, produzindo toxinas e outros compostos para repelir e deter predadores (Uriz et al., 1996; Pawlik et al., 2002), para competir por espaço com outras espécies sésseis (Porter and Targett, 1998; Davis et al., 1991; Becerro et al., 1997), bem como para comunicação e proteção contra infecção. Quando comparadas a outros organismos marinhos, as esponjas possuem a maior taxa de compostos citotóxicos, com mais de 10% das espécies investigadas apresentando alguma atividade biológica (Osinga et al., 1998; Zhang et al., 2003). Além disso, dos 15.000 produtos marinhos descritos, as esponjas respondem por mais de 5.300 desses, e, a cada ano, centenas de novos compostos de origem marinha estão sendo descobertos. A considerável diversidade química desses animais compreende além de nucleosídeos incomuns, terpenos, esteróis, peptídios cíclicos,

alcalóides, ácidos graxos, até peróxidos e derivados de aminoácidos halogenados (Sipkema et al., 2005).

Extratos naturais contêm uma excepcional diversidade de moléculas e continuam sendo uma das fontes mais promissoras para o desenvolvimento de novos fármacos (Newman et al., 2003). Ademais, estudos realizados pelo *National Cancer Institute* dos EUA, retratam as esponjas marinhas como os organismos que mais produzem moléculas de alta singularidade com possível interesse farmacológico e potencial utilização no tratamento de doenças como o câncer, a AIDS e as leucemias (Kelecom et al., 1991). Neste contexto, extratos de diferentes espécies de esponjas marinhas têm sido examinados em várias linhagens tumorais celulares, bem como em modelos animais (Ahond et al., 1988; Burres et al., 1991; Fusetani et al., 1992; Gunasekera et al., 1991; Kobayashi et al., 1989; Perry et al., 1988; Sakai et al., 1986; ter Haar et al., 1996). Trabalhos com a utilização de extratos brutos de esponjas marinhas também têm demonstrado propriedades antimicrobianas, antifúngicas e antiinflamatórias para algumas espécies da região do Caribe, Mediterrâneo, Indo-Pacífico e Atlântico Oriental.

Entre os diferentes compostos isolados até hoje, a *Psammaplin A*, por exemplo, possui propriedades anti-câncer. Da mesma forma, uma relevante atividade antiviral em HIV-1 foi observada em algumas avaronas derivadas da esponja *Dysidea cinerea* (Hirsch et al., 1991). Um outro trabalho demonstrou a inibição de CDKs, GSK-3β e CK1 por *hymenialdisine* (HD), um composto isolado de várias espécies de esponjas marinhas, sugerindo que esta molécula possa contribuir nos estudos de doenças neurodegenerativas, como o Alzheimer e o Parkinson (Meijer et al., 2000).

Alguns compostos citados anteriormente já estão sendo investigados ou mesmo sendo desenvolvidos como novos fármacos (Faulkner, 2002). O princípio antiinflamatório manoalida de *Luffariella variabilis* já está disponível no mercado

(Monks et al., 2002). Em estudos pré-clínicos de Fase I encontram-se os compostos KRN7000 de *Agelas mauritianus* e IPL 576092 de *Petrosia contignata* e, em Fase II, o agente anti-câncer halicondrina B de *Halichondria okadai*.

Entretanto, um dos principais problemas que a indústria farmacêutica tem enfrentado no desenvolvimento de programas desse gênero é a dificuldade de obtenção do material biológico, tendo em vista as altas profundidades onde as esponjas em questão se encontram, além do fato do baixo rendimento dos compostos em análise; problemas esses diretamente relacionados a fatores ambientais e geográficos. Mesmo assim, apesar da recente literatura com essa abordagem, a existência de programas de pesquisa para a proteção de esponjas em locais como o Caribe e a Austrália acaba por refletir o maior número de trabalhos científicos com espécimes dessas regiões.

I. 1. 3 Potencial de esponjas marinhas brasileiras

O Brasil possui aproximadamente 8.000 km de costa litorânea (a segunda maior do mundo depois da Austrália), sendo um dos países com o maior índice de biodiversidade do mundo. Entretanto, poucos trabalhos explorando a diversidade química da nossa fauna marinha foram realizados, uma vez que o principal foco da química de produtos naturais brasileira foi direcionado, por muitos anos, ao estudo de plantas medicinais.

Características peculiares como fatores ambientais (exposição à alta intensidade de luz e altas pressões de oxigênio) e uma geografia propícia (águas rasas) fazem das esponjas do nosso litoral candidatas ideais para a obtenção de novos fármacos, tornando-se relevante a pesquisa sobre a aplicação dos mesmos no tratamento de doenças de alto impacto sócio-econômico. Sendo assim, a utilização de produtos naturais extraídos de esponjas marinhas brasileiras pode ser considerada uma importante

ferramenta para o desenvolvimento de novos medicamentos; alvo principal da indústria farmacêutica. Ademais, é importante destacar que o crescente estudo de propriedades farmacológicas em esponjas marinhas em outros países contribuiu para a criação de programas de preservação desses animais, bem como do eco-sistema em que eles vivem, e que a existência de programas de pesquisa com esponjas nessas regiões reflete o maior número de moléculas e patentes obtidas nesses países.

No que diz respeito à pesquisa com esponjas marinhas no Brasil, a maior parte dos trabalhos tinha como objetivo principal o inventariamento faunístico e o estudo taxonômico desses animais. Entretanto, um considerável aumento no número de trabalhos explorando as propriedades farmacológicas de esponjas coletadas no litoral brasileiro pode ser observado nos últimos anos. Entre as atividades encontradas nos extratos analisados, destacam-se atividades antimicrobianas (Muricy et al., 1993), citotóxica e neurotóxica (Rangel et al., 2001), anti-câncer (Monks et al., 2002) e desorganização de microtúbulos (Prado et al., 2004). Além disso, uma importante revisão sobre produtos naturais marinhos oriundos de organismos coletados no Brasil pode ser encontrada em Berlinck et al., 2004.

I. 2 Biologia dos gliomas

De acordo com a Organização Mundial de Saúde (OMS), a cada ano surgem nove milhões de novos casos de câncer no mundo. No Brasil, segundo o Ministério da Saúde, mais de quinhentos mil novos casos de câncer surgem por ano, e aproximadamente 100 mil pessoas morrem anualmente (www.saude.gov.br). Contudo, apesar da variedade de fármacos existentes para o tratamento de diferentes tipos de câncer, problemas referentes à baixa seletividade dos antineoplásicos e ao fenótipo de

resistência a múltiplas drogas dão sustentação para a busca por novas moléculas com propriedades antiproliferativas.

De particular interesse são os gliomas. Esse tipo de neoplasia primária ocorre principalmente no cérebro, podendo afetar também outras partes do sistema nervoso central, como a medula espinhal e os nervos ópticos. São tumores que se originam de células da linhagem astrocítica, possuindo um alto grau de malignidade e, apesar do tratamento agressivo com radioterapia e quimioterapia, a sobrevida costuma ser inferior a 12 meses. Na maioria dos casos, essas neoplasias se originam de mutações espontâneas em genes que controlam o ciclo celular ou a divisão celular. Além disso, os gliomas são caracterizados por alterações em suas rotas apoptóticas (Ziegler et al., 2008). Desta maneira, adquirem uma resistência intrínseca a esse mecanismo de morte celular, dificultando ainda mais o tratamento.

Um mecanismo de morte celular em resposta a vários agentes quimioterápicos, incluindo agentes alquilantes, é a morte celular programada, também chamada de apoptose. Em contraste à morte celular por necrose, a apoptose é fisiologicamente mais vantajosa, uma vez que as células mortas são fagocitadas antes da lise celular e da liberação de mediadores pró-inflamatórios (Shacter et al., 2000; Anderson et al., 2002; Anderson et al., 2003; Fadok and Henson, 2003; Lauber et al., 2004). Nesse sentido, um recente estudo demonstrou que Temozolomide (um novo agente alquilante utilizado na terapia contra gliomas) é capaz de induzir apoptose em uma linhagem de glioblastoma humano (U87MG) (Arabinda et al., 2004). Ademais, o uso desse novo fármaco tem aumentado a sobrevida dos pacientes em dois anos quando administrado durante e após a radioterapia (Stupp et al., 2005).

I. 3 Radicais livres e morte celular programada

Um aspecto fundamental do metabolismo aeróbico é a geração de espécies parcialmente reduzidas de oxigênio molecular, conhecidas como espécies reativas de oxigênio ou radicais livres. Sob condições fisiológicas, a fosforilação oxidativa é a principal fonte endógena desses radicais.

Diversos processos biológicos são modulados por radicais livres. Entretanto, quando existe um desequilíbrio entre a produção de espécies reativas de oxigênio e os mecanismos de defesas antioxidantes, ocorre um progressivo acúmulo de danos em biomoléculas (lipídios, proteínas e DNA), levando a célula à morte. Além disso, sabe-se que o excesso de radicais livres pode causar morte celular, mas o mecanismo de morte (necrose ou apoptose) depende, entre outros fatores, do tipo e da concentração de radicais produzidos no ambiente celular.

Apoptose, ou morte celular programada, é um complexo processo que requere a integração de diferentes sinais intracelulares, sendo um dos principais mecanismos pelos quais diferentes drogas citotóxicas matam células tumorais. Esse mecanismo de morte ocorre principalmente por duas rotas referidas como extrínseca ou intrínseca, sendo a primeira dependente da ligação de ligantes nos chamados receptores de morte encontrados na membrana celular, e a segunda, da liberação de sinais de morte da mitocôndria para o citoplasma. Ambas as rotas acabam convergindo e ativando a caspase-3, uma caspase executora que cliva proteínas intracelulares e causa morte celular.

Recentes trabalhos com produtos naturais de origem marinha têm demonstrado uma correlação entre a produção de radicais livres e a morte celular por apoptose. Entretanto, os mecanismos envolvidos nessa ativação, além da rota ativada (extrínseca ou intrínseca), ainda não são conhecidos.

I. 4 A esponja marinha *Polymastia janeirensis*

A esponja marinha *P. janeirensis* (Boury-Esnault, 1973), ordem Hadromerida, família Polymastiidae, apresenta uma coloração que pode variar entre tons de marrom, na sua parte incrustante, até uma intensa coloração marrom, azul ou laranja, nas suas projeções cilíndricas, podendo ser encontrada desde a zona entre-marés, até 39 m de profundidade (Figura 1).

Figura 1: Foto *in situ* da esponja marinha *P. janeirensis*.

Embora defesas químicas não possam ser diretamente equacionadas com potentes propriedades farmacológicas, é impressionante como se correlacionam na realidade. Isso é particularmente importante para a esponja *P. janeirensis*. Conforme observações de campo, essa esponja apresenta uma aparente falta de predadores naturais. Além disso, ao serem cortadas, no momento da coleta, ocorre a liberação de um líquido laranja com propriedades antimicrobianas na água. Entretanto, poucas

informações são conhecidas sobre essa espécie, e somente dois trabalhos descrevem propriedades biológicas em extratos de *P. janeirensis*. No primeiro (Monks et al., 2002), foi observado um efeito citotóxico em extratos orgânicos contra três linhagens de câncer (HT-29, U-373 e NCI-H460), com um IC_{50} entre 50 e 100 µg/ml. Além disso, extratos aquosos retardaram significativamente a migração de leucócitos em um ensaio quimiotático. Contudo, o modo de ação dessas atividades biológicas permanece desconhecido. Já no outro trabalho (da Silva et al., 2006), uma importante atividade antiviral foi observada em extrato aquoso contra o rotavírus RV-SA11. Nesse caso, o extrato inibia os últimos estágios de replicação do rotavírus.

Os únicos compostos isolados a partir de esponjas do gênero *Polymastia* encontrados na literatura são esteróis e tetrahidroxiamidas oriundos da esponja *P. tenax*, coletada na Colômbia. Esses compostos exibiram significante atividade citotóxica contra linhagens de carcinoma humano de pulmão (A-549), carcinoma humano de cólon (HT-29 e H-116) e carcinoma humano de próstata (Faulkner, 2001; Santafe et al., 2002). No entanto, os mecanismos envolvidos no processo antiproliferativo permanecem desconhecidos.

II - OBJETIVOS

II. OBJETIVOS

Extratos de esponjas marinhas possuem uma excepcional diversidade de moléculas, sendo uma das fontes mais promissoras para o desenvolvimento de novas drogas contra o câncer. Portanto, os objetivos desta Tese são:

1- Examinar o efeito antiproliferativo de extratos brutos da esponja marinha *P. janeirensis* coletada no litoral de Santa Catarina, Brasil, utilizando como modelo de estudo uma linhagem de glioma humano (U138MG);

2- Testar a seletividade dos extratos, utilizando uma cultura de células não transformadas;

3- Examinar a produção de radicais livres, o mecanismo de morte e as rotas ativadas;

4- Isolar, dos extratos da esponja marinha *P. janeirensis*, a fração ativa responsável pela atividade antiproliferativa encontrada.

III - ARTIGOS CIENTÍFICOS

III. 1 - Artigo 1

Brazilian marine sponge *Polymastia janeirensis* induces apoptotic cell death in human U138MG glioma cell line, but not in a normal cell culture

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PRECLINICAL STUDIES

Brazilian marine sponge *Polymastia janeirensis* induces apoptotic cell death in human U138MG glioma cell line, but not in a normal cell culture

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Summary Marine sponges have been prominently featured in the area of cancer research. Here, we examined the anti-proliferative effects of crude extracts (aqueous and organic) of the Brazilian marine sponge *Polymastia janeirensis* in the U138MG human glioma cell line. Moreover, we examined the effects of extracts on selective cytotoxicity in the glioma cells in comparison with a normal cell culture. Exposure of glioma cells to treatments (24 h) resulted in cell number decrease at all doses tested, with both aqueous and organic extracts (IC_{50} <20 and <30 μg/ml, respectively). Parallel to this result, sponge extracts reduced glioma cell viability (IC_{50} <15 μg/ml for both extracts). However, higher doses (50 and 100 μg/ml) induced a stronger cytotoxic effect when compared to the lower dose tested (10 μg/ml), inhibiting more than 80% of cellular growth and viability. Propidium iodide uptake and flow cytometry analysis further showed that sponge extracts caused necrosis in the glioma cell line at higher doses, while a high percentage of apoptotic glioma cells were observed at 10 μg/ml. Moreover, apoptosis was prevented by the pan-caspase inhibitor Z-VAD, suggesting that marine sponge extracts, at lower doses, induce caspase-dependent apoptosis in U138MG glioma cells. Surprisingly the extracts herein tested were more effective than temozolomide, a potent inductor of apoptosis used for the treatment of malignant gliomas. Furthermore, our results suggested a selectivity cytotoxic effect on glioma cell line in comparison with a normal cell culture, since the effect on viability found in glioma cells was not observed in astrocyte cultures with the lower dose (10 μg/ml). Thus, this marine sponge may be considered a good candidate for development of new cancer medicines with antitumor activity against gliomas.

Keywords Cancer · Glioma cells · Apoptosis · Marine sponges · *Polymastia janeirensis* · New drugs

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Introduction

Cancer is considered a public health problem in developed and in developing countries. In Brazil, it is estimated that 100,000 people die annually [1]. Of particular interest are the malignant gliomas. These tumors arise from cells of the astrocytic lineage, and are considered the most devastating primary tumors in the brain, representing 50–60% of this type of tumor. As a result of high proliferation, invasivity, and resistance to radiation [2], the prognosis for patients with malignant gliomas is poor and the mean survival is less than 12 months [3].

Despite the intense efforts to develop treatments, effective agents are still not available. Therefore, it is of seminal importance to provide new drug leads that may be developed into new cancer medicines with antitumor activity against gliomas. In this regard, natural products extracts continue to be the most promising source of new drug leads for cancer. They contain an exceptional diversity of chemotypes, suitable for high throughput screening and further development by combinatorial synthesis, molecular modeling, and structure versus activity studies [4].

Over the last decades, the marine environment has been screened for a variety of compounds with different biological activities. Among all marine organisms, sponges represent one of the most promising source of marine bioactive compounds particularly for pharmaceutical leads [5, 6]. In order to purify new active compounds with biological activities and potential application in biomedicine, extracts from marine sponges around the world have been examined for antineoplastic activity in various tumor cell lines, as well as in animal models [7–14]. At least two drugs (Ara-A and Ara-C) synthetically derived from sponge metabolites have been clinically used in long-term cancer treatments [15]. Furthermore, sponges also have been screened for a variety of other biological activities, e.g., antimicrobial, haemolytic, hemagglutinating, ichthyotoxic, lethal properties and others [16–18]. However, there are just few reports in which the authors have screened Brazilian sponge extracts for biological activities. To date, only limited screening evaluations of extracts of Brazilian marine sponges have been reported [19–22].

In this report, we examine the anti-proliferative effects of crude extracts (aqueous and organic) of the Brazilian marine sponge *Polymastia janeirensis* in the U138MG human glioma cell line. Moreover, we examine the effects of extracts on selective cytotoxicity in the glioma cell line in comparison with a normal, untransformed cell culture. This study is part of a collaborative program among several Brazilian institutions (Centro de Estudos em Estresse Oxidativo, Universidade Federal do Rio Grande do Sul; Fundação Zoobotânica do Rio Grande do Sul, Museu de Ciências Naturais; Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul; and Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul) for the collection and screening of Brazilian marine sponges for biological activities, with the aim of identifying new sponges species and novel molecules with promising and potentially useful therapeutic activities.

Materials and methods

Sponge sampling, identification and extract preparation

Sponges samples were collected manually from exposed and semi-exposed habitats, at depths of between 0.5 and 20 m, from locations on the coastline of the Estate of Santa Catarina (southern Brazil). Taxonomic designation was based on scanning electron microscope studies and on skeletal slides and dissociated spicule mounts. Specimens of all materials are deposited in the Museu de Ciências Naturais-Porifera collection of the Fundação Zoobotânica do Rio Grande do Sul, Brazil. Aqueous and organic extracts were obtained as previously described [20].

Cell cultures

U138MG human glioblastoma cell line was obtained from the American Type Culture Collection (Rockville, Maryland, USA). Cells were grown in culture flasks in Dulbecco's modified Eagle's medium (DMEM)/15% fetal bovine serum (FBS) (*v*/*v*) (Cultilab, Campinas, SP, Brazil) and seeded in 24-well plates (TTP plates) at densities of 1×10^4 cells/well in 500 μl medium per well. Culture cells were maintained in 5% CO_2/95% air at 37 °C and allowed to grow to confluence.

Primary astrocyte cultures were prepared as previously described [23]. Briefly, hippocampus, cortex and cerebellum of newborn Wistar rats (1–2 days old) were removed, and dissociated mechanically in a Ca^{2+}- and Mg^{2+}-free balanced salt solution pH 7.4, containing 137 mM NaCl, 5.36 mM KCl, 0.27 mM Na_2HPO_4, 1.1 mM KH_2PO_4, 6.1 mM glucose. After centrifugation at 1,000 rpm for 5 min the pellet was resuspended in culture medium (pH 7.6) containing 1% DMEM, 8.39 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), (pH 7.6), 23.8 mM NaHCO3, 0.1% fungizone, 0.032% garamicine and 10% fetal calf serum from Gibco. The cells were plated at a density of 1.5×10^5 cells cm^{-2} onto 24 multiwell plates pretreated with poly-L-lysine. Cultures were maintained in 5% CO_2/ 95% air at 37 °C and allowed to grow to confluence and used at 21–28 days in vitro. Medium was changed every 3–4 days. Immunocytochemical studies (not shown) revealed that >95% of the cells in our cultures stained for glial fibrillary acidic protein.

Treatments

Immediately before experiments, both the aqueous and organic extracts were dissolved in water and dimethyl sulfoxide (DMSO), respectively, at a concentration of 1 mg/ml (*w*/*v*). The amount of DMSO (maximum 0.25%) was proven not to affect the experiments. The final concentrations of the extracts tested ranged from 10 to 100 μg/ml. After reaching subconfluence, the cultures were exposed to sponge extracts for 24 h. Control cultures were performed with DMSO (0.25% final concentration) in the absence of extracts.

Cell counting

At the end of the treatment (24 h), the medium was removed, cells were washed with phosphate-buffered saline (PBS) and 200 μl of 0.25% trypsin/ethylenediaminetetra-acetic acid (EDTA) was added to detach the cells, which were counted in a hemocytometer.

Assessment of glioma cell viability

Following treatments, cell viability was assessed by the 3-(4,5-dimethyl)-2,5 diphenyl tetrazolium bromide (MTT) assay. This method is based on the ability of viable cells to reduce MTT and form a blue formazan product. MTT solution (sterile stock solution of 5 mg/ml) was added to the incubation medium in the wells at a final concentration of 0.5 mg/ml. The cells were left for 60 min at 37 °C in a humidified 5% CO_2 atmosphere. The medium was then removed and plates were shaken with DMSO for 30 min. The optical density of each well was measured at 550 nm (test) and 690 nm (reference). Results were expressed as the percentage of cell viability against the control.

Propidium iodide assay

Cellular damage was assessed by fluorescent image analysis of propidium iodide (PI) uptake. PI is excluded from healthy cells, but following loss of membrane integrity enters cells, binds to DNA and becomes highly fluorescent. At the end of the treatment, glioma cells were incubated with PI (7.5 μmol/l) for 1 h. PI fluorescence was excited at 515–560 nm using an inverted microscope (Nikon Eclipse TE300; Nikon Inc., Melville, New York, USA) fitted with a standard rhodamine filter and a minimum of 200 cells was counted per treatment in four different fields. The presence of stained, enlarged nuclei with normal structure was scored as a necrotic cell and the results were expressed as a ratio of PI labeled cells/total number cells. Images were captured using a digital camera connected to the microscope.

Flow cytometry analysis

For annexin V/PI (AV/PI) staining, treated glioma cells (1×10^6) were washed twice in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.2H_2O$, 1.4 mM KH_2PO_4, pH 7.4) containing 1 mM EDTA (PBS-EDTA) and subsequently trypsinized with 0.13 g/L trypsin in PBS-EDTA. Trypsin was inhibited with bovine foetal serum 10%, and medium washes, and cells were combined and centrifuged (5 min, 200×g, 4 °C). Cells were allowed to recover from trypsinization in complete medium (30 min, 37 °C). Externalized phosphatidylserine was labeled (15 min, 0 °C) with 5 μl fluorescence (FITC)-conjugated annexin V in 80 μl binding buffer (10 mM HEPES pH 7.4, 145 mM NaCl, 5 mM KCl, 1.0 mM $MgCl_2.6H_2O$, 1.8 mM $CaCl_2.2H_2O$). Propidium iodide 2 μM was added 10 min prior to analysis on a FACScalibur flow cytometer (BD PharMingen). When green fluorescence was plotted against red fluorescence (PI), distinct cell populations could be detected: viable cells (FITC−/PI−), apoptotic cells (FITC+/PI− and FITC+/PI+), and necrotic cells (FITC−/PI+), as previously described [24]. Ten thousand cells were analyzed per sample, and data were reported as the percentage of apoptotic cells and necrotic cells. Temozolomide (3,4-dihydro-3 -methyl-4-oxoimidazo-(5,1-d)-1,2,3,5-tetrazin-8-carboxamide, TMZ), a cytotoxic alkylating agent [25], was used as a control of cell death.

Statistical analysis

Results were expressed as the mean±SEM of at least three independent experiments. Data were analyzed by a one-way analysis of variance (ANOVA), using a Newman Keuls test to compare mean values across groups. When appropriate, Student's *t*-test was performed. Differences were considered to be significant when $p<0.05$. Dose response curves were plotted, and the IC_{50} values (concentrations at which cellular effects are inhibited by 50%) were calculated using non-linear regression analysis.

Results

Marine sponge extracts decreased U138MG glioma cell growth and viability

In order to investigate the effect of marine sponge extracts on proliferation/viability of U138MG glioma cell line, cell cultures were treated with extracts, the cell number was counted and a MTT assay was performed. Exposure of glioma cells to treatments for 24 h resulted in cell number decrease at all doses tested, with both aqueous and organic extracts (Fig. 1), with an IC_{50} <20 and <30 μg/ml,

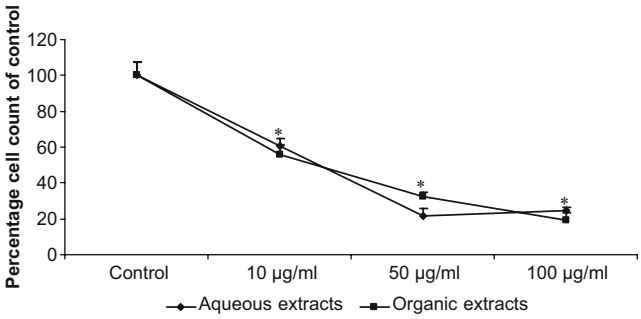

Fig. 1 Effect of marine sponge extracts on growth of U138MG glioma cells. Cells were treated for 24 h with extracts (10, 50 or 100 μg/ml) or DMSO (control), and counted in a hemocytometer as described in "Material and methods". Cell count in samples treated with DMSO was considered 100% of cell number. Data are expressed as means±S.E.M for three individual experiments. *Statistically different from control, $p<0.05$ (one-way ANOVA)

respectively. However, we observed that higher doses (50 and 100 μg/ml) of both extracts have stronger cytotoxic effects, inhibiting more than 80% of glioma cellular growth.

Parallel to this result, sponge extracts treatments also resulted in a reduction of glioma cell viability, as evidenced by a diminished ability of glioma cells to reduce MTT, with an IC_{50} <15 μg/ml for both aqueous and organic extracts (Fig. 2). Moreover, similar to cell counting, higher doses also had stronger cytotoxic effects. The reduction in MTT staining suggests not only glioma cell damage, but also a decrease in cell proliferation when compared with the control.

Cell death induced by marine sponge extracts

To determine whether the suppression of glioma cell proliferation was due to the induction of necrosis, glioma cells were treated for 24 h and then analyzed for cell membrane permeability by PI. As shown in Fig. 3, PI incorporation was higher in cells treated with both aqueous and organic extracts at 50 and 100 μg/ml, suggesting that these doses caused a loss of membrane integrity, which is an indication of cell death by necrosis. However, at 10 μg/ml, this cell death pattern was not observed.

Since higher doses decreased cell viability and induced necrosis, and the lower dose did not induce necrosis, we decided to investigate whether the decrease of glioma cell viability at 10 μg/ml was due to the induction of apoptosis. Glioma cells then were pre-incubated with membrane-permeable pan-caspase inhibitor (*N*-benzyloxycarbonyl-valyl-alanyl-aspartyl-fluoromethylketone, Z-VAD-fmk) for 1 h before treatment with extracts. At the end of treatment, Annexin V-FITC-bound phosphatidylserine and red fluorescence of DNA-bound PI in individual cells were measured using a flow cytometer as described on "Materials and methods". As shown in Fig. 4, 10 μg/ml of both aqueous and organic extracts resulted in a higher incidence of cell death that was almost entirely apoptotic. Furthermore, cell death was prevented by the pan-caspase inhibitor Z-VAD, suggesting that marine sponge extracts, at lower dose, induces caspase-dependent apoptosis in U138MG glioma cells. It is noteworthy that TMZ (100 μM), a new cytotoxic alkylating agent used in therapy for malignant gliomas and a potent inhibitor of cell growth and angiogenesis at non-toxic doses was less effective than marine sponge extracts under our assay conditions.

Selective cytotoxic effect of marine sponge extracts on U138MG glioma cells

To evaluate the selective cytotoxic effect of marine sponge extracts of *P. janeirensis* on glioma cell cultures, primary astrocyte cultures were treated for 24 h and an MTT assay was performed. Exposure of astrocyte cultures to treatments resulted in a decrease in cell viability at concentrations of 50 and 100 μg/ml (>90% for both aqueous and organic extracts), as evidenced by a decreased ability of astrocyte cells to reduce MTT. However, treatment with 10 μg/ml of aqueous and organic extracts did not decrease cell viability in astrocyte cultures, suggesting that this dose decreases cell viability only in glioma cells. Interestingly, 10 μg/ml of both aqueous and organic extracts induced apoptotic cell death in glioma cells, while 50 and 100 μg/ml induced only necrosis (Fig. 5).

Discussion

Malignant gliomas occur more frequently than other types of primary central nervous system tumors. Even with aggressive treatment using surgery, radiation, and chemotherapy, the mean reported survival is less than 1 year [2].

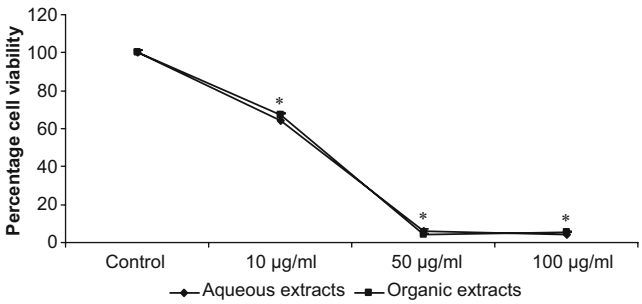

Fig. 2 Effects of marine sponge extracts on U138MG glioma cell viability. Cell cultures were exposed to extracts (10, 50 or 100 μg/ml) for 24 h and the cell viability was assessed by the MTT assay as described in "Material and methods". Glioma cultures treated with DMSO (control) was taken as 100% of cell viability. Data are the means±S.E.M for three individual experiments, and results were expressed as the percentage of cell viability against the control. *Statistically different from control, $p<0.05$ (one-way ANOVA)

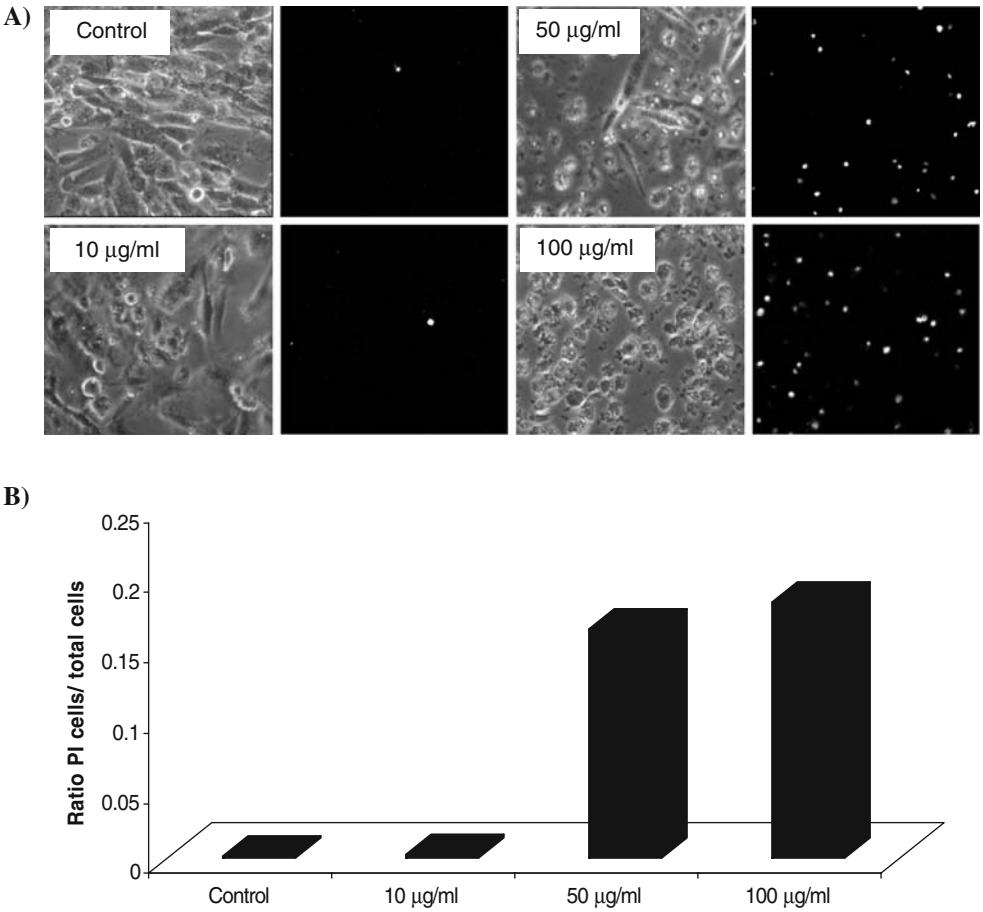

Fig. 3 Representative pictures (**a**) and analysis (**b**) of U138MG cell cultures stained with propidium iodide. At the end of the treatments, the cells were incubated with PI for 1 h. After, PI incorporation was visualized using a Nikon inverted microscope. The results were expressed as ratio PI labeled cells/total number cells and are representative for aqueous extract. Similar effects were observed in the organic extracts-treated cells

Today, current therapy for malignant glioma employs TMZ, an imidazole tetrazinone whose the methylation of DNA seems to be the principal mechanism responsible for the cytotoxicity to malignant cells. Nevertheless, it was clear from several studies that a significant proportion of tumors do not respond to TMZ therapy [26–29].

In the present study, the ability of crude extracts (aqueous and organic) of the Brazilian marine sponge *P. janeirensis* to inhibit the proliferation of U138MG glioma cells was examined. Results from cell counting showed that marine sponge extracts, at all doses tested, inhibited the proliferation of glioma cells. In addition, the glioma cell

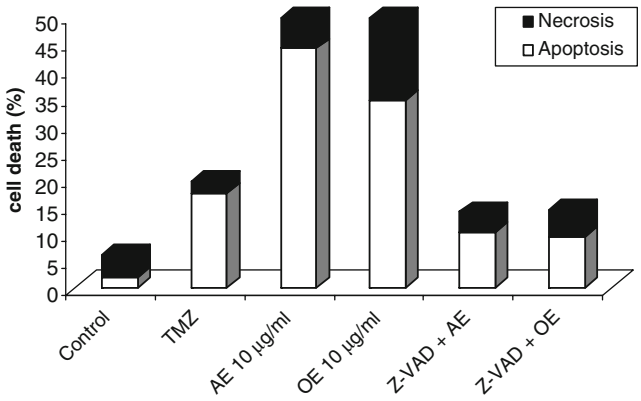

Fig. 4 Flow cytometry analysis. Annexin V-FITC-bound phosphatidylserine and red fluorescence of DNA-bound PI in individual glioma cells were measured using a flow cytometer as described under "Materials and methods". *TMZ* temozolomide, *AE* aqueous extract, *OE* organic extracts, *Z-VAD* pan-caspase inhibitor (100 µM). The data represent averages from at least three independent experiments carried out in triplicate

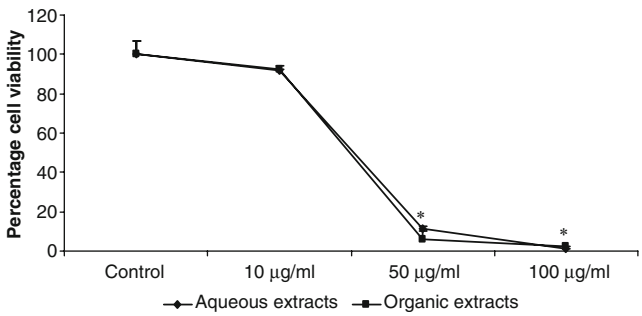

Fig. 5 Effects of marine sponge extracts on primary astrocyte cultures viability. Cells were exposed to extracts (10, 50 or 100 µg/ml) for 24 h and the cell viability was assessed by the MTT assay as described in "Material and methods". Astrocyte cultures treated with DMSO (control) was taken as 100% of cell viability. Data are the means±S.E.M for three individual experiments, and results were expressed as the percentage of cell viability against the control. *Statistically different from control and from10 µg/ml, $p<0.05$ (one-way ANOVA)

viability was estimated by MTT assay, and our data showed that extracts of *P. janeirensis* also reduced the cell viability of glioma cultures. Nevertheless, we observed that 50 and 100 μg/ml doses were more cytotoxic than 10 μg/ml, inhibiting more than 80% of cellular growth and cell viability.

Apoptosis, or programmed cell death, is characterized by a number of well-defined features which include condensation and fragmentation of the chromatin, internucleosomal DNA cleavage, membrane blebbing, caspase activation, translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane, and the ultimate formation of so called "apoptotic bodies" [30]. In contrast to necrotic cell death, apoptotic cell death is thought to be physiologically advantageous because the dying cells are cleared by phagocytosis prior to cell lysis and release of potentially inflammatory mediators [31–35].

Thus, in order to investigate whether the antiproliferative effects of the extracts were related to necrotic or apoptotic cell death, the PI uptake by glioma cells and the flow cytometry analysis were performed following treatments. Our results showed that both extracts of sponge were necrotic in the glioma cell line at higher doses (50 and 100 μg/ml), while a high percentage of apoptotic glioma cells was observed at 10 μg/ml.

Apoptotic cell death occurs through at least two overlapping pathways referred to as extrinsic and intrinsic. The extrinsic pathway is activated through ligand binding to death receptors (members of the tumor necrosis factor receptor superfamily) at the cell surface and activation of initiator caspase-8. In the intrinsic pathway, mitochondria play a pivotal early role by releasing cell death signals into the cytosol and activating caspase-9 [36]. Both pathways converge and activate executioner caspase-3, which then cleaves intracellular protein substrates and causes cell death [37].

We found that cell death was prevented by the pan-caspase inhibitor Z-VAD, suggesting that marine sponge extracts, at lower doses, induce caspase-dependent apoptosis in U138MG glioma cells. However, additional studies are necessary to determine which apoptotic pathway (extrinsic or intrinsic) was activated in response to treatments. Our preliminary data suggest that marine sponge extracts cause apoptosis through a receptor-independent pathway, since cell death was prevented by the caspase-9 inhibitor Z-LEHD, but not by the caspase-8 inhibitor Z-IETD (data not shown).

Liu et al. (2005) demonstrated that geoditin A, an isomalabaricane triterpene isolated from marine sponge *Geodia japonica*, induced reactive oxygen species, decreased mitochondrial membrane potential and mediated a caspase-3 apoptosis pathway on human promyelocytic leukemia HL60 cells [38]. However, since caspase-3 is an effector caspase that converge both pathways, the precise apoptotic rote activated in this study (receptor-dependent or receptor-independent) remains unclear. In other work, the same authors have been demonstrated that stellettin A, also isolated from *G. japonica*, induces oxidative cell death through a FasL-caspase-3-apoptotic pathway [39].

One mechanism of tumor cell death in response to various chemotherapeutic drugs including alkylating agents [40–42] is programmed cell death or apoptosis. Surprisingly, a new cytotoxic alkylating agent used in therapy for malignant gliomas (TMZ) was less effective than marine sponge extracts under our assay conditions. In addition, the cytotoxic effect of marine sponge extracts on primary astrocyte cultures was evaluated. An important feature of the present study is that the effect of sponge extracts on viability found in glioma cell line was not observed in astrocyte cultures with the lower dose (10 μg/ml), suggesting a selective cytotoxic effect of this dose on U138MG glioma cell. Interestingly, this is the same concentration that was able to induce apoptosis in the glioma cells, which is generally believed to be more physiologically beneficial than necrosis [30–33, 43].

This biphasic effect (apoptosis or necrosis) observed in glioma cells may be related to the redox state of the cells, as well as the redox properties of the extracts, and this point needs further investigation. It is clear that excess levels of free radicals can cause cell death, but the mode of death (i.e. necrotic, apoptotic, other) depends on the type, concentration, source and environment of the oxidant(s) [44]. Moreover, additional experiments should be performed to elucidate the differences between glioma and astrocyte cells effects, but one possible explanation is the distinct signaling pathways in the cells compared here.

Marine natural products have been prominently featured in the area of cancer research. It is interesting to note that out of 18 preclinical investigating anticancer agents derived from marine sources, six are from sponges [45–47], implying that marine sponges are potential resources for new anti-cancer agents. However, there are just few reports in which the authors have screened Brazilian sponge extracts for biological activities [19–22]. Furthermore, extracts from Brazilian sponges have not been examined for specific effects on the cell death-regulatory pathways.

Of particular interest is the marine sponge *P. janeirensis*. To date, little information is known about this specie, and the only detected classes of compounds already described in the reviewed literature for the genus *Polymastia* are sterols and tetrahidroxiamide [48, 49]. Moreover, only two works reported biological properties from *P. janeirensis*. In an interesting work, Monks et al. (2002) described a cytotoxic effect with organic extracts against three human tumor cell lines (HT29, U373 and NCI-H460) with IC_{50} concentrations ranging from 50 to 100 μg/ml, as deter-

mined by sulforhodamine assay. Moreover, aqueous extracts significantly retarded the migration of polymorphonuclear leukocytes in a chemotactic assay [20]. However, the mode of action underlying these biological activities remains unclear. In the other work, an in vitro antiviral activity was observed against the simian rotavirus RV-SA11 with aqueous extract of *P. janeirensis*, indicating that the compounds found in the extract inhibited the late stages of rotavirus replication [50].

To our knowledge, this is the first report demonstrating that marine sponge extracts of *P. janeirensis* induce cell death and, at lower dose, induces caspase-dependent apoptosis in U138MG glioma cells. Furthermore, our results suggested a selective cytotoxic effect on glioma cell line compared to a normal cell culture, since the effect on viability found in glioma cells was not observed in astrocyte cultures with the lower dose (10 μg/ml). Besides, the extracts herein tested were more effective than TMZ, a potent inductor of apoptosis used for the treatment of malignant gliomas. Thus, this marine sponge may be considered as a good candidate for investigations and development of news molecules, and further work to purify and characterize the chemical structure(s) of the substance (s) involved might yield new active compounds with biological activities and potential in glioma cell treatment. In this regard, bioassay-guided micro-fractionation by high performance liquid chromatography with either photodiode array or evaporative light-scattering detectors will be performed. Once founded, the chemical structure of the active constituents will be established by usual spectroscopic analysis.

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References

1. Instituto Nacional do Câncer; Ministério da Saúde. Secretaria de Atenção à Saúde. Estimativas 2008: Incidência de Câncer no Brasil. Rio de Janeiro, Brasil: INCA, 2007. Available from: http://www.saude.gov.br/
2. Avgeropoulos NG, Batchelor TT (1999) New treatment strategies for malignant gliomas. Oncologist 4:209–224
3. Holland EC (2001) Gliomagenesis: genetics alterations and mouse models. Nature 2:120–129
4. Newman DJ, Cragg GM, Snader KM (2003) Natural products as sources of new drugs over the period 1981–2002. J Nat Prod 66:1022–1037
5. Proksch P (1994) Defensive roles for secondary metabolites from marine sponges and sponge-feeding nudibranchs. Toxicon 32:639–655
6. Faulkner DJ (1998) Marine natural products. Nat Prod Rep 15 (2):113–158
7. Ahond A, Zurita MB, Colin M, Fizames C, Laboute P, Lavelle F, Laurent D, Poupat C, Pusset J, Pusset M, Thoisson O, Potier P (1988) Girolline, a new antitumour substance from sponge *Pseudaxinyssa cantharella* (Axinellidae). C R Acad Sci Paris 307:145–148
8. Burres NS, Barber DA, Gunasekera SP, Shen LL, Clement JJ (1991) Antitumor activity and biochemical effects of topsentin. Biochem Pharmacol 42:745–751
9. Fusetani N, Sugawara T, Matsunaga S (1992) Theopederins A–E, potent antitumor metabolites from a marine sponge *Theonella* sp. J Org Chem 57:3828–3832
10. Gunasekera SP, Gunasekera M, McCarthy P (1991) Discodermide: a new bioactive macrocyclic lactam from the marine sponge *Discodermia dissoluta*. J Org Chem 56:4830–4833
11. Kobayashi MJ, Murayama Y, Ohizumi Y, Sasaki T, Ohta T, Nozoe S (1989) Theonelladins a–d, novel antineoplastic pyridine alkaloids from the Okinawan marine sponge *Theonella swinhoei*. Tetrahedron Lett 30:4833–4836
12. Perry NB, Blunt JW, Munro MHG, Higa T, Sakai R (1988) Discorhabdin D, an antitumor alkaloid from the sponges *Latrunculia brevis* and *Prianos* sp. J Org Chem 53:4127–4128
13. Sakai R, Higa T, Jefford CW, Bernardinelli G (1986) Manzamine a, a novel antitumor alkaloid from a sponge. J Am Chem Soc 108:6404–6405
14. ter Haar E, Kowalski RJ, Hamel E, Lin CM, Longley RE, Gunasekera SP, Rosenkranz HS, Day BW (1996) Discodermolide, a cytotoxic marine agent that stabilizes microtubules more potently than taxol. Biochemistry 35:243–250
15. Munro MHG, Blunt JW, Lake RJ, Litaudon M, Battershill CN, Page MJ (1994) From seabed to sickbed: what are the prospects. In: van Soest RWM, van Kempen ThMG, Braekman JC (eds) Sponges in time and space. Balkema, Rotterdam, Netherlands, pp 473–484
16. Green G (1977) Ecology of toxicity in marine sponges. Mar Biol 40:207–215
17. Mebs D, Weiler I, Heinke HF (1985) Bioactive proteins from marine sponges: screening of sponge extracts for hemagglutinating, haemolytic, ichthyotoxic and lethal properties and isolation and characterization of hemagglutinins. Toxicon 23:955–962
18. Thompson JE, Walker RP, Faulkner DJ (1985) Screening and bioassays for biologically active substances from forty marine sponges species from San Diego, CA, USA. Mar Biol 88:11–21
19. Muricy G, Hajdu E, Araújo FV, Hagler NA (1993) Antimicrobial activity of southwestern Atlantic shallow-water marine sponges (Porifera). Scientia Marina 57:427–432
20. Monks NR, Lerner C, Henriques AT, Farias FM, Schapoval EES, Suycnaga ES, da Rocha AB, Schwartsmann G, Mothes B (2002) Anticancer, antichemotactic and antimicrobial activities of marine sponges collected off the coast of Santa Catarina, southern Brazil. J Exp Mar Bio Ecol 281:1–12
21. Prado MP, Torres YR, Berlink RGS, Desiderá C, Sanchez MA, Craveiro MV, Hajdu E, da Rocha RM, Machado-Santelli GM (2004) Effects of marine organisms extracts on microtubule integrity and cell cycle progression in cultured cells. J Exp Mar Bio Ecol 313:125–137
22. Rangel M, de Sanctis B, de Freitas JC, Polatto JM, Granato AC, Berlinck RGS, Hajdu EJ (2001) Cytotoxic and neurotoxic activities in extracts of marine sponges (Porifera) from southeastern Brazilian coast. Exp Mar Biol Ecol 262:31–40
23. Pinto SS, Gottfried C, Mendez A, Gonçalves D, Karl J, Gonçalves CA, Wofchuk S, Rodnight R (2000) Immunocontent and secretion of S100B in astrocyte cultures from different brain regions in relation to morphology. FEBS Lett 486(3):203–207
24. Huigsloot M, Tijdens IB, Mulder GJ, Van de Water B (2002) Differential regulation of doxorubicin-induced mitochondrial dysfunction and apoptosis by Bcl-2 in mammary adenocarcinoma (MTLn3) Cells. J Biol Chem 277:35869–35879

25. Kurzen H, Schemitt S, Naher H, Mohler T (2003) Inhibition of angiogenesis by non-toxic doses of temozolomide. Anticancer Drugs 14:515–522
26. Stupp R, Dietrich PY, Ostermann Kraljevic S, Pica A, Maillard I, Maeder P, Meuli R, Janzer R, Pizzolato G, Miralbell R, Porchet F, Regli L, de Tribolet N, MirimanoV RO, Leyvraz S (2002) Promising survival for patients with newly diagnosed glioblastoma multiforme treated with concomitant radiation plus temozolomide followed by adjuvant temozolomide. J Clin Oncol 20:1375–1382
27. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E, MirimanoV RO, European Organisation for R, Treatment of Cancer Brain T, Radiotherapy G, National Cancer Institute of Canada Clinical Trials G (2005) Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. New Engl J Med 352:987–996
28. Yung WK, Prados MD, Yaya-Tur R, Rosenfeld SS, Brada M, Friedman HS, Albright R, Olson J, Chang SM, O'Neill AM, Friedman AH, Bruner J, Yue N, Dugan M, Zaknoen S, Levin VA (1999) Multicenter phase II trial of temozolomide in patients with anaplastic astrocytoma or anaplastic oligoastrocytoma at first relapseTemodal Brain Tumor Group (erratum appears in J Clin Oncol 1999 Nov;17(11):3693). J Clin Oncol 17:2762–2771
29. Yung WK, Albright RE, Olson J, Fredericks R, Fink K, Prados MD, Brada M, Spence A, Hohl RJ, Shapiro W, Glantz M, Greenberg H, Selker RG, Vick NA, Rampling R, Friedman H, Phillips P, Bruner J, Yue N, Osoba D, Zaknoen S, Levin VA (2000) A phase II study of temozolomide vs. procarbazine in patients with glioblastoma multiforme at first relapse. Br J Cancer 83:588–593
30. Hengartner MO (2000) The biochemistry of apoptosis. Nature 407:770–776
31. Shacter E, Williams JA, Hinson RM, Senturker S, Lee YJ (2000) Oxidative stress interferes with cancer chemotherapy: inhibition of lymphoma cell apoptosis and phagocytosis. Blood 96(1):307–313
32. Anderson HA, Englert R, Gursel I, Shacter E (2002) Oxidative stress inhibits the phagocytosis of apoptotic cells that have externalized phosphatidylserine. Cell Death Differ 9(6):616–625
33. Anderson HA, Maylock CA, Williams JA, Paweletz CP, Shu H, Shacter E (2003) Serum-derived protein S binds to phosphatidylserine and stimulates the phagocytosis of apoptotic cells. Nat Immunol 4(1):87–91
34. Fadok VA, Henson PM (2003) Apoptosis: giving phosphatidylserine recognition an assist–with a twist. Curr Biol 13:R655–657
35. Lauber K, Blumenthal SG, Waibel M, Wesselborg S (2004) Clearance of apoptotic cells: getting rid of the corpses. Mol Cell 14:277–287
36. Fulda S, Meyer E, Friesen C, Susin SA, Kroemer G, Debatin KM (2001) Cell type specific involvement of death receptor and mitochondrial pathways in drug induced apoptosis. Oncogene 20:1063–1075
37. Korsmeyer SJ, Wei MC, Saito M, Weiler S, Oh KJ, Schlesinger PH (2000) Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome c. Cell Death Differ 7:1166–1173
38. Liu WK, Ho JCK, Che CT (2005) Apoptotic activity of isomalabaricane triterpenes on human promyelocytic leukemia HL60 cells. Cancer Lett 230:102–110
39. Liu WK, Cheung FWK, Che CT (2006) Stellettin A induces oxidative stress and apoptosis in HL-60 human leukemia and LNCaP prostate cancer cell lines. J Nat Prod 69:934–937
40. Eastman A, Rigas JR (1999) Modulation of apoptosis signaling pathways and cell cycle regulation. Semin Oncol 26(Suppl 16):7–16
41. Wang LG, Liu XM, Kreis W, Budman DR (1999) The effect of antimicrotubule agents on signal transduction pathways of apoptosis. Cancer Chemother Pharmacol 44:355–361
42. Konopleva M, Zhao S, Xie Z, Segall H, Younes A, Claxton DF, Estrov Z, Kornblau SM, Andreeff M (1999) Apoptosis, molecules and mechanisms. Adv Exp Med Biol 457:217–236
43. Shacter E, Weitzman SA (2002) Chronic inflammation and cancer. Oncology (Williston Park) 16(2):217–226
44. Englert RP, Shacter E (2002) Distinct modes of cell death induced by different reactive oxygen species: amino acyl chloramines mediate hypochlorous acid-induced apoptosis. J Biol Chem 277 (23):20518–20526
45. Nuijen B, Bouma M, Manada C, Jimeno JM, Schellens JH, Bult A, Beijnen JH (2000) Pharmaceutical development of anticancer agents derived from marine sources. Anticancer Drugs 11:793–811
46. Newman DJ, Cragg GM, Snader KM (2000) The influence of natural products upon drug discovery. Nat Prod Rep 17:215–234
47. Schwartsmann G (2000) Marine organisms and other novel natural sources of new cancer drugs. Ann Oncol 11:235–243
48. Faulkner DJ (2001) Marine natural products. Nat Prod Rep 18 (1):1–49
49. Santafe G, Paz V, Rodriguez J, Jimenez C (2002) Novel cytotoxic oxygenated C29 sterols from the Colombian marine sponge *Polymastia tenax*. J Nat Prod 65:1161–1164
50. da Silva AC, Kratz JM, Farias FM, Henriques AT, Dos Santos J, Leonel RM, Lerner C, Mothes B, Barardi CR, Simões CM (2006) In vitro antiviral activity of marine sponges collected off Brazilian coast. Biol Pharm Bull 2006 29(1):135–140

III. 2 - Artigo 2

Brazilian marine sponge *Polymastia janeirensis* induces oxidative cell death through a caspase-9-apoptotic pathway in human U138MG glioma cell line

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Brazilian marine sponge *Polymastia janeirensis* induces oxidative cell death through a caspase-9-apoptotic pathway in human U138MG glioma cell line

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Short running title: Effects of extracts from *Polymastia janeirensis* on glioma cells

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Abstract

In this work we examine the apoptotic rote activated in response to treatments with crude extracts (aqueous and organic) of *P. janeirensis*. Yet, the formation of intracellular ROS by glioma cells was measured. Exposure of cultures to both aqueous and organic extracts (1-100 μg/ml) resulted in cell viability decrease, with an IC_{50} <12 μg/ml for both extracts. However, 10 μg/ml resulted in a higher incidence of cell death that was almost entirely apoptotic, while a higher incidence of cell death by necrosis was observed with doses ≥25 μg/ml. Moreover, inhibition of caspase-8 had no effect on the amount of apoptosis induced by extracts, but inhibition of caspase-9 caused an inhibition of apoptosis. We also observed a dose-dependent increase on ROS production. However, 10 μg/ml is able to cause a milder oxidation that lead cells to die by apoptosis, while higher doses of sponge extracts caused a stronger increase in ROS production, leading cells to die by necrosis. This is a first report demonstrating that marine sponge extracts of *P. janeirensis* induces oxidative cell death through a caspase-9 apoptotic pathway. Further work will concentrate on bioassay-guided micro-fractionation to found the active constituents in the extracts.

Keywords: cancer; reactive oxygen species; apoptosis; marine sponges; *Polymastia janeirensis*

Introduction

Over the last years, marine organisms have been screened for a variety of compounds with different biological activities. Among all organisms screened, sponges represent one of the most promising sources of marine bioactive compounds particularly for pharmaceutical leads (Proksch, 1994; Faulkner, 1998). Ecological factors, such as competition for space with other sessile species, predation and symbiosis, have been the most important causes for this large variety of secondary metabolites (Assman et al., 2000).

In order to identify new active compounds with antitumor activity, extracts from a number of species of marine sponges have been examined in various tumor cell lines, as well as in animal models (Ahond et al., 1988; Burres et al., 1991; Fusetani et al., 1992; Gunasekera et al., 1991; Kobayashi et al., 1989; Perry et al., 1988, 1990; Sakai et al., 1986; ter Haar et al., 1996). At least two drugs (Ara-A and Ara-C) synthetically derived from sponge metabolites have been clinically used in long-term cancer treatments (Munro et al., 1994).

Of particular interest is the marine sponge *Polymastia janeirensis*. Its intense color and the apparent lack of predators suggest the presence of chemical defense mechanisms. However, little information is known about this species. In an interesting work, Monks et al. described a cytotoxic effect with organic extracts from *P. janeirensis* against three human tumor cell lines (HT29, U373 and NCI-H460). Yet, aqueous extracts significantly retarded the migration of polymorphonuclear leukocytes in a chemotactic assay (Monks et al., 2002). In other work, an *in vitro* antiviral activity was observed against the simian rotavirus RV-SA11 with aqueous extract of *P. janeirensis* (da Silva et al., 2006), and more recently, we reported that aqueous and organic extracts

of this marine sponge induces apoptotic cell death in human U138MG glioma cell line, but not in a normal cell culture (da Frota et al., 2008). However, the precise apoptotic pathway (receptor-dependent or receptor-independent) activated in response to treatments remains unclear.

In this study, we examine the apoptotic rote activated in response to treatments with crude extracts (aqueous and organic) of the marine sponge *P. janeirensis*. Moreover, in order to study if production of reactive oxygen species (ROS) was induced by extracts, the formation of intracellular ROS by U138MG cells was measured. This study is part of a collaborative program among several Brazilian institutions (Centro de Estudos em Estresse Oxidativo, Universidade Federal do Rio Grande do Sul; Fundação Zoobotânica do Rio Grande do Sul, Museu de Ciências Naturais; Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul; and Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul) for the collection and screening of Brazilian marine sponges for biological activities, with the aim of identifying new sponges species and novel molecules with promising and potentially useful therapeutic activities.

Materials and methods

Sponge sampling, identification and extract preparation

Sponges samples were collected manually from exposed and semi-exposed habitats, at depths of between 0.5 and 20 m, from locations on the coastline of the Estate of Santa Catarina (southern Brazil). Taxonomic designation was based on scanning electron microscope studies and on skeletal slides and dissociated spicule mounts.

Specimens of all materials are deposited in the Museu de Ciências Naturais-Porifera (MCNPOR) collection of the Fundação Zoobotânica do Rio Grande do Sul, Brazil. Aqueous and organic extracts were obtained as previously described (Monks et al., 2002).

Cell culture

U138MG human glioblastoma cell line was obtained from the American Type Culture Collection (Rockville, Maryland, USA). Cells were grown in culture flasks in Dulbecco's modified Eagle's medium (DMEM)/15% fetal bovine serum (FBS) (v/v) (Cultilab, Campinas, SP, Brazil) and seeded in 24-well plates (TTP plates) at densities of 1 X 10^4 cells/well in 500 µl medium per well. Culture cells were maintained in 5% CO_2/95% air at 37 ^oC and allowed to grow to confluence.

Treatments

Immediately before experiments, both the aqueous and organic extracts were dissolved in water and DMSO, respectively, at a concentration of 1 mg/ml (w/v). The amount of DMSO (maximum 0.25%) was proven not to affect the experiments. The final concentrations of the extracts tested ranged from 1 to 100 µg/ml. After reaching subconfluence, the cultures were exposed to sponge extracts for 24 h. Control cultures were performed with DMSO (0.25% final concentration) in the absence of extracts. In experiments using permeable caspase inhibitors (Z-VAD-fmk, Z-IETD-fmk, Z-LEDHfmk) (B & D Systems), cells were pre-incubated for 1 h and then treated with

extracts. None of the inhibitors used in this study had any effect on U138MG cell viability at the concentrations used.

Assessment of glioma cell viability

Following treatments, cell viability was assessed by the MTT assay. This method is based on the ability of viable cells to reduce 3-(4,5- dimethyl)-2,5 diphenyl tetrazolium bromide (MTT) and form a blue formazan product. MTT solution (sterile stock solution of 5 mg/ml) (Sigma) was added to the incubation medium in the wells at a final concentration of 0.5 mg/ml. The cells were left for 60 min at 37 ^{o}C in a humidified 5% CO_2 atmosphere. The medium was then removed and plates were shaken with DMSO for 30 min. The optical density of each well was measured at 550nm (test) and 690nm (reference). Results were expressed as the percentage of cell viability against the control.

Flow cytometry analysis

For annexin V/propidium iodide (AV/PI) staining, treated glioma cells ($1x10^6$) were washed twice in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.2H_2O$, 1.4 mM KH_2PO_4, pH 7.4) containing 1 mM EDTA (PBS-EDTA) and subsequently trypsinized with 0.13 g/L trypsin in PBS-EDTA. Trypsin was inhibited with bovine foetal serum 10%, and medium washes, and cells were combined and centrifuged (5 min, 200 x g, 4 °C). Cells were allowed to recover from trypsinization in complete medium (30 min, 37 °C). Externalized phosphatidylserine (PS) was labeled (15 min, 0 °C) with 5 µl FITC-conjugated annexin V in 80 µl binding buffer (10 mM HEPES pH

7.4, 145 mM NaCl, 5 mM KCl, 1.0 mM $MgCl_2.6H_2O$, 1.8 mM $CaCl_2.2H_2O$). Propidium iodide (PI) 2 μM was added 10 min prior to analysis on a FACScalibur flow cytometer (BD PharMingen). When green fluorescence (FITC) was plotted against red fluorescence (PI), distinct cell populations could be detected: viable cells (FITC−/PI−), apoptotic cells (FITC+/ PI− and FITC+/PI+), and necrotic cells (FITC−/ PI+), as previously described (24). Ten thousand cells were analyzed per sample, and data were reported as the percentage of apoptotic cells and necrotic cells.

Determination of intracellular ROS

Intracellular ROS were detected using an oxidation sensitive fluorescent probe, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), as previously described (LeBel et al., 1992). Briefly, after treatments with extracts, cells were incubated with DCFH-DA 10 μM for 30 min at 37 ^{o}C. The DCFH-DA was first deacetylated by cellular esterases to DCFH, which was converted to a green fluorescent product DCF by intracellular reactive oxygen species produced by treated U138MG glioma cells. The medium was discharged and cells were collected, resuspended in ice-cold PBS and sonicated. The DCFH-DA oxidation was quantified from the fluorescence emission intensity with an emission wavelength set at 535 nm and an excitation wavelength set at 485 nm.

Statistical analysis

Results were expressed as the mean ± SEM of at least three independent experiments. Data were analyzed by a one-way analysis of variance (ANOVA), using a

Newman Keuls test to compare mean values across groups. When appropriate, Student's t-test was performed. Differences were considered to be significant when $p < 0.05$. Dose response curves were plotted, and the IC_{50} values (concentrations at which cellular effects are inhibited by 50%) were calculated using non-linear regression analysis.

Results

Marine sponge extracts decreased U138MG glioma cell viability

In order to investigate the effect of marine sponge extracts on viability of U138MG glioma cell line, cell cultures were treated with extracts and a MTT assay was performed. Marine sponge extracts (1 and 5 µg/ml) did not decrease cell viability in glioma cells (Fig. 1). However, exposure of glioma cell line to doses higher than 5 µg/ml resulted in a reduction of glioma cell viability, as evidenced by a diminished ability of glioma cells to reduce MTT, with an IC_{50} <12 µg/ml for both aqueous and organic extracts. Moreover, we observed that extracts from *P. janeirensis* at doses higher than 10 µg/ml have stronger cytotoxic effects, decreasing more than 90% of glioma cell viability. The reduction in MTT staining suggests not only glioma cell damage, but also a decrease in cell proliferation when compared with the control.

Cell death induced by marine sponge extracts

To determine whether the suppression of glioma cell proliferation was due to the induction of necrosis or apoptosis, glioma cells were treated for 24 h and, at the end of

treatment, Annexin V-FITC-bound phosphatidylserine and red fluorescence of DNA-bound PI in individual cells were measured using a flow cytometer as described on "Materials and Methods". As shown in Fig. 2A, marine sponge extracts at lower doses (1 and 5 μg/ml) did not induce cell death in human U138MG glioma cell line, but exposure of cultures to doses higher than 5 μg/ml resulted in cell death. However, 10 μg/ml of both extracts resulted in a higher incidence of apoptotic cell death, while a higher incidence of cell death by necrosis was observed with doses ≥25 μg/ml. Since higher doses have stronger cytotoxic effects and induced necrosis, we decided to investigate only the effects of marine sponge extracts at 10 μg/ml.

To determine whether marine sponge extracts from *P. janeirensis* activates the extrinsic or intrinsic pathways of apoptosis, human U138MG glioma cells pre-incubated with caspase-inhibitors and exposed to treatments for 24 h. As previously described, cell death was prevented by the pan-caspase inhibitor Z-VAD (Fig. 2B), suggesting that marine sponge extracts, at 10 μg/ml, induces caspase-dependent apoptosis in glioma cells. Moreover, to determine which initiator caspase was activated in response to extracts, cells were pre-incubated with either the cell-permeable caspase-9 inhibitor Z-LEHD or the caspase-8 inhibitor Z-IETD for 1 h. We found that inhibition of caspase-8 had no effect on the amount of apoptosis induced by both aqueous and organic extracts. In contrast, inhibition of caspase-9 caused an inhibition of apoptosis.

ROS production induced by marine sponge extracts

In order to investigate the effect of marine sponge extracts on intracellular ROS production, glioma cells were treated for 24 h and a DCFH-DA assay was performed. As shown in Fig. 3, extracts at lower doses (1 and 5 μg/ml) did not have any effect.

However, exposure of cell cultures to doses higher than 5 µg/ml resulted in a dose-dependent ROS production. Similar to effects observed on viability of glioma cells, as well as in the cell death, higher doses also have stronger effects.

Discussion

Apoptosis, or programmed cell death, is characterized by a number of well-defined features (Hengartner, 2000). In contrast to necrosis, apoptotic cell death is thought to be physiologically advantageous (Shacter et al., 2000; Anderson et al., 2002; Anderson et al., 2003; Fadok and Henson, 2003; Lauber et al., 2004). In this way, we have been previously reported that Brazilian marine sponge *P. janeirensis* induces apoptotic cell death in glioma cells, but not in a normal cell culture. Moreover, extracts from this marine sponge were more effective than temozolomide, a potent inductor of apoptosis used for the treatment of malignant gliomas. However, we have not described the precise apoptotic pathway (receptor-dependent or receptor-independent) activated in response to treatments. In this work, we examine the apoptotic rote activated in response to marine sponge extracts. Moreover, the effect of extracts on intracellular ROS production also was examined. The results will be discussed further below.

Apoptotic cell death occurs through at least two overlapping pathways referred to as extrinsic and intrinsic. The extrinsic pathway is activated through ligand binding to death receptors (members of the tumor necrosis factor receptor superfamily) at the cell surface and activation of initiator caspase-8. In the intrinsic pathway, mitochondria play a pivotal early role by releasing cell death signals into the cytosol and activating caspase-9 (Fulda et al., 2001). Both pathways converge and activate executioner

caspase-3, which then cleaves intracellular protein substrates and causes cell death (Korsmeyer et al., 2000).

Our results showed that marine sponge extracts, at doses higher than 5 μg/ml, reduced the cell viability of glioma cultures. Nevertheless, we observed that higher doses were stronger cytotoxic, decreasing more than 90% of cell viability. Furthermore, higher doses caused cell death by necrosis, while a high percentage of apoptotic glioma cells were counted with 10 μg/ml. For this reason, we decided investigate only the effects of marine sponge extracts at 10 μg/ml in the apoptotic cell death. To determine whether marine sponge extracts from *P. janeirensis* activates the extrinsic or intrinsic pathways of apoptosis, human U138MG glioma cells were pre-treated with caspase inhibitors for 1 h and exposed to treatments for 24 h. We found that cell death was prevented by the pan-caspase inhibitor Z-VAD, as previously described for us. Moreover, we found that inhibition of caspase-8 with Z-IETD (a caspase-8 inhibitor) had no effect on the amount of apoptosis induced by 10 μg/ml, but inhibition of caspase-9 with Z-LEHD (a caspase-9 inhibitor) caused an inhibition of apoptosis. This result suggests that extracts from Brazilian marine sponge *P. janeirensis* cause apoptosis through a receptor-independent pathway under our assay conditions.

It is well recognized that free radicals can regulate cell death, but the mechanisms whereby oxidants activate the apoptotic machinery are not well understood. Moreover, the mode of death (i.e. necrotic or apoptotic) depends on the type, concentration, source and environment of the oxidants (Englert and Schacter, 2002). In normal conditions, there is a steady-state balance between the production of ROS and their destruction by the cellular antioxidant system. When cells are exposed to an oxidative stress various defense mechanisms are induced, including the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) (Halliwell and Gutteridge,

1999). In a previous work, we have demonstrated that there is a different modulation of oxidative stress when compared radioresistant and radiosensitive glioma cell lines, and these seem to be important for cell viability (Dal-Pizzol et al., 2003).

Some studies with marine natural products have reported a correlation between free radicals and apoptosis in cancer cell lines (Koulman et al., 1996; Liu et al., 2005; Liu et al., 2006). However, the mechanisms underlying these properties are still not well understood. In this work, we observed a dose-dependent increase on ROS production at doses higher than 5 μg/ml. Furthermore, similar to effects of marine sponge extracts on viability of cell cultures, as well as in cell death, higher doses have stronger effects on ROS production. The effect in cell viability and the strong effect in cell death observed at higher doses are most likely through the high increase of ROS production (>100% when compared to control) and a concomitant loss of membrane integrity, which is an indication of cell death by necrosis. However, 10 μg/ml is able to cause a milder oxidation that lead cells to die by apoptosis. The flow cytometry necrosis/apoptosis measurement is consistent with this consideration.

Marine natural products have been prominently featured in the area of cancer research. It is interesting to not that out of 18 preclinical investigating anticancer agents derived from marine sources, six are from sponges (Nuijen et al., 2000; Newman et al., 2000; Schwartsmann, 2000), implying that marine sponges are potential resources for new anti-cancer agents. Although Brazil has the second most extensive coastline after Australia, there are just few reports in which the authors have screened Brazilian sponge extracts for biological activities. To date, only limited screening evaluations of extracts of Brazilian marine sponges have been reported (Muricy et al., 1993; Monks et al., 2002; Prado et al., 2004; Rangel et al., 2001). Furthermore, extracts from Brazilian

sponges have not been examined for specific effects on the cell death-regulatory pathways and ROS production.

Of particular interest is the Brazilian marine sponge *P. janeirensis*. However, little information is known about this species, and the only detected classes of compounds already described for the genus *Polymastia* are sterols and tetrahidroxiamide (Faulkner, 2001; Santafe et al., 2002). These compounds exhibited significant cytotoxic activity toward human lung carcinoma (A-549), human colon carcinomas (HT-29 and H-116), mice endothelial (MS-1), and human prostate carcinoma (PC-3) cell lines in the range 0.5-10 μg/ml, but the mechanisms underlying these properties were not reported. Related compounds belonging to these groups of metabolites may be present in the tested extracts and/or could be even responsible for the antiproliferative activity described in this work. However, further studies are necessary to confirm this hypothesis.

To our knowledge, this is a first report demonstrating that marine sponge extracts of *P. janeirensis* induces oxidative cell death by apoptosis through a receptor-independent pathway. Moreover, the biphasic effect (apoptosis or necrosis) observed in response to different doses of marine sponge extracts may be related to the dose-dependent increase on ROS production, since excess levels of free radicals can cause oxidative stress, with an excessive activation of poly-ADP-ribose polymerase (PARP), and a concomitant depletion of the ATP levels in the cells, leading cells to die by necrosis. In agreement with this, some works have shown that oxidant treatment leads to increased ATP consumption and subsequent cellular ATP depletion (Filipovic et al., 1999; Schraufstatter et al., 1986). Additional studies are required to understand the exact mechanism by which sponge extracts works to regulate ROS production and cell death in glioma cells, as well as its significance in neoplasic transformation of normal

and previously injured cells. Moreover, further work will concentrate on bioassay-guided micro-fractionation to found the active constituents in the extracts.

Conflict of interest statement - The authors declare that there are no conflicts of interest.

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References

Ahond, A., Zurita, M.B., Colin, M., Fizames, C., Laboute, P., Lavelle, F., Laurent, D., Poupat, C., Pusset, J., Pusset, M., Thoisson, O., Potier, P., 1988. Girolline, a new antitumour substance from sponge *Pseudaxinyssa cantharella* (Axinellidae). Les Comptes Rendus de I`Académie des Scinces 307, 145-148.

Anderson, H.A., Englert, R., Gursel, I., Shacter, E., 2002. Oxidative stress inhibits the phagocytosis of apoptotic cells that have externalized phosphatidylserine. Cell Death and Differentiation 9(6), 616-625.

Anderson, H.A., Maylock, C.A., Williams, J.A., Paweletz, C.P., Shu, H., Shacter, E., 2003. Serum-derived protein S binds to phosphatidylserine and stimulates the phagocytosis of apoptotic cells. Nature Immunology 4(1), 87-91.

Assmann, M., Lichte, F., Pawlik, J.R., Kock, M., 2000. Chemical defenses of the Caribbean sponges *Agelas wiedenmayeri* and *Agelas conifera*. Marine Ecology Progress Series 207, 255-262.

Burres, N.S., Barber, D.A., Gunasekera, S.P., Shen, L.L., Clement, J.J., 1991. Antitumor activity and biochemical effects of topsentin. Biochemical Pharmacology 42, 745-751.

da Frota, M.L.Jr, Braganhol, E., Canedo, A.D., Klamt, F., Apel, M.A., Mothes, B., Lerner, C., Battastini, A.M., Henriques, A.T., Moreira, J.C., 2008. Brazilian marine sponge *Polymastia janeirensis* induces apoptotic cell death in human U138MG glioma cell line, but not in a normal cell culture. Investigational New Drugs, in press.

da Silva, A.C., Kratz, J.M., Farias, F.M., Henriques, A.T., Dos Santos, J., Leonel, R.M., Lerner, C., Mothes, B., Barardi, C.R., Simões, C.M., 2006. In vitro antiviral activity of marine sponges collected off Brazilian coast. Biological & Pharmaceutical Bulletin 29(1), 135-40.

Dal-Pizzol, F., Ritter, C., Klamt, F., Andrades, M., da Frota, M.L.Jr., Diel, C., de Lima, C., Braga Filho, A., Schwartsmann, G., Moreira, J.C., 2003. Modulation of oxidative stress in response to gamma-radiation in human glioma cell lines. Journal of Neuro-oncology 61(2), 89-94.

Englert, R.P., Shacter, E., 2002. Distinct modes of cell death induced by different reactive oxygen species: amino acyl chloramines mediate hypochlorous acid-induced apoptosis. Journal of Biological Chemistry 277(23), 20518-20526.

Fadok, V.A., Henson, P.M., 2003. Apoptosis: giving phosphatidylserine recognition an assist--with a twist. Current Biology 13, 655-657.

Faulkner, D.J., 1998. Marine natural products. Natural Product Reports 15(2), 113-158.

Faulkner, D.J., 2001. Marine natural products. Natural Product Reports 18(1), 1-49.

Filipovic, D.M., Meng, X., Reeves, W.B., 1999. Inhibition of PARP prevents oxidant-induced necrosis but not apoptosis in LLC-PK1 cells. American Journal of Physiology. Renal Physiology 277, 428-436.

Fulda, S., Meyer, E., Friesen, C., Susin, S.A., Kroemer, G., Debatin, K.M., 2001. Cell type specific involvement of death receptor and mitochondrial pathways in drug induced apoptosis. Oncogene 20, 1063-1075.

Fusetani, N., Sugawara, T., Matsunaga, S., 1992. Theopederins A-E, potent antitumor metabolites from a marine sponge *Theonella* sp. The Journal of Organic Chemistry 57, 3828-3832.

Gunasekera, S.P., Gunasekera, M., McCarthy, P., 1991. Discodermide: a new bioactive macrocyclic lactam from the marine sponge *Discodermia dissoluta*. The Journal of Organic Chemistry 56, 4830-4833

Halliwell, B., Gutteridge, J.M.C., 1999. Free Radical in Biology and Medicine. Clarendon Press, Oxford.

Hengartner, M.O., 2000. The biochemistry of apoptosis. Nature 407, 770-776

Kobayashi, M.J., Murayama, Y., Ohizumi, Y., Sasaki, T., Ohta, T., Nozoe, S., 1989. Theonelladins a-d, novel antineoplastic pyridine alkaloids from the Okinawan marine sponge *Theonella swinhoei*. Tetrahedron letters 30, 4833-4836.

Koulman, A., Proksch, P., Ebel, R., Beekman, A.C., Uden, W., Konings, A.W.T., Pedersen, J.A., Pras, N., Woerdenbang, H.J., 1996. Cytoxicity and mode of action of Aeroplysin-1 and a related dienone from the sponge *Aplysina aerophoba*. Journal of Natural Products 59, 591-594.

Korsmeyer, S.J., Wei, M.C., Saito, M., Weiler, S., Oh, K.J., Schlesinger, P.H., 2000. Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome c. Cell Death and Differentiation 7, 1166-1173

Lauber, K., Blumenthal, S.G., Waibel, M., Wesselborg, S., 2004. Clearance of apoptotic cells: getting rid of the corpses. Molecular cell 14, 277-287.

LeBel, C.P., Ischiropoulos, H., Bondy, S.C., 1992. Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chemical research in toxicology 5(2), 227-231.

Liu, W.K., Ho, J.C.K., Che, C.T., 2005. Apoptotic activity of isomalabaricane triterpenes on human promyelocytic leukemia HL60 cells. Cancer Letters 230, 102-110.

Liu, W.K., Cheung, F.W.K., Che, C.T., 2006. Stellettin A induces oxidative stress and apoptosis in HL-60 human leukemia and LNCaP prostate cancer cell lines. Journal of Natural Products 69, 934-937.

Monks, N.R., Lerner, C., Henriques, A.T., Farias, F.M., Schapoval, E.E.S., Suycnaga, E.S., da Rocha, A.B., Schwartsmann, G., Mothes, B., 2002. Anticancer, antichemotactic and antimicrobial activities of marine sponges collected off the coast of Santa Catarina, southern Brazil. Journal of Experimental Marine Biology and Ecology 281, 1-12.

Munro, M.H.G., Blunt, J.W., Lake, R.J., Litaudon, M., Battershill, C.N., Page, M.J., 1994. From seabed to sickbed: what are the prospects? In: van Soest RWM, van Kempen ThMG, Braekman JC (eds.), Sponges in Time and Space, A.A. Balkema Publishers, Rotterdam, The Netherlands, 1994, pp. 473-484

Muricy, G., Hajdu, E., Araújo, F.V., Hagler, N.A., 1993. Antimicrobial activity of southwestern Atlantic shallow-water marine sponges (Porifera). Scientia Marina 57, 427-432.

Newman, D.J., Cragg, G.M., Snader, K.M., 2000. The influence of natural products upon drug discovery. Natural Product Reports 17, 215-234.

Nuijen, B., Bouma, M., Manada, C., Jimeno, J.M., Schellens, J.H., Bult, A., Beijnen, J.H., 2000. Pharmaceutical development of anticancer agents derived from marine sources. Anticancer Drugs 11, 793-811.

Perry, N.B., Blunt, J.W., Munro, M.H.G., Higa, T., Sakai, R., 1988. Discorhabdin D, an antitumor alkaloid from the sponges *Latrunculia brevis* and *Prianos* sp. The Journal of Organic Chemistry 53, 4127-4128.

Prado, M.P., Torres, Y.R., Berlink, R.G.S., Desiderá, C., Sanchez, M.A., Craveiro, M.V., Hajdu, E., da Rocha, R.M., Machado-Santelli, G.M., 2004. Effects of marine organisms extracts on microtubule integrity and cell cycle progression in cultured cells. Journal of Experimental Marine Biology and Ecology 313, 125-137.

Proksch, P., 1994. Defensive roles for secondary metabolites from marine sponges and sponge-feeding nudibranchs. Toxicon 32, 639-655.

Rangel, M., de Sanctis, B., de Freitas, J.C., Polatto, J.M., Granato, A.C., Berlinck, R.G.S., Hajdu, E.J., 2001. Cytotoxic and neurotoxic activities in extracts of marine sponges (Porifera) from southeastern Brazilian coast. Journal of Experimental Marine Biology and Ecology 262, 31-40.

Sakai, R., Higa, T., Jefford, C.W., Bernardinelli, G., 1986. Manzamine a, a novel antitumor alkaloid from a sponge. Journal of the American Chemical Society 108, 6404-6405.

Santafe, G., Paz, V., Rodriguez, J., Jimenez, C., 2002. Novel Cytotoxic Oxygenated C29 Sterols from the Colombian Marine Sponge *Polymastia tenax*. Journal of Natural Products 65, 1161-1164.

Schraufstatter, I.U., Hinshaw, D.B., Hyslop, P.A., Spragg, R.G., Cochrane, C.G., 1986. Oxidant injury of cells. The Journal of clinical investigation 77, 1312-1320.

Schwartsmann, G., 2000. Marine organisms and other novel natural sources of new cancer drugs. Annals of oncology 11, 235-243.

Shacter, E., Williams, J.A., Hinson, R.M., Senturker, S., Lee, Y.J., 2000. Oxidative stress interferes with cancer chemotherapy: inhibition of lymphoma cell apoptosis and phagocytosis. Blood 96(1), 307-313.

ter Haar, E., Kowalski, R.J., Hamel, E., Lin, C.M., Longley, R.E., Gunasekera, S.P., Rosenkranz, H.S., Day, B.W., 1996. Discodermolide, a cytotoxic marine agent that stabilizes microtubules more potently than taxol. Biochemistry 35, 243-250.

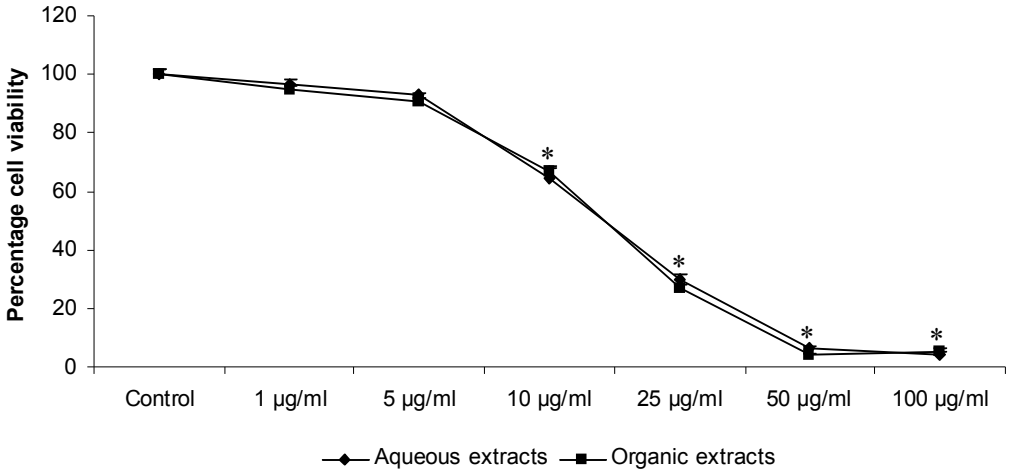
Figure legends

Figure 1. Effects of marine sponge extracts on U138MG glioma cell viability. Cell cultures were exposed to extracts for 24 h and the cell viability was assessed by the MTT assay as described in "Material and methods". Glioma cultures treated with DMSO (control) was taken as 100% of cell viability. Data are the means ± S.E.M for three individual experiments, and results were expressed as the percentage of cell viability against the control. * Statistically different from control, $p < 0.05$ (one-way ANOVA).

Figure 2. Effects of marine sponge extracts on cell death. U138MG glioma cells were treated for 24 h with different concentrations of sponge extracts **(A)** or pre-treated for 1 h with caspase inhibitors and then exposed to sponge extracts **(B)**. Annexin V-FITC-bound phosphatidylserine and red fluorescence of DNA-bound PI in individual glioma cells were measured using a flow cytometer as described in "Materials and Methods". Legend: AE, aqueous extract; OE, organic extracts; Z-VAD, pan-caspase inhibitor (100 μM); Z-LEDH, caspase-9 inhibitor (100 μM); Z-IETD, caspase-8 inhibitor (100 μM). The data represent averages from at least three independent experiments carried out in triplicate.

Figure 3. Effects of marine sponge extracts on ROS production. Cells were exposed to extracts for 24 h and intracellular ROS production was assessed by the DCFH-DA assay as described in "Material and methods". Glioma cells treated with DMSO (control) was taken as 100% of ROS production. Data are the means ± S.E.M for three individual experiments, and results were expressed as the percentage of ROS. * Statistically different from control, $p < 0.05$ (one-way ANOVA).

Figure 1


120
100
80
60
40
20
0
Percentage cell viability
*
*
*
*
Control
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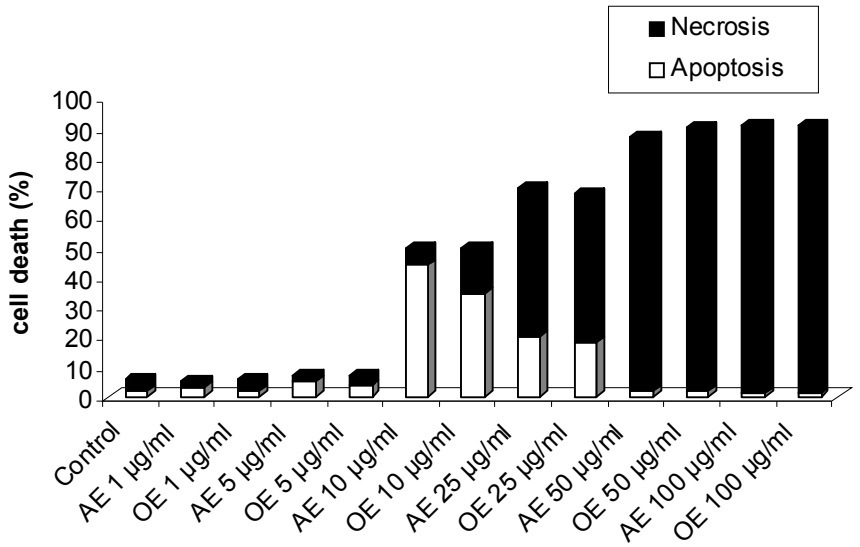
5 µg/ml
10 µg/ml
25 µg/ml
50 µg/ml
100 µg/ml
Aqueous extracts
Organic extracts

Figure 2

A)


Necrosis
Apoptosis
cell death (%)
100
90
80
70
60
50
40
30
20
10
0
Control
AE 1 µg/ml
OE 1 µg/ml
AE 5 µg/ml
OE 5 µg/ml
AE 10 µg/ml
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AE 50 µg/ml
OE 50 µg/ml
AE 100 µg/ml
OE 100 µg/ml

B)

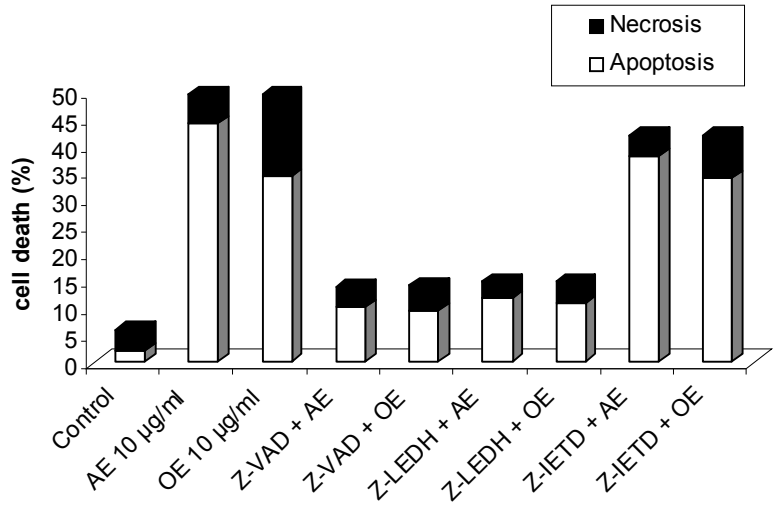
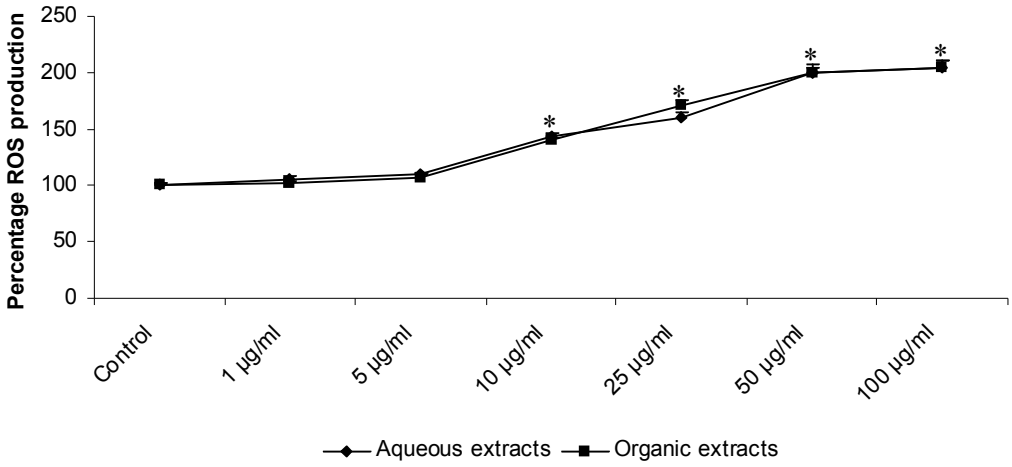

Necrosis
Apoptosis
cell death (%)
50
45
40
35
30
25
20
15
10
5
0
Control
AE 10 µg/ml
OE 10 µg/ml
Z-VAD + AE
Z-VAD + OE
Z-LEDH + AE
Z-LEDH + OE
Z-IETD + AE
Z-IETD + OE

Figure 3


Percentage ROS production
250
200
150
100
50
0
*
*
*
*
Control
1 µg/ml
5 µg/ml
10 µg/ml
25 µg/ml
50 µg/ml
100 µg/ml
Aqueous extracts
Organic extracts

III. 3 - Artigo 3

Anti-proliferative activities of extracts and isolated compounds from *Polymastia janeirensis*

Artigo submetido para publicação no periódico *The Biological Bulletin*

Anti-proliferative activities of extracts and isolated compounds from *Polymastia janeirensis*

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Marine sponges have been prominently featured in the area of cancer research. Here, we performed a bioassay-guided fractionation to found the active constituents in the extracts of the marine sponge Polymastia janeirensis. A strong cytotoxic activity was observed against the human glioma cell line U138MG with the active fraction isolated by HPLC-PDA. Furthermore, a high percentage of apoptotic glioma cells were observed after treatments. Our results suggest that this marine sponge may be considered a good source for new antiglioma metabolites. The chemical structure of the active fraction will be established by usual spectroscopic analysis in further works.

Marine sponges are considered one of the most promising sources of new active compounds with anticancer activity, and extracts from a number of species have been examined in various tumor cell lines, as well as in animal models (1-8). In particular, we have previously reported that crude extracts from Brazilian marine sponge *Polymastia janeirensis* induces apoptotic cell death in glioma cells, but not in a normal cell culture (9). This is particularly important because malignant gliomas are characterized by an intrinsic resistance to apoptosis (10). Moreover, a growing body of evidence suggests that pro-apoptotic agents have a significant antiglioma effect in preclinical models. In a continuation of our search for new anticancer leads from marine sponges, a bioassay-guided fractionation was performed to found the active constituents in the extracts of the marine sponge *P. janeirensis*. This study is part of a collaborative program among several Brazilian institutions for the collection and screening of Brazilian marine sponges for biological activities, with the aim of identifying new sponges species and novel molecules with promising and potentially useful therapeutic activities.

Sponge samples were collected and taxonomic designation was performed as previously described (11). *P. janeirensis* materials were ground with water three times

for 30 min to yield an aqueous extract (AE), and 1.002 g was subsequently extracted with 1:1 CH_2Cl_2/MeOH (4 x 20 mL). The extract was filtered, the soluble fraction (SF1) was evaporated under reduced pressure yielding 0.1468 g, and the insoluble fraction (IF1) yielded 0.9319 g. The SF1 was found to be inactive and was not pursued. The cytotoxic residue IF1 (0.006 g) was dissolved in MeOH (15 mL), the soluble fraction (SF2) was dried under reduced pressure yielding 0.0008 g, and the insoluble fraction (IF2) yielded 0.0049 g. The SF 2 was found to be inactive and was not pursued. The cytotoxic residue IF2 was dissolved in water and subjected to high performance liquid chromatography (HPLC) with a photodiode array (PDA) detector, and three regions were collected (A1, A2 and A3).

Figure 1 shows the cytotoxic activities of different fractions from aqueous extract of *P. janeirensis* against human U138MG glioma cell line. The results obtained revealed that IF2 have a strong cytotoxic effect, with an IC_{50} = 2.5 µg/ml. Taking into account the results obtained, the IF2 fractions was selected for isolation of the active constituents. HPLC-PDA analyses are shown in Figure 2. Three regions in the chromatogram, with retention time between 5, 25 and 35 min (A1, A2 and A3, respectively), were collected and tested for their cytotoxic activities. As shown in Figure 3, the A3 fraction presented a strong anti-proliferative activity, with an IC_{50} = 0.5 µg/ml. Moreover, the chromatographic profile suggests that the A3 is the active constituent in the *P. janeirensis* extracts, once only one component was observed in this fraction.

Apoptotic cell death is thought to be physiologically advantageous than necrosis, because the dying cells are cleared by phagocytosis prior to cell lysis and release of inflammatory mediators (12-16). In this way, glioma cells were exposed to A3 at 0.5 µg/ml for 24 h and stained with YOPRO-1 and propidium iodide (PI). We observed a

high content of apoptotic cells in the treated cultures (Fig. 4), suggesting that A3 fraction induces apoptotic cell death in the U138MG glioma cell line. Further investigations will concentrate on elucidation of the chemical structure of A3 by usual spectroscopic analysis.

Acknowledgements - This work was supported by FAPERGS, CNPq, CAPES and PROPESQ-UFRGS. All experiments carried out comply with current Brazilian laws.

Figure legends

Figure 1. Effects of marine sponge fractions on glioma cell viability. U138MG human glioblastoma cell line was obtained from the American Type Culture Collection (Rockville, Maryland, USA). Cells were grown in culture flasks in Dulbecco's modified Eagle's medium (DMEM)/15% fetal bovine serum (FBS) (v/v) (Cultilab, Campinas, SP, Brazil) and seeded in 24-well plates (TTP plates) at densities of 1 X 10^4 cells/well in 500 µl medium per well. Culture cells were maintained in 5% CO_2/95% air at 37 ^oC and allowed to grow to confluence. Immediately before experiments, the extracts were dissolved in water. The cultures were exposed to treatments for 24 h. Control cultures were performed with DMEM in the absence of extracts. Cell viability was assessed by the MTT assay. MTT solution (sterile stock solution of 5 mg/ml) was added to the incubation medium in the wells at a final concentration of 0.5 mg/ml. The cells were left for 60 min at 37 ^oC in a humidified 5% CO_2 atmosphere. The medium was then removed and plates were shaken with DMSO for 30 min. The optical density of each well was measured at 550nm (test) and 690nm (reference). Data are the means ± S.E.M for three individual experiments, and results were expressed as the percentage of cell

viability against the control. * Statistically different from control, $p < 0.05$ (one-way ANOVA). Dose response curves were plotted, and the IC_{50} values (concentrations at which cellular effects are inhibited by 50%) were calculated using non-linear regression analysis.

Figure 2. HPLC profile of IF2 extract. Chemical analyses were carried out with a 2695 Waters Alliance analytical chromatograph with a C_{18} columm (3.5 µm - 4.6 x 75 mm; Waters) and a PDA detector model 996 controlled by Empower Chromatograph Software (Waters Corp., Milford, MA, USA). The chromatography separation was carried out using a mobile phase, with water as solvent A and methanol as solvent B, at a flow rate of 0.5 ml/min. The gradient program was as follows: 0-30% B (20 min), 30-100% B (40 min), 100% B (50 min). The peaks were detected at 219 nm, and three regions were collected (A1, A2 and A3).

Figure 3. Effects of isolated fractions from *P. janeirensis* by HPLC on U138MG glioma cell viability. Cell cultures were exposed to A1, A2 and A3 aliquots for 24 h and the cell viability was assessed by the MTT assay as described in the legend to Fig. 3. Data are the means ± S.E.M for three individual experiments, and results were expressed as the percentage of cell viability against the control. * Statistically different from control, $p < 0.05$ (one-way ANOVA). Dose response curves were plotted, and the IC_{50} values (concentrations at which cellular effects are inhibited by 50%) were calculated using non-linear regression analysis.

Figure 4. Representative pictures of U138MG cell cultures stained with YOPRO-1 and PI for apoptosis analysis. Briefly, PI stains necrotic cells, and YOPRO-1 incorporate

into DNA only in early apoptotic cells becoming highly fluorescent. At the end of the treatment (24 h) with A3 fraction (0.5 μg/ml), glioma cells were incubated with YOPRO-1 for (1 h, 0 ^{o}C). PI (2 μM) was added 5 min prior to analysis on inverted fluorescence microscope (Nikon Eclipse TE300; Nikon Inc., Melville, New York, USA). Images were captured using a digital camera connected to the microscope.

References

1. Ahond, A., M. B. Zurita, M. Colin, C. Fizames, P. Laboute, F. Lavelle, D. Laurent, C. Poupat, J. Pusset, M. Pusset, O. Thoisson, and P. Potier. 1988. Girolline, a new antitumour substance from sponge *Pseudaxinyssa cantharella* (Axinellidae). *CR Acad Sci Paris* **307:** 145-148.

2. Burres, N. S., D. A. Barber, S. P. Gunasekera, L. L. Shen, and J. J. Clement. 1991. Antitumor activity and biochemical effects of topsentin. *Biochem Pharmacol* **42:** 745-751.

3. Fusetani, N., T. Sugawara, and S. Matsunaga. 1992. Theopederins A-E, potent antitumor metabolites from a marine sponge *Theonella* sp. *J Org Chem* **57:** 3828-3832.

4. Gunasekera, S. P., M. Gunasekera, and P. McCarthy. 1991. Discodermide: a new bioactive macrocyclic lactam from the marine sponge *Discodermia dissoluta*. *J Org Chem* **56:** 4830-4833.

5. Kobayashi, M. J., Y. Murayama, Y. Ohizumi, T. Sasaki, T. Ohta, and S. Nozoe. 1989. Theonelladins a-d, novel antineoplastic pyridine alkaloids from the Okinawan marine sponge *Theonella swinhoei*. *Tetrahedron lett* **30:** 4833-4836.

6. Perry, N. B., J. W. Blunt, M. H. G. Munro, T. Higa, and R. Sakai. 1988. Discorhabdin D, an antitumor alkaloid from the sponges *Latrunculia brevis* and *Prianos* sp. *J Org Chem* **53:** 4127-4128.

7. Sakai, R., T. Higa, C. W. Jefford, and G. Bernardinelli. 1986. Manzamine a, a novel antitumor alkaloid from a sponge. *J Am Chem Soc* **108:** 6404-6405.

8. ter Haar, E., R. J. Kowalski, E. Hamel, C. M. Lin, R. E. Longley, S. P. Gunasekera, H. S. Rosenkranz, and B. W. Day. 1996. Discodermolide, a cytotoxic marine agent that stabilizes microtubules more potently than taxol. *Biochemistry* **35:** 243-250.

9. da Frota, M. L. Jr., E. Braganhol, A. D. Canedo, F. Klamt, M. A. Apel, B. Mothes, C. Lerner, A. M. Battastini, A. T. Henriques, and J. C. Moreira. 2008. Brazilian marine sponge *Polymastia janeirensis* induces apoptotic cell death in human U138MG glioma cell line, but not in a normal cell culture. *Invest New Drugs*, in press.

10. Ziegler, D. S., A. L. Kung, and M. W. Kieran. 2008. Anti-apoptosis Mechanisms in Malignant Gliomas. *J Clin Oncol* **26(3):** 493-500.

11. Monks, N. R., C. Lerner, A. T. Henriques, F. M. Farias, E. E. S. Schapoval, E. S. Suycnaga, A. B. da Rocha, G. Schwartsman, and B. Mothes. 2002. Anticancer, antichemotactic and antimicrobial activities of marine sponges collected off the coast of Santa Catarina, southern Brazil. *J Exp Mar Biol Ecol* **281:** 1-12.

12. Shacter, E., J. A. Williams, R. M. Hinson, S. Senturker, and Y. J. Lee. 2000. Oxidative stress interferes with cancer chemotherapy: inhibition of lymphoma cell apoptosis and phagocytosis. *Blood* **96(1):** 307-313.

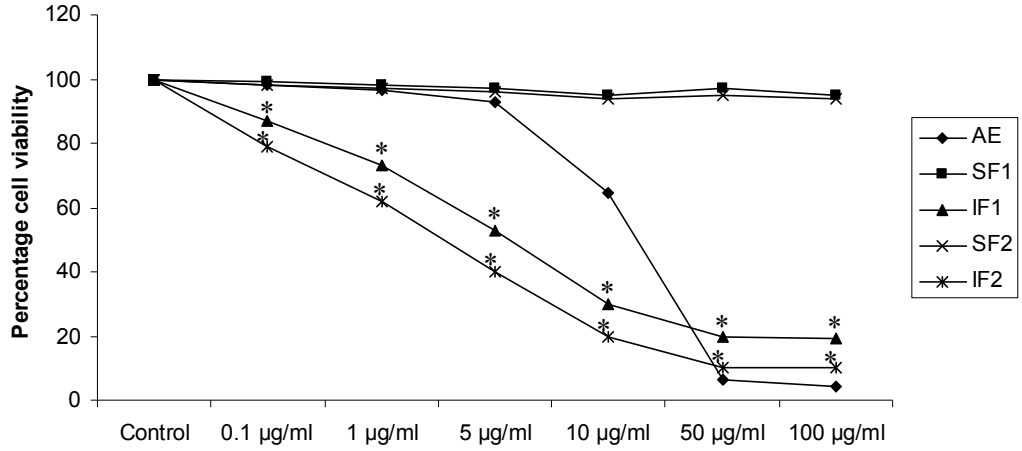
13. Anderson, H. A., R. Englert, I. Gursel, and E. Shacter. 2002. Oxidative stress inhibits the phagocytosis of apoptotic cells that have externalized phosphatidylserine. *Cell Death Differ* **9(6):** 616-625.

14. Anderson, H. A., C. A. Maylock, J. A. Williams, C. P. Paweletz, H. Shu, and E. Shacter. 2003. Serum-derived protein S binds to phosphatidylserine and stimulates the phagocytosis of apoptotic cells. *Nat Immunol* **4(1):** 87-91.

15. Fadok, V. A., and P. M. Henson. 2003. Apoptosis: giving phosphatidylserine recognition an assist--with a twist. *Curr Biol* **13:** 655-657.

16. Lauber, K., S. G. Blumenthal, M. Waibel, and S. Wesselborg. 2004. Clearance of apoptotic cells: getting rid of the corpses. *Mol Cell* **14:** 277-287.

Figure 1



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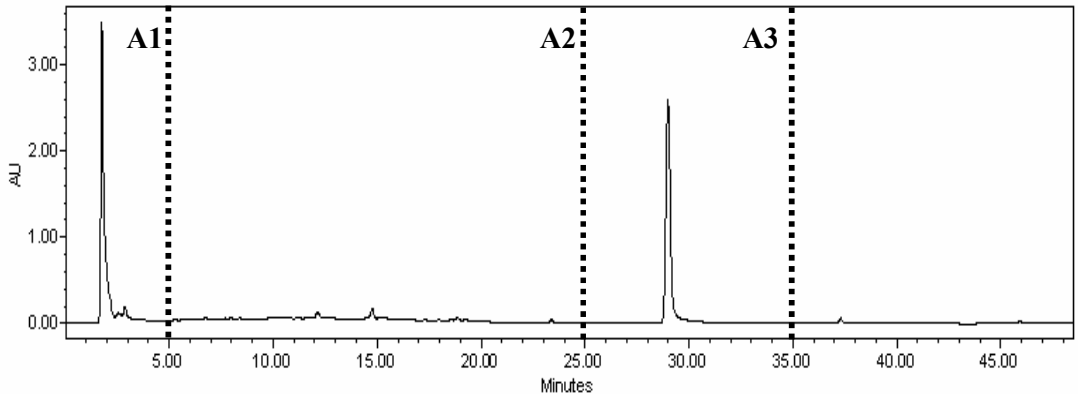


Figure 3

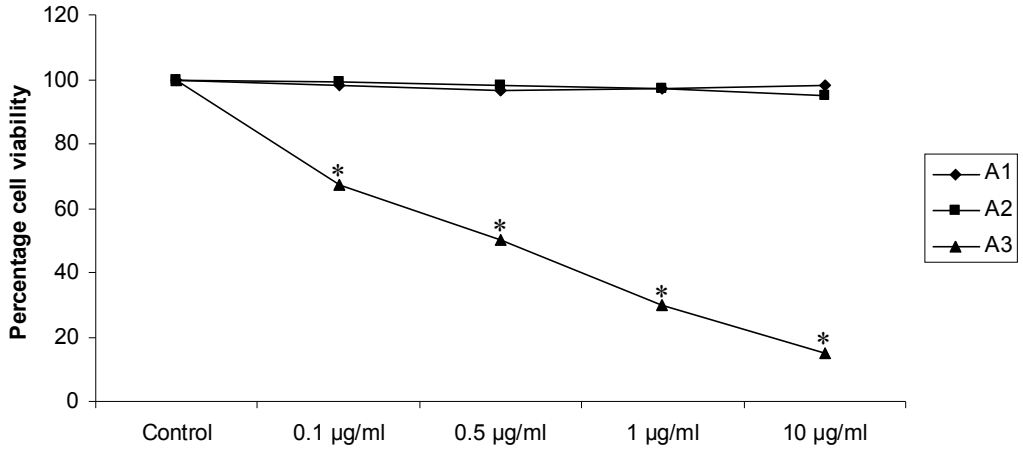
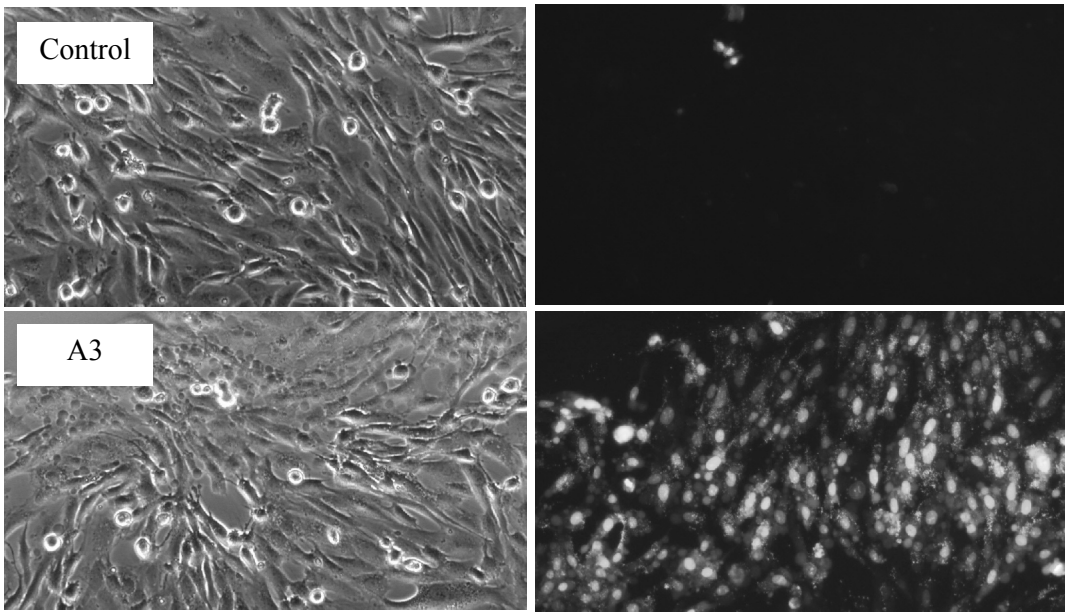


Figure 4



IV - DISCUSSÃO

IV - DISCUSSÃO

Cobrindo mais de 70% da superfície do planeta, os oceanos são uma das principais fontes de novas moléculas com diferentes atividades biológicas e com potencial aplicação no desenvolvimento de novos fármacos. Entre os diversos organismos marinhos, as esponjas (Filo Porifera) representam a principal fonte de novos metabólitos, muitas vezes inéditos na literatura e com estruturas químicas diferentes daquelas encontradas em organismos terrestres. Isso se deve ao fato das esponjas viverem fixas ao substrato. Como não podem se locomover, desenvolveram, ao longo da evolução, uma série de mecanismos de defesas químicas, muitas vezes utilizadas contra a ação de predadores, bem como na competição por espaço.

Quando comparadas a outros organismos marinhos, as esponjas apresentam a maior taxa de compostos citotóxicos, com mais de 10% das espécies investigadas apresentando alguma atividade biológica (Osinga et al., 1998; Zhang et al., 2003). Estudos recentes, realizados pelo *National Cancer Institute* dos EUA, retratam as esponjas marinhas como os organismos que mais produzem moléculas de alta singularidade com possível interesse farmacológico e potencial utilização no tratamento de doenças (Kelecom et al., 1991).

O Brasil possui a segunda maior extensão litorânea do mundo, ficando atrás apenas da Austrália, sendo um dos países com o maior índice de biodiversidade do mundo. Entretanto, poucos trabalhos explorando a diversidade química da nossa fauna marinha foram realizados, uma vez que o principal foco da química de produtos naturais no Brasil foi direcionado, por muitos anos, ao estudo de plantas medicinais. Isso mudou somente na última década, e um considerável aumento no número de trabalhos explorando as propriedades farmacológicas de esponjas coletadas no litoral brasileiro

pôde ser observado (Muricy et al., 1993; Rangel et al., 2001; Monks et al., 2002; Berlinck et al., 2004).

Neste trabalho, um efeito anti-proliferativo foi observado quando a linhagem de glioma humano U138MG foi exposta aos extratos (aquoso e orgânico) da esponja marinha *P. janeirensis* (Fig. 1 e Fig. 2, Artigo 1). Contudo, uma diferença no padrão de morte celular foi observada conforme a concentração de extrato utilizada. Nós observamos que as doses mais altas (50 e 100 µg/ml) de ambos os extratos foram extremamente citotóxicas, inibindo mais de 80% a proliferação e a viabilidade das células. Além disso, uma morte celular por necrose foi observada com essas doses (Fig. 3, Artigo 1), enquanto uma morte celular por apoptose (Fig. 4, Artigo 1) foi observada com a dose mais baixa (10 µg/ml).

Apoptose, ou morte celular programada, é caracterizada por uma série de eventos bem definidos e acredita-se ser fisiologicamente mais vantajosa que a morte por necrose. Por esse motivo, drogas que tenham como mecanismo de ação a indução de morte celular por apoptose são de grande interesse. Surpreendentemente, uma nova droga utilizada no tratamento de gliomas (Temozolomide) e que tem como principal mecanismo de ação a indução de apoptose, foi menos efetiva que os extratos aqui testados (Fig. 4, Artigo 1), nas nossas condições experimentais.

A morte celular programada pode ocorrer principalmente por duas rotas referidas como extrínseca ou intrínseca. A primeira é ativada pela ligação de ligantes nos chamados receptores de morte encontrados na superfície celular, com uma conseqüente ativação da caspase-8. Já na rota intrínseca, a mitocôndria exerce um papel fundamental, liberando sinais de morte para o citoplasma e ativando a caspase-9. Ambas as rotas convergem e ativam a caspase-3, uma caspase executora que cliva proteínas intracelulares e causa a morte celular.

Com o objetivo de elucidar a rota apoptótica ativada em resposta aos extratos, inibidores de caspase foram utilizados. A morte celular foi prevenida quando as células foram co-tratadas com Z-VAD, um inibidor geral de caspases. Além disso, quando as células foram co-tratadas com Z-IETD, um inibidor específico de caspase-8, nenhuma reversão no efeito anti-proliferativo foi observada. Entretanto, quando as células foram co-tratadas com Z-LEHD, um inibidor específico de caspase-9, uma inibição da morte apoptótica pôde ser observada (Fig. 2, Artigo 2). Esses resultados sugerem que os extratos aqui testados induzem morte celular por apoptose pela via intrínseca.

Sabe-se que radicais livres podem regular o processo de morte celular, mas os mecanismos envolvidos nessa regulação não são bem conhecidos. Além disso, o mecanismo de morte celular (necrose ou apoptose) depende muito do tipo e da concentração de radicais livres produzidos no ambiente celular (Englert e Schacter, 2002). Em um trabalho anterior do nosso grupo de pesquisa, foi demonstrada uma importante diferença na atividade das principais enzimas antioxidantes de linhagens de gliomas sensíveis ou resistentes à radiação, sugerindo um papel fundamental dos radicais livres na viabilidade celular dos gliomas estudados (Dal-Pizzol et al. 2003).

Recentes estudos com produtos naturais de origem marinha têm demonstrado uma correlação entre a geração de radicais livres e apoptose em diferentes linhagens de câncer (Koulman et al., 1996; Liu et al., 2005; Liu et al., 2006). Ademais, foi demonstrado recentemente que o composto geotidin A, um terpeno isolado da esponja marinha *Geodia japonica*, induz a produção de radicais livres e induz morte por apoptose dependente de caspase-3 na linhagem humana de leucemia promielocítica HL60 (Liu et al., 2005). Contudo, a rota de morte ativada em resposta ao tratamento com esse composto (dependente ou independente de receptor) não foi elucidada. Mais tarde, o mesmo autor demonstrou que stelletin A, um outro composto isolado da mesma

esponja marinha, induz a produção de radicais livres e causa morte celular por apoptose, com ativação da rota dependente de receptor.

Aqui, nós observamos um aumento de uma maneira dose-dependente na produção de radicais livres quando as células foram expostas aos extratos aquosos e orgânicos da esponja marinha *P. janeirensis* (Fig. 3, Artigo 2). Similar aos resultados de viabilidade celular, as doses mais altas induziram um potente aumento na produção de radicais livres (>100% comparado ao controle) e uma concomitante perda de integridade da membrana celular, um indicativo de morte por necrose. Entretanto, a dose de 10 µg/ml induziu um moderado aumento na geração de radicais livres, levando a uma morte por apoptose (Fig. 3, Artigo 2). Esses resultados explicam, pelo menos em parte, o efeito bifásico (necrose ou apoptose) observado. Níveis elevados de radicais livres podem causar estresse oxidativo, com uma super ativação da PARP, e uma conseqüente depleção dos níveis de ATP nas células, levando a uma morte por necrose. Alguns trabalhos têm demonstrado que o tratamento com oxidantes e uma produção exacerbada de radicais livres pode aumentar o consumo de ATP, causando uma depleção do mesmo (Filipovic et al., 1999; Schraufstatter et al., 1986).

De acordo com a Organização Mundial de Saúde (OMS), a cada ano surgem nove milhões de novos casos de câncer no mundo. No Brasil, segundo o Ministério da Saúde, mais de quinhentos mil novos casos de câncer surgem por ano, e aproximadamente 100 mil pessoas morrem anualmente (Instituto Nacional do Câncer, 2008). No entanto, apesar dos esforços para o desenvolvimento de novas drogas contra o câncer, ainda não existem agentes realmente efetivos contra uma grande gama de neoplasias. Por essa razão, é de extrema importância o desenvolvimento de novos fármacos. Nesse sentido, extratos naturais continuam sendo a melhor fonte de novos compostos com atividade anti-proliferativa, já que possuem uma enorme diversidade

química em suas constituições. É importante salientar que dentre 18 compostos de origem marinha que estão sendo investigados pré-clinicamente contra diferentes tipos de câncer, 6 são derivados de esponjas, ressaltando o enorme potencial desses animais como fonte de novos metabólitos com propriedades anti-proliferativas.

Características particulares como fatores ambientais (exposição à alta intensidade de luz e altas pressões de oxigênio) e uma geografia propícia (águas rasas) fazem das esponjas do nosso litoral candidatas ideais para a obtenção de novos fármacos, tornando-se relevante a pesquisa sobre a aplicação dos mesmos no tratamento de doenças de alto impacto sócio-econômico. Sendo assim, a utilização de produtos naturais extraídos de esponjas marinhas brasileiras pode ser considerada uma importante ferramenta para o desenvolvimento de novos medicamentos, alvo principal da indústria farmacêutica.

Os únicos compostos isolados a partir de esponjas do gênero *Polymastia* encontrados na literatura são esteróis e tetrahidroxiamidas, obtidos da esponja *P. tenax*, coletada na Colômbia. Esses compostos exibiram uma significante atividade citotóxica contra linhagens de carcinoma humano de pulmão (A-549), carcinoma humano de cólon (HT-29 e H-116) e carcinoma humano de próstata (Faulkner, 2001; Santafe et al., 2002). Contudo, os mecanismos envolvidos no processo anti-proliferativo permanecem desconhecidos. Além disso, somente dois trabalhos descrevem propriedades biológicas em extratos de *P. janeirensis* coletadas no Brasil. No primeiro, foi observado um efeito citotóxico em extratos orgânicos contra três linhagens de câncer (HT-29, U-373 e NCI-H460), com um IC_{50} entre 50 e 100 µg/ml. Ademais, extratos aquosos retardaram significativamente a migração de leucócitos em um ensaio quimiotático (Monks et al., 2002). Entretanto, o modo de ação dessas atividades biológicas permanece desconhecido. Já no outro trabalho, uma importante atividade antiviral foi observada em

extrato aquoso contra o rotavírus RV-SA11 (da Silva et al., 2006). Nesse caso, o extrato inibia os últimos estágios de replicação do rotavírus.

Com o objetivo de isolar da esponja marinha *P. janeirensis* os compostos responsáveis pelas atividades observadas, um fracionamento bio-guiado foi realizado. Diferentes frações foram obtidas após a extração com diferentes solventes, e a fração mais ativa foi submetida à cromatografia líquida de alta eficiência com um detector de arranjo de fotodiodos (PDA). Foram obtidas três frações, sendo que a fração A3 foi a única fração ativa (Fig. 3, Artigo 3), com uma potente atividade apoptótica (Fig. 4, Artigo 3). Além disso, o perfil cromatográfico obtido (Fig. 2, Artigo 3) sugere que a fração A3 é o composto ativo que buscávamos, uma vez que somente um pico é observado nessa região do cromatograma.

Nós também observamos uma diminuição no IC_{50} ao longo do fracionamento. Com o extrato aquoso bruto foi obtido um IC_{50} = 15 µg/ml. Já com o isolado, um IC_{50} = 0.5 µg/ml foi encontrado. Isso é extremamente importante quando a questão é o desenvolvimento de um novo fármaco, uma vez que para a indústria farmacêutica não é economicamente interessante quando a atividade desejada é encontrada apenas em grandes concentrações.

Como falado anteriormente, drogas que tenham como mecanismo de ação a indução de morte celular por apoptose são de grande interesse. Isso é particularmente importante para gliomas, uma vez que eles são caracterizados por alterações em suas rotas apoptóticas (Ziegler et al., 2008) e, mesmo com um tratamento agressivo (radiação, cirurgia e quimioterapia), adquirem uma resistência intrínseca a esse mecanismo de morte celular, dificultando ainda mais o tratamento. Nós demonstramos aqui que o composto ativo isolado da esponja marinha *P. janeirensis* induz morte

celular por apoptose na linhagem de glioma humano U138MG, nas nossas condições experimentais (Fig. 4, Artigo 3).

De uma maneira geral, o tratamento do câncer pode ser realizado através de cirurgia, radioterapia e quimioterapia. De acordo com o estágio da doença, o sucesso do tratamento geralmente é conseguido com a associação desses três tipos de tratamentos. No entanto, problemas como uma baixa seletividade dos antineoplásicos e o aparecimento de efeitos colaterais indesejados são muito freqüentes. Além disso, o surgimento de resistência aos medicamentos conduz o tratamento para o uso combinado de diferentes drogas e, conseqüentemente, aumenta a probabilidade de efeitos colaterais.

Os resultados aqui apresentados demonstram a existência de uma seletividade do efeito citotóxico. Como pode ser observado na Figura 5, Artigo 1, os extratos testados não induziram morte em culturas de astrócitos na dose em que foi observada morte por apoptose nos gliomas. Apenas as concentrações que induziram necrose foram citotóxicas para as células não transformadas. Isso é extremamente relevante quando lembramos que diversos estudos já demonstraram uma resistência à terapia com Temozolomide, o principal fármaco utilizado no tratamento de gliomas, e que tem como um dos principais mecanismos de ação a indução de apoptose.

Esse é o primeiro trabalho demonstrando que extratos e frações isoladas da esponja marinha *P. janeirensis* induzem morte celular, e que o mecanismo envolvido está, pelo menos em parte, relacionado à produção de radicais livres. Ademais, uma potente atividade apoptótica foi observada com o composto ativo isolado da esponja marinha alvo desse estudo. Assim, baseados nos dados apresentados, nós propomos que essa esponja marinha pode ser considerada uma boa candidata para futuras investigações e para o desenvolvimento de novas drogas contra o câncer. É importante salientar que o crescente estudo de propriedades farmacológicas em esponjas marinhas

em outros países contribuiu para a criação de programas de preservação desses animais, bem como do eco-sistema em que eles vivem, e que a existência de programas de pesquisa com esponjas nessas regiões reflete o maior número de moléculas e patentes obtidas nesses países.

V - CONCLUSÃO

V. CONCLUSÃO

Os resultados obtidos nesta Tese apontam a esponja marinha *Polymastia janeirensis* como uma importante fonte para o desenvolvimento de novos fármacos contra o câncer. Neste trabalho, nós demonstramos que extratos e frações isoladas da esponja marinha *P. janeirensis* induzem morte celular em uma linhagem de glioma humano, e que o mecanismo envolvido está, pelo menos em parte, relacionado à produção de radicais livres. Além disso, uma potente atividade apoptótica foi observada com o composto ativo isolado da esponja marinha alvo desse estudo. Com esse trabalho, esperamos contribuir de alguma maneira para o desenvolvimento de novas metodologias para o tratamento dos gliomas. Por isso, ressalta-se a importância de trabalhos como esse, uma vez que espécies podem ser extintas antes mesmo de serem descritas e pesquisadas quanto às suas propriedades biológicas.

VI - PERSPECTIVAS

VI. PERSPECTIVAS

As principais perspectivas desse trabalho são 1) a elucidação estrutural do composto ativo isolado da esponja marinha *P. janeirensis* por análises espectroscópicas usuais, 2) desvendar quais os componentes da via intrínseca estão envolvidos na morte celular programada observada em resposta aos tratamentos, 3) desvendar os mecanismos pelos quais os radicais livres gerados pelos extratos induzem a morte celular por necrose ou apoptose na linhagem estudada e 4) examinar o efeito antitumoral do composto ativo isolado em outras linhagens tumorais.

REFERÊNCIAS BIBLIOGRÁFICAS

Ahond A., Zurita M.B., Colin M., Fizames C., Laboute P., Lavelle F., Laurent D., Poupat C., Pusset J., Pusset M., Thoisson O., Potier P. (1988). *Les Comptes Rendus de I`Académie des Scinces* 307, 145-148.

Anderson H.A., Englert R., Gursel I., Shacter E. (2002). *Cell Death and Differentiation* 9(6), 616-625.

Anderson H.A., Maylock C.A., Williams J.A., Paweletz C.P., Shu H., Shacter E. (2003). *Nature Immunology* 4(1), 87-91.

Das A., Banik N.L., Patel S.J., Ray S.K. (2004). *Molecular Cancer* 3, 36-45.

Becerro M.A., Turon X., Uriz M.J. (1997). *Journal of Chemical Ecology* 23,1527–1547.

Bergmann W., Feeney R.J. (1950). *Journal of the American Chemical Society* 72(6), 2809-2810.

Bergquist P.R. Sponges**.** London, Hutchinson, 268p., 1978.

Berlinck R.G.S., Hajdu E., da Rocha R.M., de Oliveira J.H.H.L., Hernández I.L.C., Seleghim M.H.R., Granato A.C., de Almeida E.V.R., Nuñez C., Muricy G., Peixinho S., Pessoa C., Moraes M.O., Cavalcanti B.C., Nascimento G.G.F., Thiemann O., Silva M.,

Souza A.O., Silva C.L., Minarini P.R.R. (2004). *Journal of Natural Products* 67, 510-522.

Burres N.S., Barber D.A., Gunasekera S.P., Shen L.L., Clement J.J. (1991). *Biochemical Pharmacology* 42, 745-751.

da Silva A.C., Kratz J.M., Farias F.M., Henriques A.T., Dos Santos J., Leonel R.M., Lerner C., Mothes B., Barardi C.R., Simões C.M. (2006). *Biological & Pharmaceutical Bulletin* 29(1), 135-40.

Davis A.R., Butler A., Altena I. (1991) *Mar Ecol Prog Ser* 72, 117-123.

Dorée C. (1909). *Biochemical Journal* 4, 72-106.

Ebel R., Brenzinger M., Kunze A., Gross H., Proksh P. (1997) *Journal of Chemical Ecology* 23(5), 1451-1461.

Englert RP, Shacter E. (2002). Journal of Biological Chemistry 277(23), 20518-20526.

Fadok V.A., Henson P.M. (2003). *Current Biology* 13, 655-657.

Faulkner D.J. (2000) *Natural Product Reports* 17, 1-6.

Faulkner D.J. (2001). *Natural Product Reports* 18(1), 1-49.

Faulkner D.J. (2002) *Natural Product Reports* 19, 1-48.

Fusetani N., Sugawara T., Matsunaga S. (1992). *The Journal of Organic Chemistry* 57, 3828-3832.

Gunasekera S.P., Gunasekera M., McCarthy P. (1991). *The Journal of Organic Chemistry* 56, 4830-4833.

Henze M. (1909). *Physiol Chem* 55, 427-432.

Hirsch S., Rudi A., Kashman Y., Loya Y. (1991). *Journal of Natural Products* 54, 92-97.

Hooper J.N.A., Levi C. (1994). Biogeography of Indo-west Pacific sponges: microcionidae, raspailiidae, axinellidae. In: van Soest, R.W.M., van Kempen, T.M.G., Braekman, J.C. (Eds.), Sponges in Time and Space. Balkema, Rotterdam, pp. 191–212, 1994.

Instituto Nacional do Câncer; Ministério da Saúde. Secretaria de Atenção à Saúde. Estimativas 2008: Incidência de Câncer no Brasil. Rio de Janeiro, Brasil: INCA, 2007. Available from http://www.saude.gov.br/

Kelecom A. (1991). *Mem Inst Oswaldo Cruz* 86(2), 99-106.

Kijjoa A., Sawangwong P. (2004). *Marine Drugs* 2, 73-82.

Kobayashi M.J., Murayama Y., Ohizumi Y., Sasaki T., Ohta T., Nozoe S. (1989). *Tetrahedron letters* 30, 4833-4836.

Koulman A., Proksch P., Ebel R., Beekman A.C., Uden W., Konings A.W.T., Pedersen J.A., Pras N., Woerdenbang H.J. (1996). *Journal of Natural Products* 59, 591-594.

Lauber K., Blumenthal S.G., Waibel M., Wesselborg S. (2004). *Molecular Cell* 14, 277-287.

Lederer E. (1938). *Bull Soc Chem Biol* 20, 567-342.

Liu WK, Ho JCK, Che CT. (2005). *Cancer Letters* 230,102-110.

Liu WK, Cheung FWK, Che CT. (2006) *Journal of Natural Products* 69, 934-937.

Meijer L., Thunnissen A.M., White A.W., Garnier M., Nikolic M., Tsai L.H., Walter J., Cleverley K.E., Salinas P.C., Wu Y.Z., Biernat J., Mandelkow E.M., Kim S.H., Pettit G.R. (2000). Chem Biol 7, 51-63.

Monks N.R., Lerner C., Henriques A.T., Farias F.M., Schapoval E.E.S., Suyenaga E.S., Rocha A.B., Schwartsmann G., Mothes B. (2002). *Journal of Experimental Marine Biology and Ecology* 281, 1-12.

Munro M.H.G., Blunt J.W., Lake R.J., Battershill C.N., Page M.J. (1993). From seabed to sickbed: What are the propects? In: Soest, R.W.M. Van; Kempen, T.M.G. Van; Braekman, J.C. Sponges in time and spaces: Biology, Chemistry, Paleontology. Amsterdam, Brookfield, 503p.

Muricy G., Hajdu E., Araújo F.V., Hagler N.A. (1993). *Scientia Marina* 57, 427-432.

Newman D.J., Cragg G.M., Snader K.M. (2000). *Natural Product Reports* 17(3), 215-234.

Newman DJ, Cragg GM, Snader KM. (2003). *Journal of Natural Products* 66, 1022-1037.

Newman D.J., Cragg G.M. (2004). *Journal of Natural Products* 67, 1216-1238.

Osinga R., Tramper J., Wijiffels R.H. (1998). *TIBTECH* 16, 130-134.

Pawlik J.R. (1993). *Chem Rev* 93, 1911-1922.

Pawlik J.R., McFall G., Zea S. (2002). *Journal of Chemical Ecology* 28(6), 1103-15.

Perry N.B., Blunt J.W., Munro M.H.G., Higa T., Sakai R. (1988). *The Journal of Organic Chemistry* 53, 4127-4128.

Pettit G.R., Lippert J.W., Taylor S.R., Tan R., Willians M.D. (2001). *Journal Natural Products* 64(7), 883-891.

Porter J.M., Targett W.M. (1988). *The Biological Bulletin* 175, 230-239.

Prado M.P., Torres Y.R., Berlink R.G.S., Desiderá C., Sanchez M.A., Craveiro M.V., Hajdu E., da Rocha R.M., Machado-Santelli G.M. (2004). *Journal of Experimental Marine Biology and Ecology* 313, 125-137.

Proksch P. (1994). *Toxicon* 6, 639-655.

Rangel M., de Sanctis B., de Freitas J.C., Polatto J.M., Granato A.C., Berlinck R.G.S., Hajdu E. (2001). *Journal of Experimental Marine Biology and Ecology* 262, 31-40.

Sakai R., Higa T., Jefford C.W., Bernardinelli G. (1986). *Journal of the American Chemical Society* 108, 6404-6405.

Santafe G., Paz V., Rodriguez J., Jimenez C. (2002). *Journal of Natural Products* 65, 1161-1164.

Shacter E., Williams J.A., Hinson R.M., Senturker S., Lee Y.J. (2000). *Blood* 96(1), 307-313.

Simpson T.L. The Cell Biology of Sponges. Springer-Verlag, New York, 1984.

Sipkema D., Franssen M.C., Osinga R., Tramper J., Wijffels R.H. (2005). *Marine Biotechnololy* 7, 142-162.

Stupp R., Mason W.P., van den Bent M.J. et al (2005). *New England Journal of Medicine* 352, 987-996.

Tahara Y. (1910). *Biochemische Zeitschrift* 30, 255-275.

ter Haar E., Kowalski R.J., Hamel E., Lin C.M., Longley R.E., Gunasekera S.P., Rosenkranz H.S., Day B.W. (1996). *Biochemistry* 35, 243-250.

Uriz M.J. et al. (1996). *Journal of Experimental Marine Biology and Ecology* 205, 187-204.

Van; Braekman, J. C. Sponges in the time and space: Biology, Chemistry, Paleontology. Amsterdam, Brookfield, 503p.

Vogel S. (1977). *Proceedings of the National Academy of Sciences USA* 74, 2069-2071.

Zhang W., et al. (2003). *Biomolecular Engineering* 20, 413-419.

Ziegler D.S., Kung A.L., Kieran M.W. (2008). *Journal of Clinical oncology* 26(3), 493-500.

ANEXOS

- Carta de confirmação de envio do artigo científico “Brazilian marine sponge *Polymastia janeirensis* induces oxidative cell death through a caspase-9-apoptotic pathway in human U138MG glioma cell line” para publicação no periódico *Toxicology in Vitro*;

- Carta de confirmação de envio do artigo científico “Anti-proliferative activities of extracts and isolated compounds from *Polymastia janeirensis*” para publicação no periódico *The Biological Bulletin*;

- Artigo complementar “Antioxidant and procoagulant activities of extracts from brazilian marine sponge *Polymastia janeirensis*” submetido para publicação no periódico *The Biological Bulletin*, e carta de confirmação do envio.

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Authors: Mario Luiz Conte Frota Jr, Ph.D.; Elizandra Braganhol; Fabio Klamt; Miriam Apel; Beatriz Mothes; Clea Lerner; Ana Battastini; Amelia Henriques; Jose Moreira
Article Type: Research Paper

Dear Dr. Mario Luiz Conte Frota Jr,

Your submission entitled "Brazilian marine sponge Polymastia janeirensis induces oxidative cell death through a caspase-9-apoptotic pathway in human U138MG glioma cell line" has been received by Toxicology in Vitro.

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Condensed title: Bioactivities from marine sponge

Manuscript title: Antioxidant and procoagulant activities of extracts from Brazilian marine sponge *Polymastia janeirensis*

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Keywords: Brazilian marine sponges; Biological activities; Reactive oxygen species; Natural products.

Abstract

This paper describes the antioxidant and procoagulant activities of extracts (aqueous and organic) from Brazilian marine sponge *Polymastia janeirensis*. Moreover, the total phenolic content of the extracts is evaluated. Both aqueous and organic extracts inhibited hydroxyl production and prevented 2-deoxyribose degradation (IC_{50} =163 μg/ml and 129.5 μg/ml, respectively). The extracts also diminished nitric oxide production. Aqueous extracts were 2.3 times more effective than orgainc extracts in inhibiting this radical formation; the IC_{50} of aqueous extract was 150.95 μg/ml and the IC_{50} of organic extract was 345 μg/ml. Yet, both aqueous and organic extracts diminished the TBARS (an index of lipid peroxidation) content induced by ABAP (IC_{50} =186.4 μg/ml and 158.9 μg/ml, respectively). The determination of total phenolic content showed that extracts presented an equally high content of phenolics, where phenolic compounds constitue 6.79 ± 0.31% dry weight of aqueous extract and 6.90 ± 0.29% dry weight of organic extract. In addition, extracts of *P. janeirensis* displayed a potent effect upon blood coagulation, indicating a procoagulant activity. This is the first report demonstrating antioxidant and procoagulant activities of extracts from Brazilian marine sponge *P. janeirensis*, suggesting that this marine sponge should be considered as a good source of active compounds.

Introduction

Marine organisms have provided a large proportion of the bioactive natural products reported over the last 20 years. Among the many phyla found in the oceans, the best sources of pharmacologically active compounds are sponges (Munro *et al*., 1999), with 10% of the investigated species showing some biological activity. Ecological factors, such as competition for space with other sessile species, predation and symbiosis, have been the most important causes for this large variety of secondary metabolites (Assman *et al*., 2000).

Reactive oxygen species (ROS) and oxidative stress play an important role in the etiology and progression of major human degenerative diseases. Moreover, oxidative stress affects circulating proteins and is associated with an abnormal coagulative pattern (Shacter *et al*., 1995). For this reason, there is a great interest in substances that act as endogenous and exogenous antioxidants with potential application in biomedicine. In this context, analysis of marine organisms chronically exposed to high levels of solar UV radiation often reveals active antioxidants of unknown composition (Dunlap *et al*., 2003). This is particularly significant for tropical marine organisms. During daylight exposure, the air saturation come across > 250% (hyperoxic), which, in combination with high light intensities, can cause photooxidative toxicity to the organisms via the photodynamic production of cytotoxic ROS (Dunlap *et al*., 1999). Tropical marine sponges thus provide a rich resource for the discovery of novel products with redox properties.

Although Brazil has the second most extensive coastline after Australia, there are just few reports in which the authors have screened Brazilian sponge extracts for biological activities. To date, only limited screening evaluations of extracts of Brazilian marine sponges have been reported (Muricy *et al.*, 1993; Monks *et al.*, 2002; Prado *et al.*, 2004; Rangel *et al.*, 2001).

Furthermore, extracts from Brazilian sponges have not been examined for specific effects on the blood coagulation and free radicals production.

Of particular interest is the marine sponge *Polymastia janeirensis* Boury-Esnauls, 1973. Its intense color and the apparent lack of predators suggest the presence of chemical defense mechanisms. However, little information is known about this species, and the only detected class of compounds already described for the genus *Polymastia* are sterols and tetrahidroxiamide (Faulkner, 2001; Santafe *et al.*, 2002). Moreover, only two works reported biological properties from *P. janeirensis* (Monks *et al.* 2002; da Silva *et al.*, 2006).

In this report, we investigate the *in vitro* effects of crude extracts from *P. janeirensis* (aqueous and organic) collected from the Brazilian coastline on blood coagulation and their redox properties. Moreover, the phenolic content of extracts was evaluated. This study is part of a collaborative program among several Brazilian institutions (Centro de Estudos em Estresse Oxidativo, Universidade Federal do Rio Grande do Sul; Fundação Zoobotânica do Rio Grande do Sul, Museu de Ciências Naturais; and Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul) for the collection and screening of Brazilian marine sponges for biological activities, with the aim of identifying new sponges species and novel molecules with promising and potentially useful therapeutic activities.

Materials and methods

Reagents

All drugs were purchased from Sigma Chemicals (St. Louis, MO, USA).

Sponge sampling and identification

Sponge samples were collected manually from exposed and semi-exposed habitats, at depths between 0.5 and 20 m, from locations on the coastline of Santa Catarina (southern Brazil). Taxonomic designation was based on scanning electron microscope studies and on skeletal slides

and dissociated spicule mounts. Specimens of all materials are deposited in the Museu de Ciências Naturais–Porifera (MCNPOR) collection of the Fundação Zoobotânica do Rio Grande do Sul, Brazil.

Extract preparation

Aqueous extracts were produced by the following procedure. Sponge materials were ground together with sand and water three times for 30 min. The resulting extract (collected after each 30 min) was subsequently filtered and freeze-dried. The remaining material was sequentially extracted five times with a methanol/toluene mixture (3:1, v/v) by maceration over 5 days. The resulting extract solution was then filtered and concentrated in a Rotavapor (organic extracts). Immediately before experiments, both the aqueous and organic extracts were dissolved in water and DMSO, respectively, at a concentration of 1 mg/ml (w/v). The amount of DMSO (maximum 0.25%) present was proven not to affect the experiments. The final concentrations of the extracts tested ranged from 10 to 100 μg/ml.

Hydroxyl scavenging activity

The formation of hydroxyl radicals ($^{\bullet}OH$) from Fenton reagents was quantified using 2-deoxyribose oxidative degradation. The principle of the assay is the quantification of the 2-deoxyribose degradation product, malondialdehyde, by its condensation with TBA (Hermes-Lima *et al*., 1994). Briefly, typical reactions (control) were started by the addition of Fe(II) (6μM final concentration) to solutions containing 5 mM 2-deoxyribose, 100 μM H_2O_2 and 20 mM phosphate buffer (pH 7.2). To measure extracts redox activity, different concentrations of extracts (10 - 1000 μg/ml) were added to the system before Fe (II) addition. Trolox (water-soluble vitamin E analogue) and ascorbic acid were used as antioxidant standards. Reactions were carried out for 15 min at room temperature and were stopped by the addition of 4% phosphoric acid (v/v)

followed by 1% TBA (w/v, in 50 mM NaOH). After boiling for 15 min, the absorbance of solutions was measured at 532 nm. All tests were performed in triplicate. Percent values were determined by comparing the absorbance of control (Fenton reagents) and test samples. Results were expressed as IC_{50}.

Scavenging activity of nitric oxide

Nitric oxide (NO) generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which were measured by the Griess reaction (Green *et al*., 1982). The reaction mixture (3 ml) containing 10 mM sodium nitroprusside in phosphate-buffered saline (control) and extracts at different concentrations (10 - 1000 µg/ml) were incubated at 37ºC for 60 min. Trolox and ascorbic acid were used as reference antioxidant molecules. A 0.3 ml aliquot of the incubated sample was removed and 0.3 ml Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride in 2% H_3PO_4) was added. The absorbance of the chromophore formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with naphthylethylene diamine was measured after 15 minutes at 540 nm. All tests were performed in triplicate. Percent values were measured by comparing the absorbance of control (10 mM sodium nitroprusside in phosphate-buffered saline) and test preparations. Results were expressed as IC_{50}.

TBARS assay

The degree of lipid peroxidation was assayed by estimating the thiobarbituric acid reactive substances (TBARS) by using the standard method with minor modifications (Draper and Hadley, 1990). In brief, different concentrations of extracts (10 - 1000 µg/ml) were added to a solution rich in lipids - yolk of egg 1% (w/v) in buffer (Na_2HPO_4 20 mM, NaH_2PO_4 20 mM, KCl 140 mM, pH 7,4). Trolox and ascorbic acid were used as antioxidant standards. Lipid

peroxidation was initiated by adding 2,2'-azobis 2-metilpropionamidina (ABAP) 10 mM (control). After 60 min at 37ºC, 0.3 ml of this reaction mixture was taken in a tube containing 0.6 ml of 15% TCA, centrifuged (10.000g, 10 min) and 0.5 ml from supernatant was separated and mixed with 0.5 ml of 0.67% TBA (w/v). The mixture was heated in a hot water bath at 100 °C for 20 min to complete the reaction. The intensity of pink coloured complex formed was measured at 532 nm. Percent values were determined by comparing the absorbance of control (ABAP) and test samples. Results were expressed as IC_{50}.

Total phenolic content

The total phenolic content of the extracts was determined by an adapted colorimetric assay in which tannic acid was used as standard (Singleton *et al.*, 1999). Briefly, a 100 µl aliquot of extracts was assayed with 100 µl of Folin reagent and 200 µl of sodium carbonate (35%, w/v). The mixture was vortexed and diluted with distilled water to a final volume of 2 ml. After 10 min the absorption at 725 nm was measured and the total phenolic content was expressed as tannic acid equivalents (TAE µg/mg extract).

Clotting assay

A plasma coagulation assay adapted to the SpectraMax microplate ELISA reader was used as described (Ribeiro *et al*., 1995). The procedure allows to follow clot formation and to use kinetics parameters for the coagulation process. Briefly, 160 µl reactions containing 50 µl of human citrated plasma were incubated for 5 min, with 80 µl of 20 mM HEPES, pH 7.4, with or without varied amounts of extract (10, 25, 50 and 100 µg). Coagulation was triggered by adding $CaCl_2$ to a final concentration of 10 mM, and clot formation was monitored at 37 ^{o}C in the SpectraMax system at 650 nm. To access calcium-independent procoagulant activity, EDTA was added, in place of $CaCl_2$, to a final concentration of 10 mM.

Statistical analysis

Results were expressed as the mean ± SEM of at least three independent experiments. Data were analyzed by a one-way analysis of variance (ANOVA), using a Newman Keuls test to compare mean values across groups. When appropriate, Student's t-test was performed. Differences were considered to be significant when $P < 0.05$. Dose response curves were plotted, and the IC_{50} values (concentrations at which effects are inhibited by 50%) were calculated using non-linear regression analysis.

Results

Hydroxyl scavenging activity

Hydroxyl radicals were formed in solution and were detected by their ability to degrade 2-deoxyribose into fragments that formed a pink chromogen upon heating with TBA. When extracts were added to the reaction mixture, they removed $^{\bullet}OH$ and prevented sugar degradation. The IC_{50} of aqueous extract was 163 μg/ml and the IC_{50} of organic extract was 129.5 μg/ml (Table 1).

Scavenging activity of the nitric oxide

The results of the inhibitory effect of aqueous and organic extracts on NO production are shown in Table 1. Aqueous extract was 2.3 times more effective in attenuating nitric oxide production than organic extract. The IC_{50} of aqueous extract was 150.95 μg/ml and the IC_{50} of organic extract was 345 μg/ml.

TBARS assay

Table 1 shows the antioxidant capacity of extracts and reference compounds in a lipoperoxidative system. Both aqueous and organic extracts prevented lipid peroxidation induced by ABAP at different concentrations, with an IC_{50} of 186.4 μg/ml for aqueous extract and 158.9 μg/ml for organic extract.

Total phenolic content

The determination of total phenolic content in aqueous and organic extracts of marine sponge *P. janeirensis* showed that both extracts presented an equal content of phenolics, where 6.79 ± 0.31% dry weight of aqueous extract and 6.90 ± 0.29% dry weight of organic extract are phenolic compounds (Fig. 1).

Blood coagulation

To analyze the effect of marine sponge extracts upon clotting, human citrated plasma was incubated with extracts at different doses prior to the induction of coagulation with calcium. As shown in fig.2, extracts of marine sponge *P. janeirensis* displays a potent effect upon blood coagulation. While the control plasma (with no sample added) took over 4 min to initiate coagulation after the addition of calcium, plasma incubated with aqueous extract of the *P. janeirensis* took around 1.5 min to start coagulation (fig.2A), and 3 min with organic extract (fig.2B), indicating procoagulant activity. To access calcium-independent procoagulant activity, EDTA was added, in place of $CaCla_2$, but any effect was observed (data not shown). Moreover, normal plasma was not capable of clotting when neither extract nor calcium was added (data not shown).

Discussion

Diverse biological processes are modulated by free radicals, and ROS are recognized as a cause of immediate cellular injury leading to cell death or apoptosis. ROS can also lead to progressive accumulation of biomolecular damage and, consequently, are involved in many physiological (i.e. aging) and pathological (i.e. cancer) processes (Ames *et al*., 1993). Moreover, oxidative stress may promote an abnormal coagulative pattern and alter the structure and the function of coagulative proteins, as observed in several diseases (de Cristofaro *et al*., 2002; Abraham, 2000).

The hydroxyl ($^{\bullet}$OH) radical is probably the most potent ROS, and the probable initiator of the chain reactions which form lipid peroxides and organic radicals. Peroxidation of biomembrane lipids is known to cause serious damage to tissues and cells. This damage in the cellular membrane in turn leads to an increase in calcium leakage from internal storage sites in the cell. This will trigger an increase in nitric oxide synthesis via the activation of calmodulin-dependent nitric oxide synthase (Lai and Singh, 2004). Nitric oxide can further amplify iron-mediated free radical formation via its effects on iron metabolism and release of iron from ferritin (Richardson and Ponka, 1997; Reif, 1990). Thus, the effects will be amplified. Natural products that specifically target free radical production and blood coagulation are, therefore, promising for experimental approaches to the treatment of various malignancies.

Our work suggests that extracts of marine sponge *P. janeirensis* have antioxidant activity. In general, both aqueous and organic extracts decreased the $^{\bullet}$OH production, attenuated NO generation and prevented lipid peroxidation. Nevertheless, the scavenging activity of the nitric oxide was clearly greater in the aqueous extract (2.3 times more effective than organic extract).

Metabolites consisting of benzenoid and terpenoid parts are some of the most active marine metabolites. However, the antioxidant activity of marine phenolic metabolites has been poorly studied. From sponges, a few examples of antioxidants have been reported; terpenoid phenols and sesquiterpenequinones isolated from *Sarcotragus spinulosus* have been shown to possess antioxidant activity (Utkina *et al.*, 2004).

It is know that phenolic compounds may act as antioxidants by scavenging reactive oxygen and nitrogen species and chelating redox-active transition metal ions *in vitro*, however, it has been demonstrated that the performance of these secondary metabolites in oxidative systems depends on activity-structure relationships (Cao *et al.*, 1997; Rice-Evans *et al.*, 1996). In this

work, we used total phenolic content to estimate the contribution of these substances in sponge extracts to the performance in different antioxidant assays. Although this method could be overestimating total phenolics, it is so far the only single and widely used method for estimating total phenols (Deepa *et al.*, 2007). Our results demonstrated that total phenolic content of aqueous extract was equal to organic extract. However, the differences observed in the scavenging activity of the nitric oxide suggest qualitative differences in the phenolic composition of the extracts, and this point needs further investigations.

In a recent work, da Silva *et al*. (2007) reported that the antioxidant activities of *Bauhinia microstachya* extracts (a brazilian native plant widely used in folk medicine to treat various ailments) are linked to their polyphenol content. Interestingly, our results demonstrated a high content of phenolic compounds in both aqueous and organic extracts from *P. janeirensis*, suggesting that extracts of the Brazilian marine sponge *P. janeirensis* should be considered as a good source of natural antioxidants. In this context, there are some studies showing that the phenolic content of an extract could be positively correlated to the antioxidant potential (Hukkanen et al., 2006; Kuti and Konuru, 2004).

We also investigated the action of sponge extracts upon clotting. Our findings show, for the first time, the presence of procoagulant activity in marine sponge extracts. However, the mechanism by which this works is unknown, and studies are being performed in order to better characterize the pathway (extrinsic or intrinsic) here activated.

Sponges are known to produce a wide variety of chemical compounds and chemical analyses are the subject of an impressive body of literature. However, there are just few reports in which the authors have screened Brazilian sponge extracts for biological activities. To our knowledge, this is the first report demonstrating that marine sponge extracts from *P. janeirensis* have antioxidant activities and affect blood coagulation *in vitro* . Moreover, our findings suggest

that this marine sponge should be considered a good source of natural antioxidants due to their high phenolic content. Additional studies are required to understand the exact mechanism by which these extracts works, and further work to purify and characterize the chemical structure(s) of the substance(s) involved might yield new active compounds with biological activities and potential application in biomedicine.

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References

Munro, M. H. G., J. W. Blunt, E. J. Dumbei, S. J. H. Hickford, R. E. Lill, S. Li, C. N. Battershill, and A. R. Duckworth. 1999. The discovery and development of marine compounds with pharmaceutical potential. *J. Biotechnol.* **70:** 15-25.

Assmann, M., F. Lichte, J. R. Pawlik, and M. Kock. 2000. Chemical defenses of the Caribbean sponges *Agelas wiedenmayeri* and *Agelas conifera*. *Mar. Ecol. Prog. Ser.* **207:** 255-262.

Shacter, E., J. A. Williams, and R. L. Levine. 1995. Oxidative modification of fibrinogen inhibits thrombin-catalyzed clot formation. *Free Radic. Biol. Med.* **18:** 815-21.

Dunlap, W., L. Llewellyn, J. Doyle, and Y. Yamamoto. 2003. A microtiter plate assay for screening antioxidant activity in extracts of marine organisms. *Mar. Biotechnol.* **5(3):** 294-301.

Dunlap, W. C, M. Shick, and Y. Yamamoto. 1999. Sunscreens, oxidative stress and antioxidant functions in marine organisms of the Great Barrier Reef. *Redox Rep.* **4:** 301-306.

Muricy, G., E. Hajdu, F. V. Araújo, and A. N. Hagler. 1993. Antimicrobial activity of southwestern Atlantic shallow-water marine sponges (Porifera). *Scientia Marina* **57:** 427-432.

Monks, N. R., C. Lerner, A. T. Henriques, F. M. Farias, E. E. S. Schapoval, E. S. Suycnaga, A. B. da Rocha, G. Schwartsman, and B. Mothes. 2002. Anticancer, antichemotactic and

antimicrobial activities of marine sponges collected off the coast of Santa Catarina, southern Brazil. *J. Exp. Mar. Biol. Ecol.* **281:** 1-12.

Prado, M. P., Y. R. Torres, R. G. S. Berlink, C. Desiderá, M. A. Sanchez, M. V. Craveiro, E. Hajdu, R. M. da Rocha, and G. M. Machado-Santelli. 2004. Effects of marine organisms extracts on microtubule integrity and cell cycle progression in cultured cells. *J. Exp. Mar. Biol. Ecol.* **313.** 125-137.

Rangel, M., B. de Sanctis, J. C. de Freitas, J. M. Polatto, A. C. Granato, R. G. S. Berlink, and E. J. Hajdu. 2001. Cytotoxic and neurotoxic activities in extracts of marine sponges (Porifera) from southeastern Brazilian coast. *J. Exp. Mar. Biol. Ecol.* **262:** 31-40.

Faulkner, D. J. 2001. Marine natural products. *Nat. Prod. Rep.* **18(1):** 1-49.

Santafe, G., V. Paz, J. Rodriguez, and C. Jimenez. 2002. Novel Cytotoxic Oxygenated C29 Sterols from the Colombian Marine Sponge *Polymastia tenax*. *J. Nat. Prod.* **65:** 1161-1164.

da Silva, A. C., J. M. Kratz, F. M. Farias, A. T. Henriques, J. Dos Santos, R. M. Leonel, C. Lerner, B. Mothes, C. R. Barardi, and C. M. Simões. 2006. In vitro antiviral activity of marine sponges collected off Brazilian coast. *Biol. Pharm. Bull.* **29(1):** 135-40.

Hermes-Lima, M., E. M. Wang, H. M. Schulman, K. B. Storey, and P. Ponka. 1994. Deoxyribose degradation catalyzed by Fe(III)EDTA: kinetics aspects and potential usefullness for submicromolar iron measurements. *Mol. Cell. Biochem.* **137:** 65-73.

Green, L. C., D. A. Wagner, J. Glogowski, P. L. Skipper, J. K. Wishnok, and S. R. Tannenbaum. 1982. Analysis of nitrate, nitrite and 15N nitrate in biological fluids. *Anal. Biochem.* **126:** 131-138.

Draper, H. H., and M. Hadley. 1990. Malondialdehyde determination as index of lipid peroxidation. *Methods Enzymol.* **186:** 421-431.

Singleton, V. L., R. Orthofer, and R. M. Lamuela-Raventós. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods Enzymol.* **299:** 152-178.

Ribeiro, J. M., M. Schneider, and J. A. Guimarães. 1995. Purification and characterization of prolixin S (nitrophorin 2), the salivary anticoagulant of the blood-sucking bug Rhodnius prolixus. *Biochem. J.* **308:** 243-249.

Ames, B. N., M. K. Shigenaga, and T. M. Hagen. 1993. Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Natl. Acad. Sci. USA.* **90:** 7915-7922.

de Cristofaro, R., B. Rocca, E. Vitacolonna, A. Falco, P. Marchesani, G. Ciabattoni, R. Landolfi, C. Patrono, and G. Davi. 2002. Lipid and protein oxidation contribute to a prothrombotic state in patients with type 2 diabetes mellitus. *J. Thromb. Haemost.* **1:** 250-256.

Abraham, E. 2000. Coagulation abnormalities in acute lung injury and sepsis. *Am. J. Respir. Cell Mol. Biol.* **22:** 401-404.

Lai, H., and N. P. Singh, 2004. Magnetic-field-induced DNA strand breaks in brain cells of the rat. *Environ. Health Perspect.* **112(6):** 687-693.

Richardson, D. R., and P. Ponka. 1997. The molecular mechanisms of the metabolism and transport of iron in normal and neoplastic cells. *Biochim. Biophys. Acta.* **1331:** 1-40.

Reif, D. W. 1990. Nitric oxide mediates iron release from ferritin. *Arch. Biochem. Biophys.* **283:** 537-541.

Utkina, N. K., A. E. Makarchenko, O. V. Shchelokova, and M. V. Virovaya. 2004. Antioxidant activity of phenolic metabolites from marine sponges. *Chem. Nat. Comp.* **40:** 373-377.

Cao, G., E. Sofic, and R. L. Prior. 1997. Antioxidant and Prooxidant Behavior of Flavonoids: Structure-Activity Relationships. *Free Rad. Biol. Med.* **22:** 749-760.

Rice-Evans, C. A., N. J. Miller, and G. Paganga. 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Rad. Biol. Med.* **20:** 933-956.

Deepa, N., C. Kaur, B. George, B. Singh, and H. C. Kapoor. 2007. Antioxidant constituents in some sweet pepper (Capsicum annuum L.) genotypes during maturity. *LWT.* **40:** 121-129.

Da Silva, E. G., G. A. Behr, A. Zanotto-Filho, R. Lorenzi, M. A. B. Pasquali, L. G. Ravazolo, C. L. Bordigon-Jr, F. A. da Silva, A. L. Aboy, V. L. Bassani, A. T. Henriques, F. H. Reginatto, F. Dal-Pizzol, and J. C. F. Moreira. 2007. Antioxidant activities and free radical scavenging potential of Bauhinia microstachya (Raddi) Macbr. (Caesalpinaceae) extracts linked to their polyphenol content. *Biol. Pharm. Bull.* **30:** 1488-1496.

Hukkanen, A. T., S. S. Pölönen, S. O. Kärenlampi, and H. I. Kokko. 2006. Antioxidant capacity and phenolic content of sweet rowanberries. *J. Agric. Food Chem.* **54:** 112-119.

Kuti, J. O., and H. B. Konuru. 2004. Antioxidant capacity and phenolic content in leaf extracts of tree spinach (Cnidoscolus spp.). *J. Agric. Food Chem.* **52:** 117-121.

Table 1. Antioxidant potential of extracts from *P. janeirensis* in different *in vitro* systems

Sample test	Scavenging activity against hydroxyl radical in IC_{50}	Scavenging activity against nitric oxide radical in IC_{50}	Inhibition of lipoperoxidation in IC_{50}
Aqueous extract	163 ± 0.1	150.95 ± 0.35	186.4 ± 0.1
Organic extract	129.5 ± 0.2	345 ± 0.4	158.9 ± 0.1
Trolox	1.15 ± 0.02	0.20 ± 0.01	13.6 ± 0.08
Ascorbic acid	6.56 ± 0.01	N.D	4.1 ± 0.02

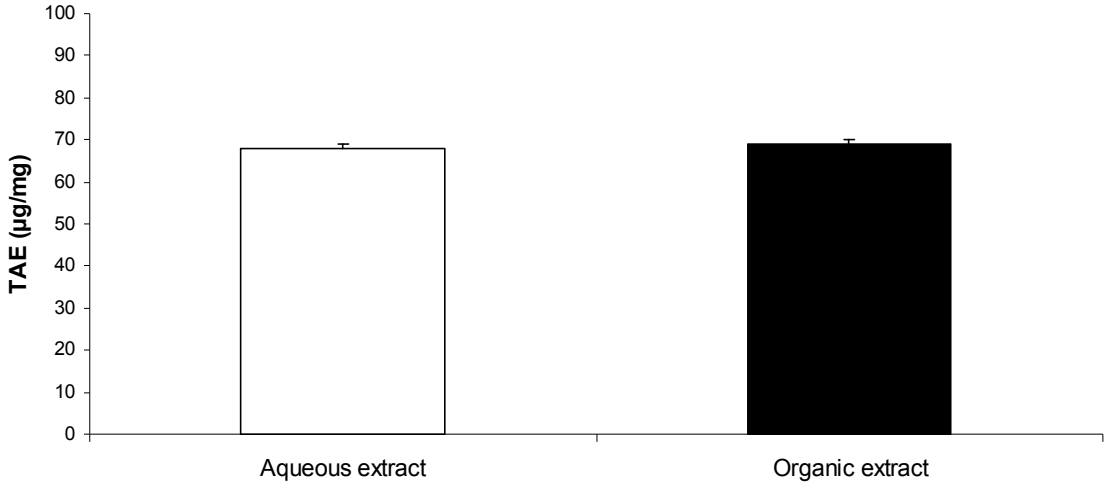
a) IC_{50}: sample concentration required for 50% inhibition. Aqueous and organic extracts are µg/ml. References standards are mM in hydroxyl and nitric oxide assay; in lipoperoxidation assay standards are µM. N.D: not detected. The results are expressed as means ± SEM of at least three independent experiments.

Figure legends

Figure 1. Total phenolic content of aqueous and organic extracts from Polymastia janeirensis. The results are expressed as tannic acid equivalents (TAE µg/mg extract). Bars represent the means ± SEM of at least three independent experiments.

Figure 2. Effect of sponge extracts from *Polymastia janeirensis* upon coagulation of human plasma. (A) Aqueous extracts; (B) Organic extracts. Fifty microliters of normal citrated human plasma were incubated with 80 µl of 20 mM HEPES, pH 7.4, with or without extract (10, 25, 50 and 100 µg/ml). Coagulation was triggered by adding 10 µl of 100 mM $CaCl_2$. In the control reaction (■) no extract was added. In the experimental reactions, plasma was incubated with either 10 (▲), 25 (×), 50 (✳) or 100 µg (●) of extract prior to the addition of calcium. (♦) Plasma was incubated with extract, and 10 Mm EDTA was added instead of $CaCl_2$. Reactions (clot formation) were monitored in the SpectraMax system at 650 nm and 37 ^{o}C. The data are representative of 3 independent experiments carried out in triplicate.

Figure 1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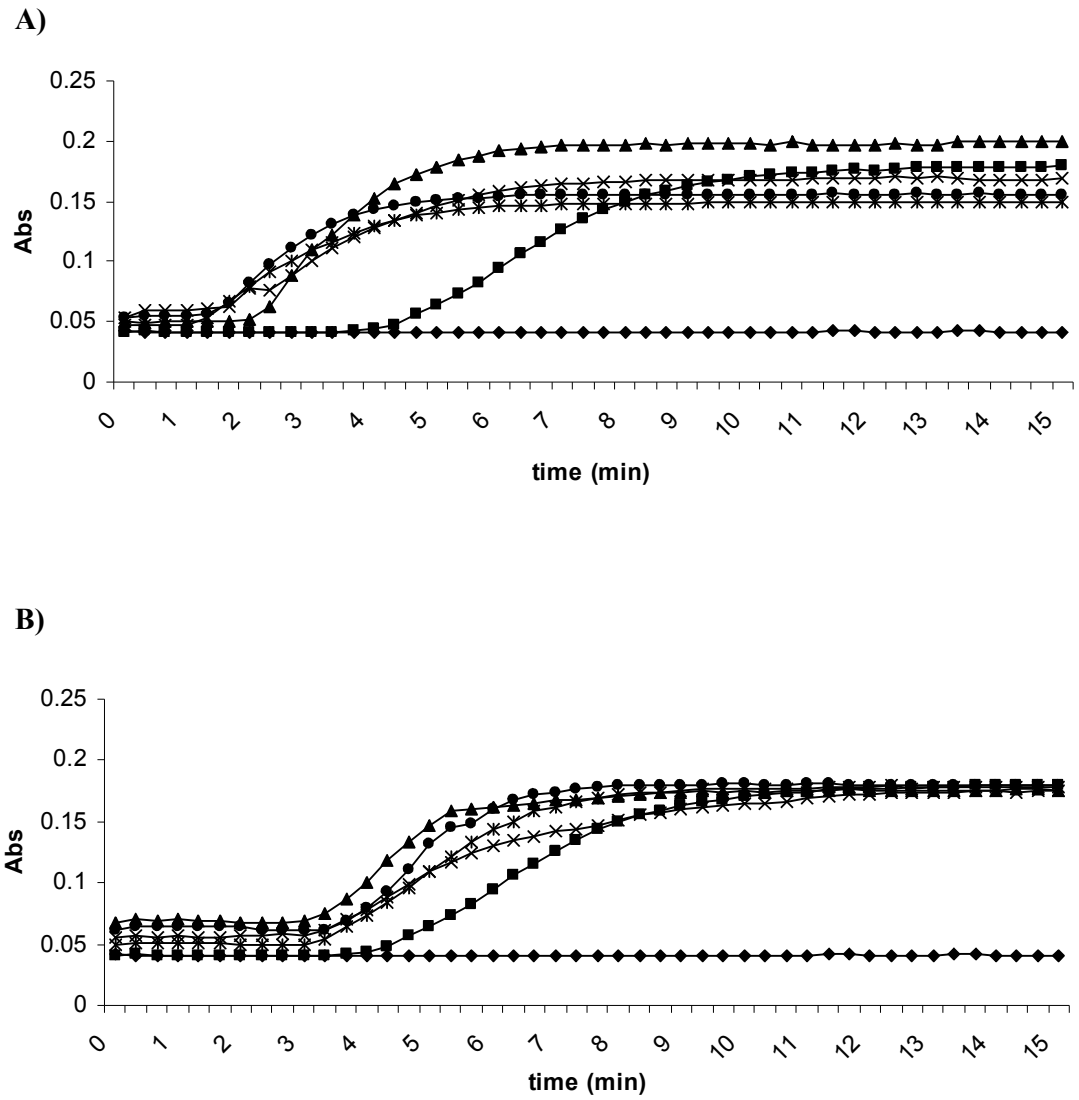
TAE (µg/mg)
Aqueous extract
Organic extract

Figure 2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