UNIVERSIDADE SÃO FRANCISCO
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AMANDA GOMES DA SILVA

EFEITO DO LÍQUIDO CELÔMICO DE *Echinometra lucunter* NA DIMINUIÇÃO DA NEUROTOXICIDADE CAUSADA PELO PEPTÍDEO AMILOIDE Aβ42

Bragança Paulista
2023

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Dissertação apresentada ao Programa de Pós Graduação Stricto Sensu em Ciências da Saúde da Universidade São Francisco, como requisito final para obtenção do Título de Mestre em Ciências da Saúde.

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Dedico este trabalho
À minha querida filha Giovanna Gomes Pavanello de Campos

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O Tempo

A vida é o dever que nós trouxemos para fazer em casa.
Quando se vê, já são seis horas!
Quando de vê, já é sexta-feira!
Quando se vê, já é natal…
Quando se vê, já terminou o ano…
Quando se vê perdemos o amor da nossa vida.
Quando se vê passaram 50 anos!
Agora é tarde demais para ser reprovado…
Se me fosse dado um dia, outra oportunidade, eu nem olhava o relógio.
Seguiria sempre em frente e iria jogando pelo caminho a casca dourada e inútil das horas…
Seguraria o amor que está a minha frente e diria que eu o amo…
E tem mais: não deixe de fazer algo de que gosta devido à falta de tempo.
Não deixe de ter pessoas ao seu lado por puro medo de ser feliz.
A única falta que terá será a desse tempo que, infelizmente, nunca mais voltará.

Mário Quintana

RESUMO

A Doença de Alzheimer (DA) é uma doença neurodegenerativa crônica e progressiva, manifestada por deterioração da memória e perda significativa das funções cognitivas, que acomete principalmente pacientes idosos. É considerado o principal tipo de demência, e devido ao aumento da expectativa de vida, o número de casos em 2015 foi de 46,8 milhões de pessoas, sendo hoje a 7ª causa de morte no mundo. A projeção é que em 2050 se tenha 131,5 milhões de casos. A doença é caracterizada pela formação de placas amiloides, que leva a uma série de disfunções neuronais, incluindo encurtamento de neuritos e morte celular, inflamação crônica e dano oxidativo. Acredita-se que a atividade de secretases sobre a Proteína Precursora Amiloide (APP) gera certos peptídeos β-amiloides (Aβ) que se agregam e acumulam em regiões do cérebro, causando toxicidade celular. Até o momento, não há cura específica para a doença, sendo que o tratamento clínico disponível apenas prolonga o tempo de vida dos pacientes em alguns anos. Assim, se faz necessária a busca por novas moléculas para um possível tratamento ou prevenção da doença. Neste trabalho, o líquido celômico do ouriço-do-mar *Echinometra lucunter* e suas frações, obtidas por extração em fase solida, foram testados em neurônios expostos ao Aβ42 oligomerizado (Aβ42o) para avaliação da redução de toxicidade neuronal, pelo método do MTT, em efeito tratamento e prevenção. A redução da formação ou eliminação de Aβ42o foi avaliada por espectrometria de massas ou tioflavina e o estresse oxidativo foi determinado pela quantificação de antioxidantes e poder antioxidante da amostra pelo reagente trolox. Foi visto que 1 h de incubação do líquido celômico preveniu a morte neuronal causada pelo Aβ42o e 24 horas de tratamento reverteu a morte celular, e algumas frações reproduziram tal efeito. As frações El 0, El 50 e El 100 reduziram a quantidade de peptídeo agregado no meio de cultura das células, e quando incubadas diretamente com o peptídeo já agregado, demostraram efeito de reversão da oligomerização, o que explica a diminuição da toxicidade celular. A forma monomérica do peptídeo, a forma menos tóxica, foi encontrada nessas amostras, confirmando a redução da oligomerização. Além desse mecanismo de remoção de Aβ42o, foi estudado o estresse oxidativo, pois o líquido celômico possui moléculas antioxidantes, confirmadas pelo método do DPPH e peróxido de hidrogênio. Foi visto que a fração El 50 aumentou significativamente o poder antioxidante do meio de cultura dos neurônios após a exposição com Aβ42o, reduzido, portanto, o estresse oxidativo. Essa fração foi então analisada por espectrometria de massas para determinação de sua composição, em comparação com banco de produtos naturais (GNPS), que possui moléculas provenientes de animais marinhos. Foram identificados 6 compostos, e 2 deles com conhecida atividade antioxidante, além de 2 peptídeos previamente identificados pelo grupo. Em conclusão, moléculas do líquido celômico de *Echinometra lucunter* representam uma fonte de compostos capazes de atuar em vários mecanismos, que podem conferir uma neuroproteção e/ou tratamento da doença multifatorial, que é a Doença de Alzheimer.

Palavras-chave: Doença de Alzheimer. *Echinometra lucunter.* peptídeo Aβ42. Neuroproteção. tratamento.

ABSTRACT

Alzheimer's Disease (AD) is a chronic and progressive neurodegenerative disease, manifested by memory deterioration and significant loss of cognitive functions, mainly affecting elderly patients. It is considered the main type of dementia, and due to the increase in life expectancy, the number of cases in 2015 was 46.8 million people, currently ranking the 7th leading cause of death worldwide. The expectation is that in 2050 there will be 131.5 million cases. The disease is characterized by the formation of amyloid plaques, which leads to a series of neuronal dysfunctions, including neurites shortening, cell death, chronic inflammation, and oxidative damage. It is believed that the activity of secretases on Amyloid Precursor Protein (APP) generates certain β-amyloid peptides (Aβ) that aggregate and accumulate in regions of the brain, causing cellular toxicity. Currently, there is no specific cure for the disease, and the available clinical treatment only prolongs the patient's lifespan by a few years. Thus, the search for new molecules for a possible treatment or prevention of the disease is necessary. In this study, the coelomic fluid of the sea urchin *Echinometra lucunter* and its fractions obtained by solid-phase extraction were tested on neurons exposed to oligomerized Aβ42 (Aβ42o) to evaluate the reduction of neuronal toxicity by the MTT method in both treatment and prevention effects. The reduction of Aβ42o formation or elimination was evaluated by mass spectrometry or thioflavin, and oxidative stress was determined by the quantification of antioxidants and the antioxidant power of the sample by the trolox reagent. It was observed that 1 h of coelomic fluid incubation prevented neuronal death caused by Aβ42o, and 24 hours of treatment reversed cell death, and some fractions reproduced such an effect. The El 0, El 50, and El 100 fractions reduced the amount of aggregated peptide in the cell culture medium, and when incubated directly with the already aggregated peptide, they demonstrated a reversal effect of oligomerization, which explains the reduction of cellular toxicity. The monomeric form of the peptide, the least toxic form, was found in these samples, confirming the reduction of oligomerization. In addition to this mechanism of Aβ42o removal, oxidative stress was studied because the coelomic fluid contains antioxidant molecules, confirmed by the DPPH and hydrogen peroxide methods. It was observed that the El 50 fraction significantly increased the antioxidant power of the neuron culture medium after exposure to Aβ42o, thereby reducing oxidative stress. This fraction was then analyzed by mass spectrometry to determine its composition, compared to the Natural Product Database (GNPS), which has molecules from marine animals. Six compounds were identified, and two of them had known antioxidant activity, in addition to two peptides previously identified by the group. In conclusion, molecules from the coelomic fluid of *Echinometra lucunter* represent a source of compounds capable of acting on various mechanisms, which may confer neuroprotection and/or treatment of the multifactorial disease that is Alzheimer's Disease.

Keywords: *Alzheimer's disease. Echinometra lucunter. Aβ42 peptide. Neuroprotection. treatment.*

LISTA DE SÍMBOLOS E ABREVIAÇÕES

Aβ: peptídeo beta amiloide

Aβ42o: peptídeo beta amiloide 42 oligomerizado

AChE :acetilcolinaesterase

ACN: Acetonitrila

ANVISA: Agência Nacional de Vigilância Sanitária

APP (*Amyloid precursor protein*): Proteína precursora do peptídeo β-amiloide

AUF (*arbitrary units of fluorescence*): unidades arbitrárias de fluorescência

CCL: Comprometimento Cognitivo Leve

DA: Doença de Alzheimer

DMSO (*Dimethyl Sulfoxide Solution*): Solução de dimetilsulfóxido

EI: *Echinometra lucunter*

FBS (*fetal bovine serum*): Soro Fetal Bovino

FDA: Food and Drug Administration

ICMBio: Instituto Chico Mendes de Conservação da Biodiversidade

MTT: brometo de 3-(4,5-Dimetiltiazol-2-il)2,5-difenil-2 H-tetrazólio

µg: micrograma

µL: microlitro

µM: micromolar

mL: mililitros

m/z: massa sobre carga

nm: nanômetro

PET: Tomografia por emissão de pósitrons

PBS (*phosphate-buffered saline*): tampão fosfato-salina

ROS: Espécies Reativas de Oxigênio

SCN: Sistema Nervoso Central

SUS: Sistema Único de Saúde

TFA: Ácido Trifluoracético

λ: comprimento de onda

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1. INTRODUÇÃO

1.1. Doença de Alzheimer

A Doença de Alzheimer (DA) é o principal tipo de demência, que acomete cerca de 45 milhões de pessoas por ano em todo o mundo, sendo 7ª causa de morte. A expectativa é que em 2050 se tenha 131,5 milhões de casos (ALZHEIMER'S DISEASE INTERNATIONAL, 2018).

A DA é considerada um transtorno neurodegenerativo crônico, progressivo e irreversível, caracterizada por deterioração da memória e perda significativa das funções cognitivas, que acomete principalmente pacientes idosos (ARAUJO; PONDE, 2006).

A teoria mais aceita é que a DA tem início após a formação de placas amiloides e hiperfosforilação da proteína Tau, que levam a morte neuronal significativa em regiões específicas do cérebro. Como consequência da morte neuronal, há perda de contato sináptico, além de inflamação crônica pela ativação de microglias e dano oxidativo (HARDY; SELKOE, 2002). O primeiro local acometido é hipocampo, local onde há formação de memória e cognição, e por isso a memória a curto prazo é o primeiro sinal clínico observado. A medida em que a doença avança, há comprometimento da memória de longo prazo, além de danos a outras partes do cérebro, levando ao comprometimento da linguagem, cognição espacial e funções executivas (SCHILLING et al., 2022).

As placas amiloides ocorrem após o acúmulo de peptídeos amiloides, que quanto atinge níveis elevados começa a causar disfunção neuronal, e com o passar dos anos, danos à estrutura do cérebro, diminuição da cognição e comprometimento de vários aspectos funcionais do paciente. A Figura 1 mostra o gráfico correlacionando o curso temporal da doença, desde fases pré-clínicas, após comprometimento cognitivo leve (CCL) e com o quadro de demência instalado (SPERLING et al., 2011).

A proteína Tau, também implicada na DA, é uma proteína solúvel, associada a microtúbulos expressa principalmente nos axônios dos neurônios. É codificado pelo gene MAPT, localizado no cromossomo 17 (locus17q21), e contém 16 éxons. Existem 6 isoformas principais de tau no ser humano adulto, variando em tamanho de 352 aminoácidos. Cada uma das isoformas tem um papel fisiológico, pois funciona de maneira diferentes durante o desenvolvimento do cérebro. A primária função da Tau é promover a formação e montagem de microtúbulos, mas também exerce funções no transporte axonal, neuroplasticidade, regulação celular e transmissão sináptica.

Quando a tau é fosforilada, ela se desprende dos microtúbulos, transportando organelas intracelulares como mitocôndrias e vesículas. A hiperfosforilação impede que essa função ocorra (MOTA et al., 2023).

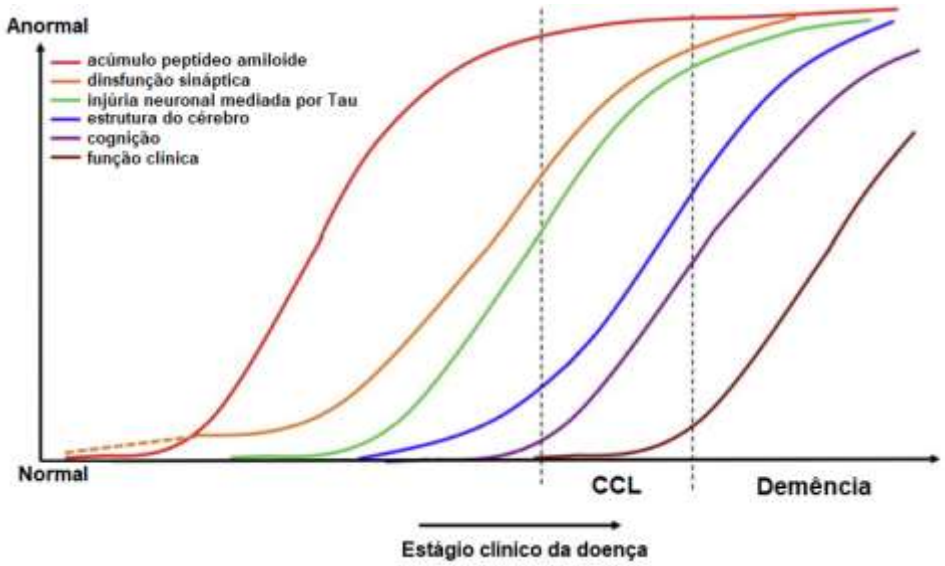

FIGURA 1 – Gráfico da Progressão da DA após a formação do peptídeo amiloide e proteína Tau, detectados no fluído cerebroespinhal de pacientes ou por imagem por PET. CCL = comprometimento cognitivo leve. Fonte: adaptado de SPERLING et al., 2011.

As placas amiloides se formam após o processamento da proteína precursora amiloide (APP). Essa proteína é composta por uma família de genes APP APLP1 (*Amyloid Beta Precursor Like Protein 1*) e APLP2 (*Amyloid Beta Precursor Like Protein 2)*, e possui funções fisiológicas que incluem desenvolvimento do sistema nervoso, formação de junção neuromuscular, sinaptogênese, complexidade dendrítica e densidade de espinhas, crescimento e orientação axonal, e funções sinápticas, incluindo plasticidade sináptica, aprendizado e memória (MÜLLER; DELLER, 2017).

Em condições fisiológicas, a porção C-terminal da APP é processada pela via não-amiloidogênica, que consiste na clivagem por enzimas α-secretases, liberando o fragmento sAPPalfa e um peptídeo de 83 aminoácidos (C83) para o meio extracelular, que é neuroprotetor e neurotrófico (WAKABAYASHI; STROOPER, 2008).

No entanto, a APP pode ser processada pela via amiloidogênica, pela ação da enzima β-secretase, liberando o fragmento sAPPbeta e um peptídeo de 99 aminoácidos (C99), que é

posteriormente clivado pela γ-secretase, gerando um novo peptídeo, o beta-amiloide, de 40 ou 42 aminoácidos (Aβ40 e Aβ42) (LOPEZ; GOZÁLEZ; LÉGER, 2019) (Figura 2).

Foram identificadas mutações nos genes da APP, da presenilina 1 e presenilina 2 (do complexo da γ-secretase), de forma a deixar a enzima mais ativa, e consequentemente liberar uma quantidade maior de Aβ40 e Aβ42. Portanto, acredita-se que a doença tenha um caráter genético, além de aspectos ambientais que parecem contribuir com o seu desenvolvimento, como a poluição, dieta rica em componentes pró-inflamatórios, infecções crônicas, metais pesados e poluição (BREIJYEH; KARAMAN, 2020)

Os peptídeos Aβ40 e Aβ42 são altamente hidrofóbicos, e por isso se oligomerizam em soluções aquosas, como o meio extracelular do tecido cerebral. Esses peptídeos insolúveis agregados, conhecidos como placas amiloides, se acumulam em regiões extracelulares e intracelulares específicas do cérebro e causam a DA (Figura 2). Peptídeos oligomerizados são mais tóxicos que em sua forma monomérica (HARDY; SELKOE, 2002).

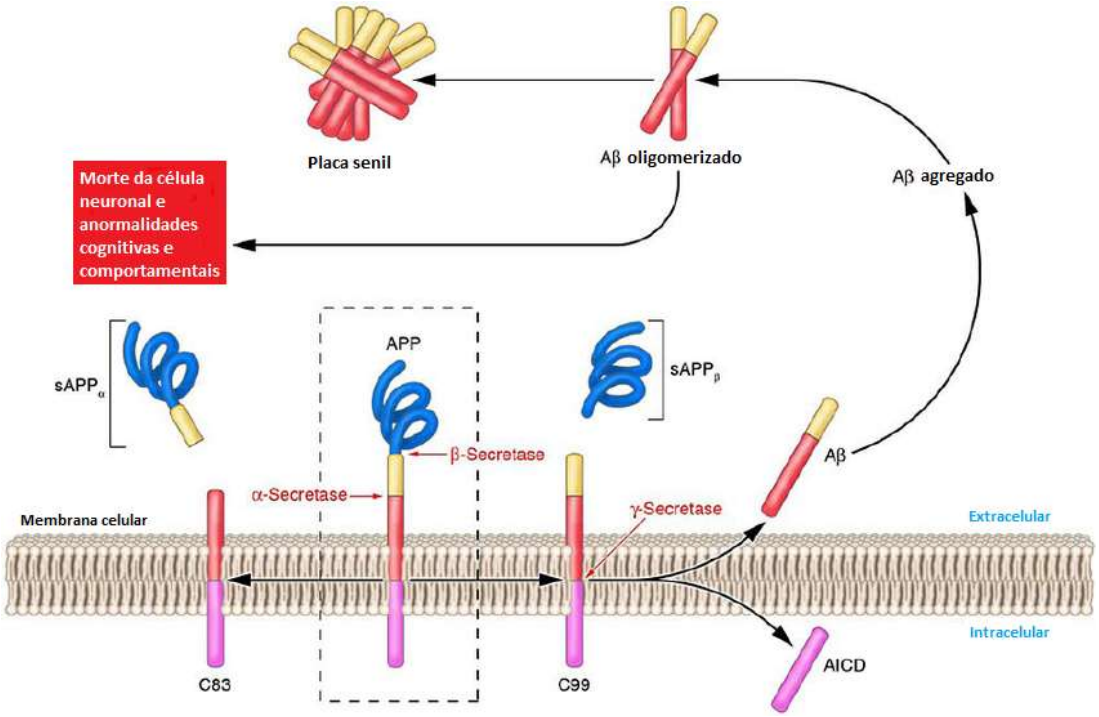

FIGURA 2 – Esquema representativo da clivagem proteolítica de secretases e geração de agregados de Aβ. APP = proteína precursora amiloide; C83 = APP processada, com os 83 aminoácidos da porção N-terminal; C99 = APP processada, com os 99 aminoácidos da porção N-terminal Fonte: IMBIMBO; LOMBARD;POMARA., 2005.

Os peptídeos amiloides Aβ40 e o Aβ42 são nomeados dessa forma por conta da quantidade de aminoácidos presentes, conforme a sequência de resíduos mostrada na Figura 3. O Aβ40 é o mais abundante (80 a 90%), porém o Aβ42 tem se demonstrado mais tóxico por ser mais hidrofóbico e fibrilogênico, sendo depositado mais facilmente no cérebro (MURPHY; LEVINE, 2010).

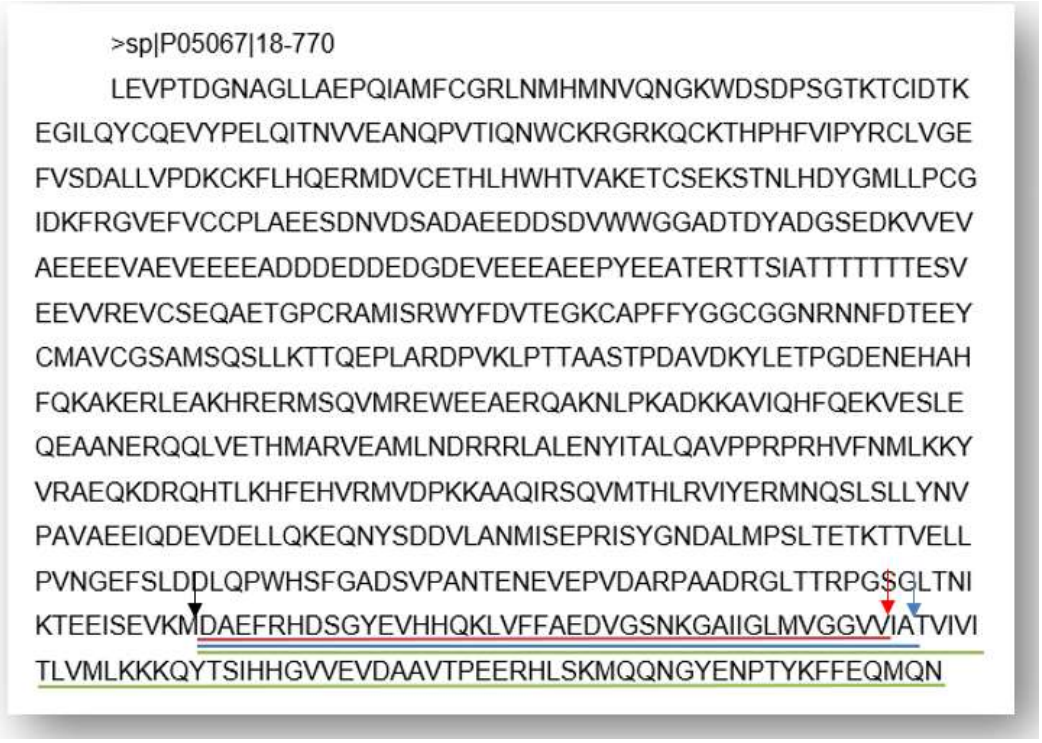

FIGURA 3 – Sequência da APP (P05067) com representação dos peptídeos gerados após clivagem proteolítica por secretases. As setas indicam os pontos de clivagem. Linha vermelha = Aβ40 (sp|P05067|672-711), linha azul = Aβ42 (sp|P05067|672-713), linha verde = C99 (sp|P05067|672-770). Fonte: BANAGOURO, 2022.

Proteínas disformes e agregados são frequentemente removidos por um complexo catalítico denominado sistema ubiquitina-proteassomo. Na DA, devido ao tamanho dos produtos agregados, a eliminação pelo sistema de ubiquitina-proteassomo é inviável, uma vez que os produtos não são capazes de atingir o sítio catalítico desse complexo (DOMENICO; TRAMUTOLA; PERLUIGI, 2016).

Uma alternativa para a degradação desses agregados é o sistema autofagia-lisossomal, em que proteínas indesejáveis são dirigidas aos lisossomos, onde são clivadas por enzimas proteolíticas (catepsinas) e eliminadas (STOKA; TURK; TURK, 2016).

A inflamação crônica está relacionada à DA, cuja progressão consiste em agregação de peptídeos amiloides, que gera placas amiloides, e hiperfosforilação da proteína Tau, que causam ativação da glia do SNC, infiltração de células imunes periféricas, dano, morte neuronal e atrofia neuronal ao longo do tempo (BUCCIANTINI; GIANNONI; CHITI, 2002; SOKOLOVA et al. 2009).

A ativação da catepsina B e do inflamassomo ainda induzem a produção de espécies reativas de oxigênio (ROS) e levam a um estresse oxidativo da DA, o que causa uma série de alterações mitocondriais, que resultam também em morte celular (TANEO et al., 2015).

Sendo assim, a DA consiste em múltiplos mecanismos, disparados pela presença de peptídeos amiloides oligomerizados, sendo, portanto, importante a sua eliminação para o controle da doença.

1.2. Animais marinhos como fonte de novas moléculas

Animais marinhos possuem uma vasta diversidade de moléculas para defesa química contra predadores, microrganismos e digestão da presa. Neste sentido, podemos utilizar essa "biblioteca de moléculas" previamente selecionada ao longo de anos de evolução, ainda pouco exploradas, em favor da descoberta de novas estruturas funcionais (HENDLER et al., 1995).

Cerca de um terço das drogas mais vendidas no mundo são derivadas de produtos naturais, dentre elas plantas e animais. Animais marinhos têm contribuído para o fornecimento de moléculas que hoje são comercializadas para várias indicações terapêuticas, como câncer, infecções virais e dor (MALVE, 2016; RAWAT et al., 2006; NEWMAN; CRAGG, 2007).

Um exemplo é o peptídeo ziconotídeo, também conhecido como ω-conotoxina MVIIA, comercialmente conhecido como Prialt®. Este peptídeo possui 25 aminoácidos e é obtido do molusco *Conus magnus*. Ele é utilizado como analgésico para o tratamento de dores crônicas em pacientes que não respondem a tratamentos com opioides. O peptídeo é cerca de 50 vezes mais potente do que a morfina, mas não tem efeitos colaterais de dependência (OLIVEIRA, et al., 1987; COSTA, et al., 2015).

O alcaloide trabectidina (Youndelis®) e o indolocarbazol midostaurina são medicamentos aprovados pela ANVISA para o tratamento de tumores sólidos e leucemia, respectivamente, que

foram desenvolvidos a partir de moléculas de tunicados. Eribulina (Halaven®), citarabina e nelarabina (Arranon® nos Estados Unidos ou Atriance® na Europa) são medicamentos provenientes de esponjas do mar para o tratamento de leucemia (BARRECA et al., 2020).

1.3. Moléculas de origem animal com ação neuroprotetora

Estudos mostram moléculas proveniente de animais marinhos ou terrestres que agem na neuroproteção ou em mecanismos de remoção de peptídeo amiloide.

A octovespina é um peptídeo proveniente do veneno da vespa *Polybia occidentalis*, e se mostrou capaz de reduzir a agregação de Aβ em modelos *in vitro* e *in vivo* (CAMARGO et al., 2022).

Outra molécula, a exenatida, foi inicialmente desenvolvida para o tratamento de diabetes tipo 2 e agora está sendo estudada por sua atividade neuroprotetora em neurônios hipocampais de rato, após a morte celular causada por 6-hidroxidopamina (6-OHDA), relacionada à Doença de Parkinson, Aβ e agentes de estresse oxidativo (LI et al., 2010).

Peptídeos da anêmona do mar *Heteractis crispa* aumentaram a viabilidade de neurônios N2A, após serem lesionados por 6-OHDA (SINTSOVA et al., 2021). Efeito neuroprotetor utilizando 6-OHDA também foi observado pelos peptídeos do hexacoral *Palythoa caribaeorum*, em peixes-zebra (LIAO et al., 2018).

1.4. Echinometra lucunter

O *Echinometra lucunter* é o ouriço-do-mar mais abundante da costa brasileira, conhecido como ouriço preto ou pindá. É encontrado no costão rochoso ao longo de toda a costa, até a Flórida, nos Estados Unidos da América (TAVARES, 2004).

O animal possui uma carapaça rígida, com espinhos imersos, facilmente quebradiços, e capazes de causar um grande número de acidentes em banhistas (HADDAD JR, 2003). A penetração do espinho na pele causa dor intensa, inflamação local (edema e eritema), e sangramento, e se não removidos podem levar a sinovite granulomatosa (WADA et al., 2008).

Os espinhos possuem grânulos em sua estrutura, ricos em moléculas que são liberadas após o contato com a pele (SCIANI et al., 2011). Essas moléculas são capazes de causar inflamação, caracterizada por aumento de adesão e migração leucocitária em camundongos e diminuição do limiar de dor de ratos (SCIANI et al., 2017). Portanto, esses sintomas observados

nos acidentes são decorrentes da presença de toxinas, e não somente um trauma mecânico da entrada dos espinhos na pele.

O líquido celômico do animal, um fluido interno que circunda os órgãos, tem a função de transporte de gases, moléculas e excreção. O líquido contém células do sistema imune inato, capazes de fagocitar substâncias estranhas ao animal, além de bactérias patogênicas (HAUG et al., 2002).

Moléculas provenientes do líquido celômico de *E. lucunter* foram descritas pelo grupo. A echinometrina, um peptídeo de 8 aminoácidos, demonstrou um importante efeito inflamatório, com desgranulação de mastócitos (SCIANI et al., 2014). Outros peptídeos foram descritos no líquido celômico, além de outros compostos de baixa massa (SCIANI et al., 2016).

Portanto, esse animal possui uma vasta fonte de moléculas bioativas, que podem ser utilizadas para bioprospecção.

2. JUSTIFICATIVA

A Doença de Alzheimer (DA), dentre as diversas formas de demência, é a mais frequente, correspondendo a até 70% do total da prevalência das demências. É uma doença caracterizada por perda de memória e déficit cognitivo. É comum em idosos: aproximadamente 1% nos indivíduos com idade entre 60-65 anos e 30 – 35% na população acima dos 80 anos. Devido ao aumento da expectativa de vida, o número de casos tem aumentado consideravelmente, sendo previsto que em 2050 se tenha 131,5 milhões de acometidos pela doença (ALZHEIMER'S DISEASE INTERNATIONAL, 2018).

Em 2010, o SUS gastou 2,4 bilhões de reais com internações hospitalares por doenças crônicas (diabetes, hipertensão arterial, neoplasias, doenças respiratórias, cardiovasculares, do aparelho locomotor, endócrinas nutricionais e metabólicas) (SOTO et al., 2015). No período de 2008 a 2013 foram gastos pelo SUS mais de R$ 90 milhões em medicamentos para o tratamento da DA (COSTA et al., 2015).

Atualmente, apenas 2 grupos farmacológicos estão aprovados para o tratamento da DA: os inibidores da acetilcolinaesterase (AChE) e os antagonistas do receptor glutaminérgico N-metil-Daspartato (NMDA) (DIAS, L. 2022). No mercado, estão disponíveis quatro medicamentos

licenciados pela ANVISA para o tratamento da DA, são eles: tacrina, rivastigmina, donepezil e galantamina (ENGELHARDT et al. 2005).

Recentemente, dois anticorpos monoclonais foram aprovados pelo Food and Drug Administration (FDA) para o tratamento da DA, o aducanumab e o lecanemab, que se ligam às placas amiloides, reduzindo os seus níveis no cérebro. Isso levou a redução do declínio cognitivo da DA, em pacientes com grau leve da doença (MUKHOPADHYAY; BANERJEE, 2021; VAN DYCK et al., 2023).

A partir desse cenário, a identificação de terapêuticas antiamilóide pode oferecer uma estratégia terapêutica para combater as doenças neurodegenerativas humanas, tendo como pressuposto teórico a hipótese da cascata do amiloide. As terapêuticas modificadoras com alvo na acumulação de Aβ mais estudadas hoje incluem os inibidores e moduladores das enzimas β-secretase e γ-secretase e a imunoterapia (HUNG; FU,2017).

Com relação aos inibidores da β-secretase, devido à toxicidade que resulta da sua ação, poucos estão em desenvolvimento clínico, entre eles: Verubecestat, Lanabecestat, Elenbecestat, Atabecestat e CNP520 (SAHOO et al., 2018; PANZA et al., 2019). A principal toxicidade associada a esses inibidores foi a hepática, embora a inibição completa da enzima tenha mostrado prejuízo na plasticidade sináptica funcional e estrutural, não resultando em benefícios para os pacientes (ZHU et al., 2018).

Já, com relação ao desenvolvimento de terapêutica com ação inibitória da γ-secretase, que tem como objetivo reduzir a quantidade de Aβ que é formado, atualmente, em ensaio clínico de fase II encontra-se o avagacestat e em ensaio clínico de fase III, que terminou recentemente devido a ineficácia clínica, o semagacestat (MAIA, M., SOUSA. E, 2019).

Como mencionado, anteriormente, a biodiversidade brasileira, e em particular os animais marinhos, podem fornecer novas entidades moleculares que promovam possibilidades para a redução das placas amiloides que causam a DA, em um efeito antiamiloide direto, o que é apresentado nessa dissertação. É importante ressaltar que, ao conhecimento do grupo, nenhum estudo foi publicado com a ação de novas moléculas de *E. lucunter* na Doença de Alzheimer.

3. OBJETIVO

Avaliar o potencial de moléculas do líquido celômico do ouriço-do-mar *Echinometra lucunter* em diminuir ou prevenir a toxicidade causada pelo peptídeo Aβ42 da Doença de Alzheimer, em

cultura de neurônios, e estudar mecanismos de eliminação de placa amiloide e redução do estresse oxidativo.

3.1 Objetivos específicos

- Obter o líquido celômico ouriço-do-mar *Echinometra lucunter* e frações;

- Tratar neurônios com frações do líquido celômico e verificar se causam prevenção ou redução da toxicidade neuronal induzida por Aβ42 oligomerizado.

- Verificar se a redução da toxicidade foi devido à remoção ou inibição da formação de Aβ42 oligomerizado.

- Verificar a presença de compostos antioxidantes e avaliar os efeitos das moléculas no estresse oxidativo.

4. CAPÌTULO 1

Artigo submetido à revista Journal of Venomous Animals and Toxins Including Tropical Diseases (JVAT)

O artigo foi feito em colaboração com pesquisadores do Instituto Butantan, e mostra que uma fração do líquido celômico de *E. lucunter* foi capaz de prevenir e reverter a toxicidade causada pelo Aβ42 oligomerizado em neurônios SH-SY5Y diferenciados. Essa diminuição da toxicidade foi devido à sua capacidade de desestabilizar as placas amiloides, tornando-as monômeros de peptídeos, ou seja, a forma menos tóxica. Além disso, a fração reduziu o estresse oxidativo, devido à presença de moléculas antioxidantes, detectadas por testes de oxidação in vitro e identificadas por espectrometria de massas.

Echinometra lucunter molecules in the reduction of neurotoxicity caused by Aβ42– effects on disaggregation and oxidative stress

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Abstract: *Echinometra lucunter* is a sea urchin commonly found in the America's rocky shore. Its coelomic fluid contains molecules that are used for defense and biological processes, some of which may have therapeutic potential for the treatment of amyloid-based neurodegenerative diseases, such as Alzheimer's, which currently have few drug options available. In this study, we demonstrated that *E. lucunter* coelomic fluid (ELCF) and certain fractions can prevent and treat toxicity caused by oligomeric amyloid peptide Aβ42 in SH-SY5Y neuron-like cells. To understanding the cause of toxicity reduction, we founded that one fraction (El50) reduced the oligomerized Aβ42 and the oxidative stress caused by the amyloid peptide through its antioxidant molecules, which in turn reduced cell death. Mass spectrometry analysis revealed that the fraction was composed of small molecules containing flavonoids antioxidants and two peptides.

Our results suggest that these molecules may interact with various molecular targets, resulting in a global reduction of neurotoxicity, which may be useful in treating dementia.

Keywords: sea urchin; amyloid peptide 42; Alzheimer's disease; protein clearance, oxidative stress.

1. Introduction

Animal venoms are a rich source of bioactive compounds, and many of them have neuroactive molecules, which can be used as pharmacological tools for the treatment of several disorders, including Alzheimer's Disease, Parkinson's Disease, Huntington's Disease, amyotrophic lateral sclerosis, among others (Yang et al., 2019).

Alzheimer's disease is the main type of dementia, characterized by extracellular plaques of amyloid peptides (Aβ), generated after precursor amyloid protein (APP) processing by beta and gamma-secretases, besides the formation of intraneuronal neurofibrillary tangles. Both features cause neurotoxicity and neurological damage in the affected area, which spreads through the brain, over time. Moreover, Aβ activates microglia to release pro-inflammatory mediators and reactive oxygen species (Mota et al., 2021).

Aβ have been detected in other neurological diseases, such as Parkinson's (Lim et al., 2019), vascular dementia (Liu et al., 2015), Lewy body dementia (Biundo et al., 2021), amyotrophic lateral sclerosis (Calingasan et al., 2005) and Down syndrome (Head and Lott, 2004).

Thus, molecules that reduce neurotoxicity by impairing amyloid plate formation or inducing degradation of amyloid peptides are being searched from animal venoms.

Octovespin, a peptide from *Polybia occidentalis* wasp venom, was able to reduce the aggregation of Aβ in both *in vitro* and *in vivo* models (Camargo et al., 2022). A molecule names exenatide, which was firstly developed for type 2 diabetes treatment, now is being studied for its neuroprotective activity on rat hippocampal neurons, after cell death caused by 6-hydroxydopamine (6-OHDA), Aβ and oxidative stress agents (Li et al., 2010). Neuroprotective effect has also been detected in other animal venoms. Peptides from the sea anemone *Heteractis crispa* showed increasing in the N2A viability, after being injured by 6-OHDA (Sintsova et al., 2021), and neuroprotection was observed by peptides from *Palythoa caribaeorum* in zebrafishes (Liao et al., 2018).

Molecules that increase the activity of enzymes which can degrade amyloid peptides have also been studied. Metalloproteases from snake venoms were shown to degrade the Aβ peptide in an animal model of AD (Nalivaeva et al., 2012). Phospholipase A_2 from bee venom caused reduction of Aβ deposits in the AD transgenic mouse brain (Ye et al., 2016). Smith et al. (Smith et al., 2016) reported that a peptide from *Bothrops asper* venom could increase the Vmax of both neprilysin and endothelin converting enzyme-1, two enzymes that degrade amyloid peptides.

The venom from the scorpion *Buthus martensii Karsch*, containing peptides resistant to heat, increased neurogenesis and, in a *Caenorhabditis elegans* model that expresses Aβ1 42, could reduce Aβ plaque deposition, in comparison to an untreated group (Zhang et al., 2016).

Dendrotoxins from *Dendroaspis* venom, by blocking K+ channel, increased acetylcholine release, useful to restore neurotransmission in the hippocampus. An acetylcholinesterase-activity inhibitor was identified in the *P. bundokalbo* spider venom (Lopez et al., 2021).

Thus, animal venoms are a library of compounds able to interfere with some aspect of amyloid plaque removal, contributing to the obtention of new prototypes for treatment of neurodegenerative diseases.

Echinometra lucunter is an abundant sea urchin living in Americas intertidal rocky shore, which secrete several molecules for chemical defense and homeostasis (Sciani et al., 2013, 2011). Our group has been studying secretions from *E. lucunter* spines and coelomic fluid, and has described several biomolecules (Sciani et al., 2017, 2016, 2014). Thus, this animal can be used as a source of molecules therapeutically relevant.

In this study, we show molecules from *E. lucunter* sea urchin coelomic fluid that reduced the neurotoxicity caused by Aβ42 in SH-SY5Y neuron-like, in both preventive and treatment approaches.

2. Materials and Methods

2.1. Sample

Echinometra lucunter sea urchin was collected in São Sebastião SP, Brazil (23°49′53″S; 45°31′18″W), under license number 13852-1 from the Brazilian Environmental Agency (IBAMA), without distinction of sex, age or size. The coelomic fluid was extracted by puncturing the peristomial membrane and added to acetic acid 0.05% (v:v; final concentration). The fluid was kept

in ice bath until further processing. The coelomic fluid (ELCF) was then centrifuged at 1248 × g for 5 min, at 4°C and the supernatant was processed by solid phase extraction (SPE) using C18 cartridges (Strata®, 55 m, 70˚A, 5 g/20 mL, Phenomenex Inc., Torrance, CA, USA) and elution was performed with increased concentration of acetonitrile (0, 25, 50, 75 and 100%), in a solution containing 0.1% trifluoroacetic acid (TFA). Thus, fractions named El 0, 25, 50, 75 and 100 corresponds to elution with acetonitrile.

Aβ42 was purchased from FastBio (Ribeirão Preto, Brazil), manufactured by Biomatik (Canada). The peptide was diluted in DMSO to get a 1 mM solution, and then diluted in phosphate-saline buffer 50 mM pH 7.2 to 100 µM. This solution (named Aβ42o) was maintained at 4°C for 24 hours for oligomerization, which was confirmed by thioflavin-T fluorescence (see section 2.3).

2.2. Cell viability

SH-SY5Y cells (ECACC, Sigma Aldrich, St. Louis, MO, USA) were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (1:1) (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 U/mL of penicillin/streptomycin (Gibco Life Technologies, Grand Island, NY, USA) in a humidified atmosphere of 5% CO_2 at 37°C. In the 15^{th} passage, after adhesion, cells (1×10^4 cells/well, 96 wells) were differentiated by adding 10 µM all-trans retinoic acid (Sigma Aldrich, Saint Louis, MO) in a media containing 1% FBS. This cell media was replaced every 2 days, until the 8^{th} day, when neurons were used in the experiments.

E. lucunter coelomic fluid was tested in a range of 0.1 to 100 µg/mL to test its potential to cause cell death. SPE fractions were tested in a concentration of 100 µg/mL. After that, *E. lucunter* sample was tested in neuron-like, in 96-well plates, differentiated and maintained in a humidified 5% CO_2 incubator at 37°C in a density of 1×10^4 cells/well. Two approaches were used: (1) prevention: incubation of *E. lucunter* coelomic fluid (10 µg/mL) for 1 or 6 h and then addition of Aβ42 (5 µM) for 48 hours; (2) treatment: incubation of Aβ42 (5 µM) for 48 hours and then addition of *E. lucunter* coelomic fluid (10 µg/mL) for 3 or 24 hours. The treatments were performed in triplicate.

After treatment, the cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, where the medium was removed, and the reagent was incubated for 3 hours in a concentration of 0.5 mg/mL. The blue formazan product was dissolved in dimethyl sulfoxide (DMSO), and the absorbance was measured at 540 nm.

The results were plotted in a graph of % viable cells, being the negative control (the same volume of sample, but PBS) the 100%, in a triplicate experiment.

2.3. Identification and quantification of Aβ42

The cell culture media was collected, and 60% acetonitrile in ultrapure water was added. The solution was centrifuged at 10000 rpm at 4°C for 10 min. The supernatant was inserted into a C18 column (Titan, 80Å, 5 x 2.1 mm, 1.9 µm, Supelco) coupled to the mass spectrometry (QToF Xevo GS-XS, Waters Co., USA). Chromatography was performed by elution with acetonitrile containing 0.1% formic acid, in 3 steps: 0% B, 60% B and 100% B, in a constant flow of 0.2 mL/min. The ions corresponding to Aβ42 (m/z 1129.0 and m/z 1505, to 3 and 2 charges respectively) as well as the fragments, generated after argon collision, were monitored after positive ionization, in a range of 300 to 1800 m/z and FWHM 40000 resolution at 500 m/z. For the MS/MS analysis. The instrument control and data acquisition were conducted by MassLinx 4.2.

Alternatively, 2 µL cell culture media collected from negative control (cells treated with PBS), positive control (cells treated with oligomeric Aβ42) and samples were added to 196 µL phosphate-saline (PBS) buffer 50 mM pH 7.2 and 2 µL thioflavin-T 1 mM, in a 96-well plate. The fluorescence was read in λex = 450/λem = 490 nm and values of samples, in triplicate, were compared to the positive control, in triplicate.

Thioflavin-T was also used to determine the oligomerization of Aβ42 in contact with ELCF and its fractions. Samples (10 µg/mL) was incubated with 50 mM pH 7.2 PBS buffer and 100 µM Aβ42. Thioflavin-T (1 mM) was added and after 5 minutes, the fluorescence was read in λex = 450/λem = 490 nm. Experiments were performed in triplicate and samples were compared to a negative control (without Aβ42) and a positive control (Aβ42 without sample).

2.4. Antioxidant activity

The antioxidant activity was assessed by the DPPH and hydrogen peroxide (H_2O_2) methods, both conducted on 96-well plate.

For DPPH, ELCF and fractions (10 μg) were diluted in 180 μL methanol and incubated with 0.1 mM DPPH reagent, diluted in methanol, in the dark. After 5 minutes at room temperature, the mixture had its absorbance read by a spectrophotometer at $\lambda = 515$ nm.

For hydrogen peroxide assay, samples were added to 0.1 M pH 7.0 phosphate buffer, containing 89 mM NaCl and hydrogen peroxide 0.2 mM. The mixture was incubated for 10 minutes at 37°C, and after that, 0.5 mL of HRPO (0.05 mg) and phenol (0.1 mg) diluted in 0.1 M pH 7.0 phosphate buffer was added for 15 minutes at room temperature. Then, 1.3 M NaOH was added for 10 minutes, and the absorbance was read at $\lambda = 610$ nm.

As a positive control, ascorbic acid (1 mM) was used instead samples for both assays. The % of oxidant activity was calculated by: $[(Ac - As)/ Ac)] \times 100$, where Ac = absorbance of the control and As = absorbance of the test sample, being the control the reagent, without sample. The tests were performed in triplicate and calculated as average ± SD.

The antioxidant power of cells was measured using the Antioxidant Assay Kit (709001, Cayman Chemicals, MI, USA), following the manufacturer's instructions. After being treated in the prevention or treatment approach, the cell culture media was collected and submitted to the test. The test was performed in triplicate and calculated as average ± SD.

2.5. Compounds identification

The fraction 50 of the ELCF was analyzed by mass spectrometry for compounds identification. The fraction was analyzed using reverse-phase ultraperformance liquid chromatography with a C18 column (1.7 μm, 100 Å, 2.0 mm × 50 mm). The elution was done using a binary gradient of 0-100% B over 40 min, being A = formic acid (FA)/H_2O (1:1000) and B = FA/acetonitrile/H_2O (1:900:100), at a constant flow rate of 0.2 mL/min. Mass spectrometry

(Q-ToF Xevo GS, Waters Co.) was used for automatic monitoring of the column eluates in a positive ionization mode, with MS/MS analysis performed using argon collision energy. The scan was performed in a range of 100 to 1800 m/z for MS and 50 to 1500 m/z for MS/MS, and FWHM 40000 resolution at 500 m/z.

Equipment control and data acquisition were conducted using the MassLynx 4.2. Raw files were processed using GNPS-MassIVE public data repository for untargeted MS^2 data using compounds identification and molecular networking with MS^2 and spectral similarity (http://gnps.ucsd.edu). Data was set as 0.5 Da of precursor ion mass tolerance, 0.2 Da of fragment ion mass tolerance, 6 min matched peaks and 0.7 score threshold. It was used all public spectra at GNPS, curated by natural product scientific community (Wang et al., 2016).

2.6. Statistical analysis

Data are presented as mean (SD). Statistical analysis was performed using one-way ANOVA, with Tukey's posttest, by comparing all groups, considering p value 0.05.

3. Results

3.1. Cell toxicity

Neuron-like cells were incubated with *Echinometra lucunter* coelomic fluid (ELCF) to verify if it caused neurotoxicity. The coelomic fluid did not cause toxicity in neuron-like cells even at high concentrations (Figure 4A), nor did the *E. lucunter* coelomic fluid fractions, which were generated after solid phase extraction (Figure 4B) - in fact, El 100 even increased cell viability.

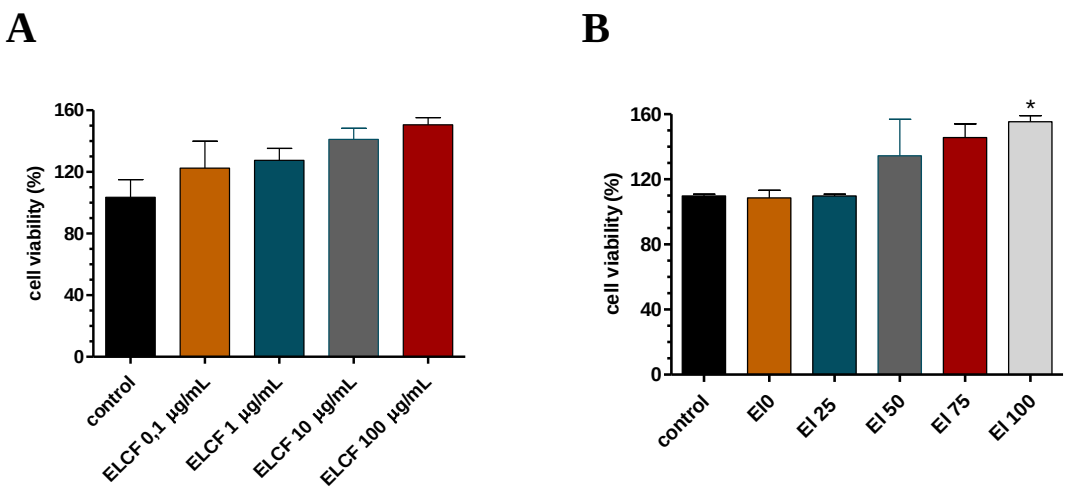

Figure 4. Cell viability of SH-SY5Y neuron-like after *Echinometra lucunter* coelomic fluid (ELCF) and its fractions. (**A**) Cell viability of different concentrations of coelomic fluid; (**B**) cell viability of *E. lucunter* coelomic fluid SPE fractions (100 µg/mL); Control = cells treated with PBS. Data are presented as mean (SD). *indicates $p<0.05$ of samples compared to control, by one-way ANOVA test, followed by Tukey's test.

After confirming the lack of toxicity, the *Echinometra lucunter* coelomic fluid (ELCF) was incubated in neuron-like cells to verify its ability to prevent or treat cell damage, caused by the oligomerized amyloid peptide Aβ42.

Aβ42o induced toxicity of neuron-like 48h after incubation (Figure 5A, B, C and D). When ELCF was added 1h or 6h before the Aβ42o, it could prevent neurotoxicity (Figure 5A), being 1h more prominent, although statistically the same as control group. In a treatment effect, the addition of ELCF for 3 or 24h after Aβ42o incubation was able to reverse the toxic effect caused by the peptide (Figure 5B).

When we analyzed ELCF fractions, we could see interesting results in both prevention and treatment effects. All fractions prevented cell death caused by Aβ42o, and El50, 75 and 100 significantly increased the cell viability (Figure 5C). Regarding treatment, only fraction El25 could not reverse the toxic effects caused by Aβ42o, while other fractions reproduced the observed effects of raw sample (Figure 5D).

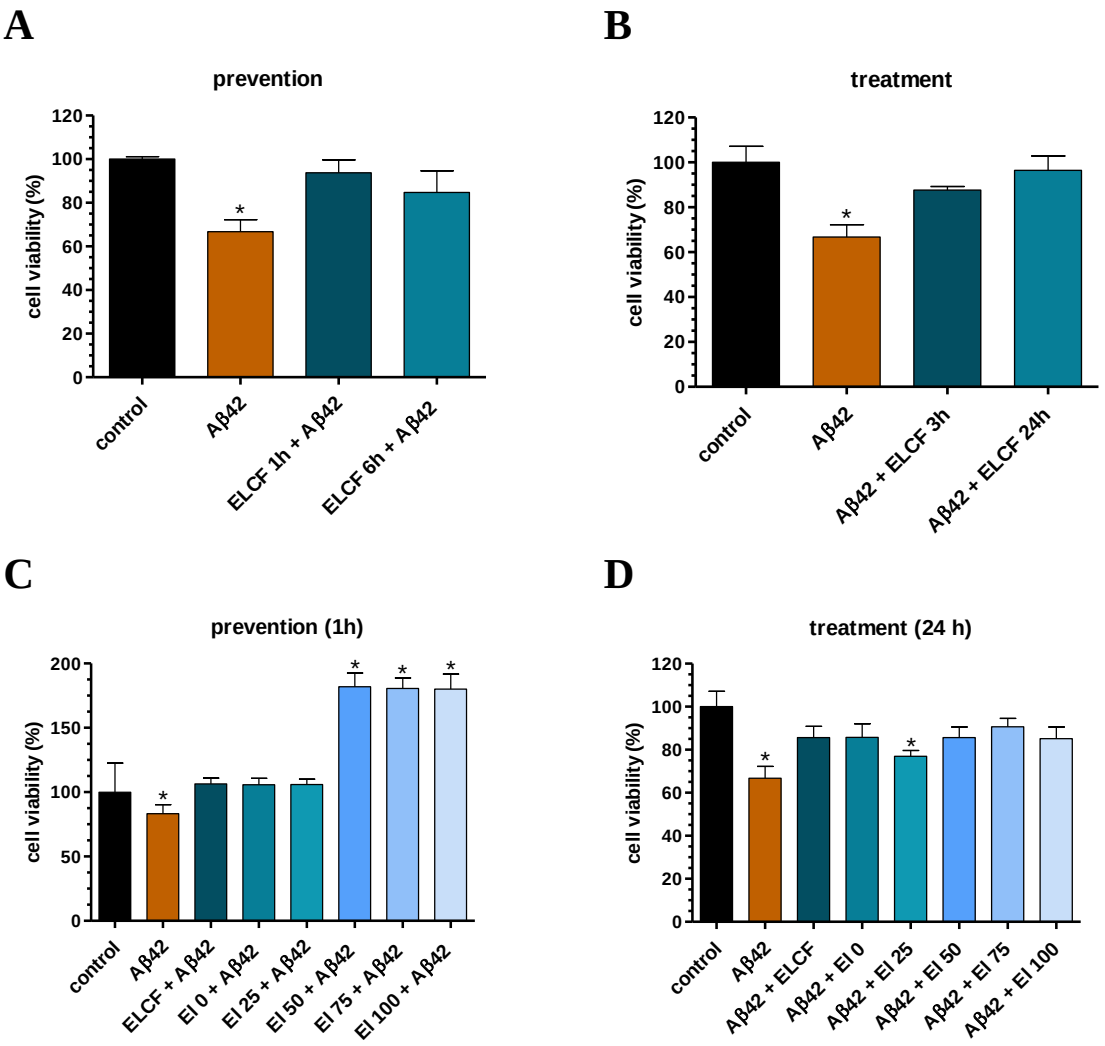

Figure 5. Cell viability of SH-SY5Y neuron-like after Aβ42o and *Echinometra lucunter* coelomic fluid (ELCF) and its fractions. (**A)** prevention evaluation (addition of sample for 1 or 6h before the incubation of Aβ42o); (**B**) treatment evaluation (sample addition after the incubation of Aβ42o for 3 or 24h); (**C**) prevention evaluation of ELCF fractions; (**D)** treatment evaluation with ELCF fractions. Control = cells treated with PBS. Data are presented as mean (SD). *indicates $p<0.05$ of samples compared to control, by one-way ANOVA test, followed by Tukey's test.

3.2. Aβ42 identification and quantification

Mass spectrometry analysis was used to verify the presence of monomeric amyloid peptide in the cell culture media, after the incubation of samples in both prevention and treatment approaches. The ion corresponding to Aβ42 (1129.0 or m/z 1505) was absent in cells without any treatment (table 1, control PBS) and present in cells in which the peptide was added (table 1, Aβ42). When ELCF and El 0 or El 25 fractions were incubated in the prevention approach, no ion was not

identified in the media (table 1, prevention), while they were observed with El 50, 75 and 100. When ‘treatment samples’ were analyzed, the ion was identified in all samples, indicating that the monomeric amyloid peptide was in the cell media.

Table 1. Presence or absence of Aβ42 ion (m/z 1129.0 or m/z 1505) in the culture media of SH-SY5Y neuron-like treated with the amyloid peptide, *E. lucunter* coelomic fluid and its SPE fractions before (1h = prevention) or after (24h = treatment).

	Control	**Aβ42**	**prevention**	**treatment**
ELCF	—	✓	—	✓
El 0	—	✓	—	✓
El 25	—	✓	—	✓
El 50	—	✓	✓	✓
El 75	—	✓	✓	✓
El 100	—	✓	✓	✓

Fluorescence emission analysis of the cell culture media was performed after incubation with thioflavin-T to detect Aβ42 in its oligomeric form. We observed that ELCF and fractions El 0, 50, and 100 were effective in reducing Aβ42o in the prevention approach, and in the treatment approach, fraction El 50 significantly reduced Aβ42o levels (Figure 6).

To verify if the reduction of Aβ42 oligomerization is caused by the direct action of ELCF and its fractions, we added the amyloid peptide to each fraction and then incubated thioflavin-T. We could see that ELCF did not cause any effect, but when fractions were evaluated, fractions El 0, 50 and 100 impaired the oligomerization of amyloid peptide (Figure 7), in agreement with the previous results of Aβ42 oligomerization, observed in the cell media.

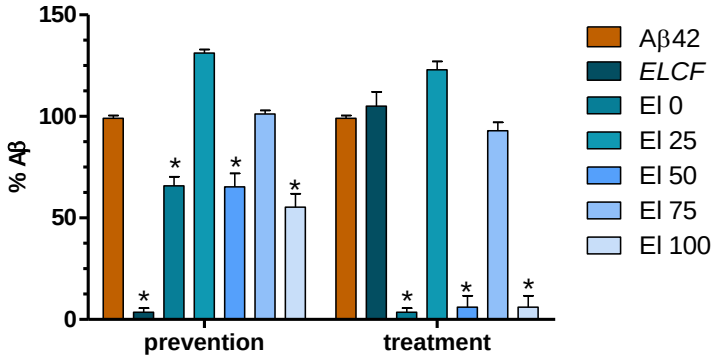

Figure 6. Percent of aggregated Aβ detected by fluorescence, in the presence of thioflavin-T, in SH-SY5Y neuron-like culture media treated with Aβ, *Echinometra lucunter* coelomic fluid (ELCF) and its fractions, obtained by solid-phase extraction. Prevention = samples incubated 1 h before Aβ42; treatment = samples incubated 24h and then Aβ42. Data are presented as mean (SD). *indicates $p<0.05$ of samples compared to control, by one-way ANOVA test, followed by Tukey's test.

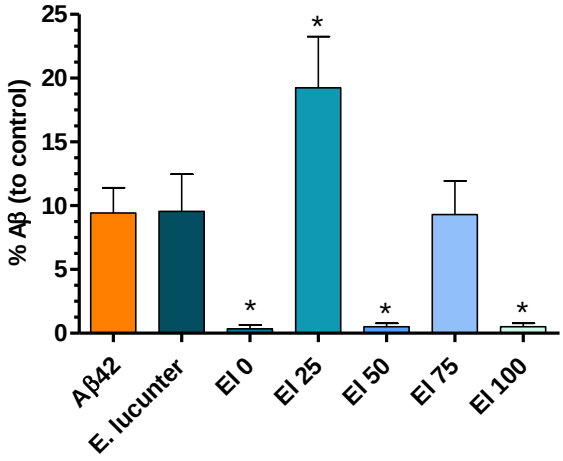

Figure 7. Percent of aggregated Aβ detected by fluorescence, in the presence of thioflavin, in *Echinometra lucunter* coelomic fluid and its fractions added to Aβ42. Data are presented as mean (SD). *indicates $p<0.05$ of samples compared to control, by one-way ANOVA test, followed by Tukey's test.

3.3. Oxidative stress

E. lucunter coelomic fluid and its fractions were tested to verify the presence of antioxidant molecules by DPPH and peroxide assays. It was verified that the coelomic fluid, El 25 and 50 were

able to reduce the oxidant effect of the reagent, acting as an antioxidant, as well as ascorbic acid, used as a positive control (Figures 8A and B).

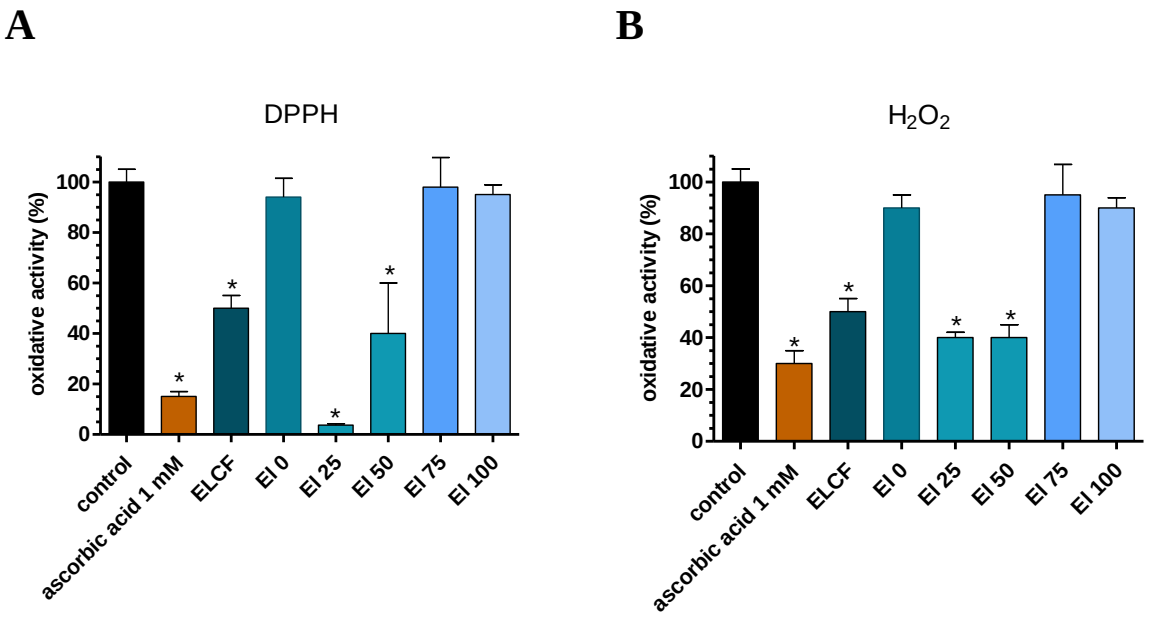

Figure 8. Oxidative activity of *Echinometra lucunter* coelomic fluid (ELCF) and its fractions. (A) DPPH assay; (B) peroxide assay. Control = reagent without samples; ascorbic acid is the positive control (antioxidant). Data are presented as mean (SD). *indicates $p<0.05$ of samples compared to control, by one-way ANOVA test, followed by Tukey's test.

The cell culture media from SH-SY5Y neurons-like treated with ELCF and fractions was collected to test its antioxidant capacity. It was observed that Aβ42o reduced the antioxidant power for both prevention and treatment approaches, compared to a control group, with neurons treated with PBS. On the other hand, *E. lucunter* coelomic fluid presented more antioxidant, in mM than Aβ42, similar to control group. The fraction El 25 was able to reproduce the effects observed with the raw sample, while the others have diminished antioxidant power, as shown in figure 9A (prevention) and B (treatment).

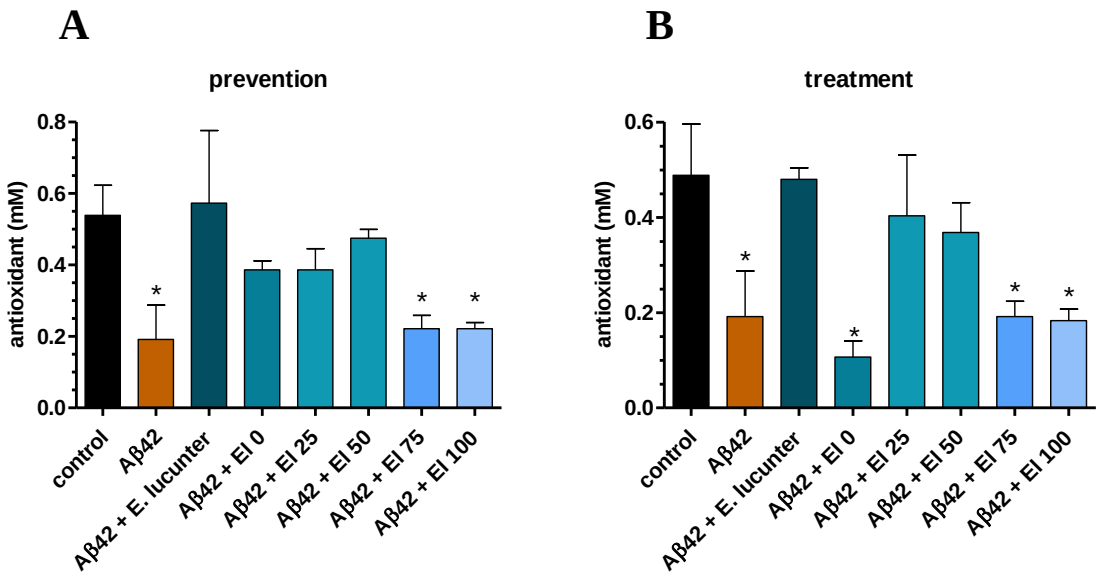

Figure 9. Antioxidant power of SH-SY5Y neuron-like media after treatment with Aβ42 or *Echinometra lucunter* coelomic fluid or its fractions, obtained by solid phase extraction. (A) Prevention approach = samples incubated 1 h before Aβ42; (B) treatment approach = samples incubated 24h and then Aβ42. Data are presented as mean (SD). *indicates $p<0.05$ of samples compared to control, by one-way ANOVA test, followed by Tukey's test.

3.4. Compounds identification

Due to its interesting effect of preventing or reversing cell death caused by Aβ42o, through a mechanism of reducing olimgomerization and controlling oxidative stress, fraction El 50 was analyzed by mass spectrometry to determine its composition. After comparing the mass spectra to the GNPS database, it was possible to identify 6 small molecules (Table 2), besides 2 peptides previously identified by our group, with sequences LLHA and AAPCPDVEVSEQF, corresponding to this fraction (Sciani et al., 2016). No proteins were detected in this sample (data not shown).

Table 2 – Compound identified in GNPS database

Compound name	Spectrum ID	Exact mass	Precursor m/z
Taxifolin (dihydroquercetin)	CCMSLIB00006410892	304.058	305.07
Benzalkonium chloride (C12)	CCMSLIB00000531495	304.29	304.29
C17-Sphinganine	CCMSLIB00009943587	287.50	288.276

DAPG	CCMSLIB00004679387	210.053	211.061
Minaprine	CCMSLIB00003137768	298.179	299.188
phenazine-1-carboxylic acid	CCMSLIB00000839196	224.059	225.066

4. Discussion

The amyloid cascade hypothesis is the most acceptable to explain Alzheimer's disease. This hypothesis postulate that the APP protein, present in neurons, is cleaved by secretases, generating amyloid peptides. (Mota et al., 2021).

The Aβ40 is the most abundant amyloid peptide (80 to 90%), but the Aβ42 is the most toxic, due to its hydrophobic nature, which allows plaque formation and deposit into the neurons (Murphy and LeVine, 2010). That is the reason we chose to study Aβ42 instead other amyloid peptides, and we could verify its neurotoxicity in our cell model. SH-SY5Y cell line has been widely used as a model for neurodegenerative diseases study. After differentiation with agents, such as retinoic acid, it changes its morphology in order to be similar to primary neurons, with neuritic process, electrical excitability and synaptophysin-positive synapses, characteristics of cholinergic and dopaminergic neurons (Kovalevich and Langford, 2013).

Using this model, we demonstrated that *E. lucunter* coelomic fluid prevents neuron-like death when added 1h before the Aβ42o and impaired cell death when incubated for 24 h after the addition of oligomeric amyloid peptide 42 (48 h in contact with the cells). Some fractions could reproduce such effects – fractions El 50, 75 and 100.

At physiologically relevant concentrations, monomeric Aβ is not associated with cellular toxicity. However, soluble oligomers, which exhibit considerable heterogeneity in terms of size and structure, have been demonstrated to have significant neurotoxicity (Cremades and Dobson, 2018). The oligomers induce synaptic defunction and neuron apoptosis, besides inducing oxidative stress and release of inflammatory mediators, contributing to the amyloid-based neurodegenerative diseases progression (Reddy and Beal, 2008; Ruan et al., 2009). In our cell model, we could demonstrate neuron-like death after incubation with Aβ42o.

To understand how ELCF and fractions reduced the toxicity caused by Aβ42o, we verified if the amyloid peptide was removed from the cells in its monomeric or aggregated form. Moreover, we checked the reduction of oxidative stress, as sea urchins are a rich source of antioxidants. For that, we opted to work with fractions, instead of the raw fluid, to reduce the number of molecules and get an assertive response.

Fractions El 25 and 75 were not able to reduce the oligomerized Aβ42 in both cellular mechanisms and by direct action on the peptide. Regarding fraction 25, only oligomerized form of the peptide was found, which explains the lack of effect on either prevention or treatment approach on cell death.

On the other hand, fractions El 0, 50 and 100 were effective in reducing oligomerized Aβ42 levels in cell media, in both prevention and treatment approaches, as evidenced by thioflavin-T quantification, a reagent that binds only to oligomeric amyloid peptides. While oligomeric peptides were still detected in the preventive approach, the results were more pronounced in the treatment with *E. lucunter* fractions 0, 50, and 75, suggesting that the molecules act on the oligomeric peptides after their formation without affecting the aggregation process.

Our findings confirmed that these three fractions were able to reduce the oligomers even in the absence of cells, indicating a direct action on the peptide structure. These results suggest the possibility of removing amyloid plaques that have already formed and deposited in the brain of patients, preventing neuron loss, and potentially controlling the progression of the disease.

Using this same thioflavin-T assay, Camargo et al. (2022) verified that a synthetic peptide optimized by the one isolated from wasp venom was able to prevent Aβ aggregation, and consequently reduced the toxicity caused by the amyloid peptide, confirmed by animal's tests, in which memory impairment was reversed (Camargo et al., 2022).

Several natural compounds, especially polyphenols, have shown to inhibit the oligomerization of amyloid peptide, acting stabilizing the monomers by nucleation or disaggregation (Phan et al., 2019). Epigallocatechin gallate and myricetin are structures similar to the one found here (in El 50), with antiaggregating effects on Aβ peptide (Pagano et al., 2020).

Most of the evidence suggests that the N-terminal and β1 region of Aβ are crucial in disrupting the aggregation process and controlling the toxicity of stabilized oligomers. Changes in the recognition of the monomer/membrane are associated with alterations in the accessibility of the hydrophobic β1-turn region and charged N-terminus, a probable site of inhibitory molecules, and

this is one mechanism of disaggregation and reduction of toxicity by oligomers (Ahmed et al., 2019).

Therefore, one mechanism of reduction of amyloid peptide-toxicity by fractions El 0, 50 and 100 is the disaggregation of oligomeric amyloid peptide, especially in a treatment approach, making it less toxic to neurons (Sengupta et al., 2016).

Another mechanism demonstrated here was the oxidative stress caused by Aβ42 and its reversion by *E. lucunter* samples. We observed that the *E. lucunter* coelomic fluid and fractions El 25 and 50 have intense antioxidant propriety, evaluated by two assays. When these samples were incubated in neuron-like cells, they could increase the antioxidant power in SH-SY5Y-treated cell culture media, in both prevention and treatment approaches, indicating reactive oxygen species (ROS) removal.

Antioxidants have already been described in sea urchins. The first report was from McClendon, in 1912, in which it was reported an antioxidant effect in the coelomic fluid from *Arbacia punctulate* (McClendon, 1912). A pigment from the *Anthocidaris crassispina* shell showed important antioxidant activity by the DPPH method (Kuwahara et al., 2009). Pigments derived from naphtoquinone, with high antioxidant activity, were isolated from *Echinometra mathaei*, a sea urchin specie related to *Echinometra lucunter*, used in this work (Soleimani et al., 2016).

Echinochromes were described in sea urchins, being the most abundant the Echinochrome A, clinically used in cardiology and ophthalmology, without causing adverse effects (Kim et al., 2021). Several free radicals scavenging mechanisms have been related to this molecule (Utkina and Pokhilo, 2012)

Sintsova et al. showed similar results: peptides from *Heteractis crispa* sea anemone reduced reactive oxygen species (ROS) production in Neuro-2A cells, induced by 6-OHDA, due to the ability of molecules to scavenge free-radicals (Sintsova et al., 2021).

Antioxidants are molecules that scavenge free radicals, able to restore mitochondrial damage. Mitochondrial abnormalities, such as the generation and release of ROS, cause lipid and protein peroxidation, and DNA damage. Oxidative stress and consequent neuron apoptosis have been observed in Alzheimer's Disease, closely related to Aβ deposition (Carrillo-Mora et al., 2014; Kumar and Singh, 2015). The release of inflammatory mediators caused by Aβ also induces the

production of ROS. Thus, the control of oxidative stress would impair the toxicity caused by Aβ42, which we observed in this work.

Taxifolin, a molecule identified in El 50, belongs to the flavanonols class, and possesses powerful antioxidant properties (Das et al., 2021), which may be responsible for controlling the oxidative stress observed in this study.

Other identified molecules could have important biological effects. Phenazine-1-carboxylic acid, an aromatic carboxylic acid, has antimicrobial and antifungal activity, which could be important for the maintenance of coelomic fluid in sea urchin, and has already been found in jellyfish (Yue et al., 2022). Minaprine is a phenylpyridazine, used for treatment of depression due to its action as a reversible inhibitor of MAO-A, serotonin and dopamine uptake inhibitor. Moreover, it has been found that minaprine weakly inhibits acetylcholinesterase, being considered for Parkinson's Disease (EDWARDS et al., 1996).

In conclusion, we identified a fraction (El50) from *E. lucunter* coelomic fluid that was able to prevent and reduce cell death caused by oligomerized Aβ42 by disaggregating the oligomer and reducing oxidative stress, two important factors in amyloid peptide-related diseases such as Alzheimer's. Our findings suggest that a sample that interacts with several molecular targets, resulting in a global reduction of neurotoxicity, could be a promising approach for a prototype therapy to treat neurodegenerative multifactorial dementias.

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5. CONCLUSÃO

O líquido celômico de *Echinometra lucunter* possui moléculas que contribuem para a eliminação do peptídeo amiloide oligomerizado do meio extracelular da cultura de neurônios antes e após a sua incubação com as células, em um efeito prevenção e tratamento, respectivamente.

Além disso, moléculas antioxidantes presentes no líquido celômico foram capazes de reduzir o estresse oxidativo, impedindo a morte dos neurônios.

Assim, o líquido celômico de *Echinometra lucunter* representa uma fonte de compostos capazes de atuar em vários mecanismos, e que podem conferir uma neuroproteção e/ou tratamento da doença multifatorial, que é a Doença de Alzheimer. Desta forma, foi possível se obter soluções relacionadas à causa do doença, o que pode resultar em um tratamento efetivo, que poderá contribuir para a melhora dos sintomas dos pacientes acometidos por essa doença sem cura.

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