

UNIVERSIDADE SÃO FRANCISCO
Programa de Pós-Graduação *Stricto Sensu* em Ciências da Saúde

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**ATIVIDADE DE FRAÇÕES E MOLÉCULAS ISOLADAS
DE *Eudendrium carneum* NA INIBIÇÃO DA MORTE
NEURONAL CAUSADA PELO PEPTÍDEO AMILÓIDE 42**

Bragança Paulista
2026

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RESUMO

A doença de Alzheimer (DA) é um distúrbio neurodegenerativo caracterizado por um declínio progressivo das funções cognitivas e funcionais. Um dos principais marcadores patológicos da doença é a formação de placas de beta-amiloide ($A\beta$), derivadas da proteína precursora amiloide (APP), no tecido cerebral. O acúmulo dessas placas amiloides leva ao comprometimento da proteostase e à desregulação da dinâmica do cálcio intracelular, incluindo disfunção lisossomal e inativação de enzimas proteolíticas, como a catepsina D. Esses efeitos contribuem para o aumento do acúmulo de placas, ativação de vias apoptóticas e neurodegeneração. Este estudo investiga os efeitos de frações e moléculas isoladas do extrato metanólico do hidrozóario *Eudendrium carneum* na inibição da morte neuronal causada pelo peptídeo amiloide, com o potencial de elucidar mecanismos envolvidos na restauração da atividade lisossomal. Inicialmente, o extrato foi fracionado por HPLC, e as frações resultantes foram incubadas com neurônios SH-SY5Y diferenciados, previamente expostos ao peptídeo beta-amiloide 42 oligomerizado ($oA\beta 42$), para avaliar o impacto na viabilidade neuronal. Quatro frações reduziram a morte celular induzida por $oA\beta 42$, conforme determinado pelos ensaios de MTT e LDH. Uma dessas frações foi analisada, e verificada pureza, e a molécula identificada como Sarmentosídeo B por meio de comparação com banco de dados, após análise por espectrometria de massa. O Sarmentosídeo B apresentou previsão de baixa toxicidade oral e poucos efeitos adversos, nenhum deles envolvendo o sistema nervoso central. Sendo um glicosídeo esteroidal, a molécula foi testada em um modelo neuronal para avaliar a dinâmica do cálcio intracelular, e não foram observados efeitos nos canais de cálcio ou sódio. A integridade lisossomal foi avaliada por citometria de fluxo após marcação com Lysotracker, revelando que o Sarmentosídeo B restaurou a morfologia lisossomal degradada pelo $oA\beta 42$. A expressão da proteína mTOR, aumentada com o $oA\beta 42$, foi diminuída com o tratamento com o Sarmentosídeo B, indicando aumento da via de autofagia. Essa inibição foi confirmada por *docking* molecular, na ligação da molécula ao sítio de ligação à rapamicina, na proteína. Além disso, a atividade catalítica da catepsina D, prejudicada por $oA\beta 42$, foi restaurada após o tratamento com a molécula. Essa restauração lisossomal levou à remoção do $A\beta 42$ agregado nos neurônios, evidenciada pela coloração com Tioflavina-T. Esses achados mostram o efeito do Sarmentosídeo B na restauração da autofagia e remoção dos agregados proteicos, com consequente efeito neuroprotetor, que podem contribuir para a o desenvolvimento de novos protótipos e alternativas medicamentosas para o tratamento da doença de Alzheimer.

Palavras-chave: Doença de Alzheimer. Peptídeo amiloide. Novas moléculas. *Eudendrium carneum*. Lisossomos.

ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive decline in cognitive and functional abilities. One of the primary pathological hallmarks of the disease is the formation of amyloid-beta ($A\beta$) plaques in brain tissue, which are derived from the amyloid precursor protein (APP). The accumulation of these amyloid plaques leads to the impairment of proteostasis and the dysregulation of intracellular calcium dynamics, including lysosomal dysfunction and the inactivation of proteolytic enzymes, such as cathepsin D. These effects contribute to increased plaque accumulation, activation of apoptotic pathways, and neurodegeneration. This study investigates the effects of fractions and isolated molecules from the methanolic extract of the hydrozoan *Eudendrium carneum* on the inhibition of neuronal death caused by the amyloid peptide, with the potential to elucidate mechanisms involved in the restoration of lysosomal activity. Initially, the extract was fractionated by HPLC, and the resulting fractions were incubated with differentiated SH-SY5Y neurons, previously exposed to oligomerized amyloid-beta 42 (oA β 42), to evaluate the impact on neuronal viability. Four fractions reduced oA β 42-induced cell death, as determined by MTT and LDH assays. One of these fractions was analyzed for purity, and the molecule was identified as Sarmentoside B through database comparison following mass spectrometry analysis. Sarmentoside B showed a prediction of low oral toxicity and few adverse effects, none of which involved the central nervous system. As a steroidal glycoside, the molecule was tested in a neuronal model to evaluate intracellular calcium dynamics, and no effects were observed on calcium or sodium channels. Lysosomal integrity was assessed by flow cytometry after LysoTracker staining, revealing that Sarmentoside B restored the lysosomal morphology degraded by oA β 42. The expression of the mTOR protein, which increased with oA β 42, was decreased with Sarmentoside B treatment, indicating an upregulation of the autophagy pathway. This inhibition was confirmed by molecular *docking*, in the binding of the molecule on the rapamycin binding site, in the protein. Furthermore, the catalytic activity of cathepsin D, impaired by oA β 42, was restored after treatment with the molecule. This lysosomal restoration led to the removal of aggregated A β 42 in neurons, as evidenced by Thioflavin-T staining. These findings demonstrate the effect of Sarmentoside B in restoring autophagy and removing protein aggregates, resulting in a neuroprotective effect that may contribute to the development of new prototypes and medicinal alternatives for the treatment of Alzheimer's disease.

Keywords: Alzheimer's disease. Amyloid peptide. Novel molecule. *Eudendrium carneum*. Lysosomes.

LISTA DE SÍMBOLOS E ABREVIACÕES

Aβ	Peptídeo beta-amiloide.
Aβ42	Peptídeo beta-amiloide composto por 42 aminoácidos.
APP/PPA	Proteína precursora amiloide.
ATP	Trifosfato de adenosina.
CatD / CTSD	Catepsina D.
CTSB	Catepsina B.
DA	Doença de Alzheimer.
EROs	Espécies reativas de oxigênio.
EVs	Vesículas extracelulares.
FDA	<i>Food and Drug Administration.</i>
H₂O₂	Peróxido de hidrogênio.
mTOR	<i>mammalian target of rapamycin.</i>
NFTs	Emaranhados neurofibrilares.
oAβ	Oligômeros beta-amiloides solúveis.
oAβ42	Peptídeo beta-amiloide 42 oligomerizado.
p38 MAPK	Proteína quinase ativada por mitógenos p38.
PNM	Produtos naturais marinhos.
pTau	Proteína Tau fosforilada.
SNC	Sistema Nervoso Central

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1. INTRODUÇÃO

1.1. Doença de Alzheimer

A doença de Alzheimer (DA) é uma condição neurodegenerativa progressiva que se manifesta por meio de modificações sinápticas, degeneração neuronal e comprometimento cognitivo (HAUT; ARGYROUSI; ARANCIO, 2023).

Do ponto de vista fisiopatológico, a DA é caracterizada pela presença de depósitos extra e intracelulares de placas contendo peptídeo beta-amiloide ($A\beta$) e aglomerados intraneuronais fibrilares contendo proteína tau hiperfosforilada (GIRI; LÜ; ZHANG, 2016; SHINOHARA et al., 2014).

Existe uma hipótese que abrange os eventos patológicos na DA, conhecida como "Hipótese da Cascata Amiloide", a qual sugere que os depósitos de $A\beta$ são o ponto inicial e os promotores para o desenvolvimento da DA. A concepção de avaliar e modificar a $A\beta$ em seus estágios iniciais pode se mostrar como uma abordagem terapêutica promissora. Os peptídeos amiloides são gerados após o processamento da proteína precursora amiloide (APP) (GULISANO et al., 2018; HAUT; ARGYROUSI; ARANCIO, 2023).

A APP é uma proteína transmembranar de passagem única, altamente expressa no Sistema Nervoso Central (SNC). O gene que representa a APP localiza-se no cromossomo 21 (ZHENG; KOO, 2011). Uma significativa fração desta proteína está localizada no complexo de Golgi, onde será encaminhada para ser incorporada na membrana plasmática ou direcionada para o interior dos lisossomos (CAPORASO et al., 1994).

A APP desempenha um papel significativo ao facilitar a diferenciação e o crescimento das células neurais em indivíduos adultos, modulação das atividades pré-sinápticas, particularmente na regulação das vesículas pré-sinápticas e na subsequente exocitose de neurotransmissores (MULLER; ZHENG, 2012; YANG et al., 2005). De maneira específica, a APP é movida dos axônios em direção aos terminais sinápticos por meio do transporte axonal anterógrado, utilizando vesículas tubulares como veículo de transporte (MULLER; ZHENG, 2012).

O processamento da APP ocorre através da ação de α -, β - e γ -secretases. A ação coordenada da β - e γ -secretases resulta na liberação de $A\beta_{1-42}$, o peptídeo anômalo composto por 42 aminoácidos, enquanto a α -secretase desempenha um papel primordial no processamento

metabólico fisiológico da APP (TILLEMENT; LECANU; PAPADOPOULOS, 2011).

A clivagem da APP nos terminais sinápticos leva ao acúmulo de A β 42 nas sinapses. Após sua formação, os monômeros de A β são liberados no espaço extracelular ou mantidos intracelularmente, onde ocorre sua agregação. É notável que o acúmulo intraneuronal de A β precede a formação de placas amiloides (WIRTHS; MULTHAUP; BAYER, 2004).

A agregação de A β é favorecida pela configuração da folha β , as quais se organizam ao longo do eixo da fibrila, e propicia interações hidrofóbicas (EISENBERG; JUCKER, 2012; TYCKO, 2003). Essas fibrilas são rígidas e altamente ordenadas, além de serem insolúveis em água. Apesar de serem os marcadores histopatológicos da DA, múltiplos estudos sugerem que os oligômeros A β solúveis (oA β) também são indutores de neurotoxicidade (CHITI; DOBSON, 2017; DETOMA et al., 2012).

À medida em que a pesquisa avança, torna-se cada vez mais evidente que os agregados solúveis de menor porte, intermediários e metaestáveis, denominados oligômeros proteicos mal dobrados, frequentemente demonstram maior toxicidade do que suas formas fibrilares maduras (CASCELLA et al., 2021). As características desses elementos podem apresentar variações, abrangendo diferenças em termos de tamanho, hidrofobicidade e grau de estabilidade (CHITI; DOBSON, 2017).

O peptídeo A β 42, quando agregado, resulta em uma agregação fibrilar, que culmina na formação de placas extracelulares entre as células neuronais, exercendo um efeito adverso sobre as células neurais saudáveis adjacentes (BURNS; ILIFFE, 2009; GANGULY et al., 2017).

Outra proteína tóxica está presente na DA, a proteína tau hiperfosforilada, e juntamente com o A β 42 desencadeiam influências em distintos momentos temporais da doença, sendo o acúmulo de A β relacionado ao estágio inicial da doença, enquanto a Tau fosforilada (pTau) é considerada um marcador para estágios posteriores da enfermidade (D'ERRICO; MEYER-LUEHMANN, 2020; ATLANTE et al., 2021).

1.2. Autofagia e a Doença de Alzheimer

A autofagia é um processo catabólico essencial para a degradação e reciclagem de componentes celulares, no qual organelas e proteínas defeituosas ou de longa duração são

degradadas pelos lisossomos (NUNES, 2015). Esse mecanismo permite a reutilização de aminoácidos e outras moléculas, garantindo a renovação celular e a manutenção da homeostase. Nos neurônios, a autofagia é crucial para a proteostase e pode ser ativada em resposta à escassez de nutrientes, estresse celular ou acúmulo de proteínas mal dobradas, mutadas ou agregadas (MANÇANO et al., 2024).

Na DA, esse sistema encontra-se comprometido, o que prejudica a eliminação das placas amiloides e contribui para a morte neuronal. Estudos indicam que vacúolos autofágicos imaturos são observados no cérebro de pacientes diagnosticados com DA, sugerindo uma deficiência no processo de maturação dessas estruturas, essenciais para a remoção de resíduos celulares. Além disso, a autofagia tem um papel importante na degradação de subprodutos do processamento da proteína precursora amiloide (APP), incluindo os peptídeos A β 40/ β A42 e a proteína Tau fosforilada (JANGE, 2020).

Outro fator relevante é a hiperativação da via mTOR, que ocorre paralelamente à progressão temporal e regional dos agregados de Tau e A β . Acredita-se que essa ativação exacerbada seja induzida pelo A β , resultando na redução da autofagia e contribuindo para a hiperfosforilação de Tau (NUNES, 2015). Assim, estratégias terapêuticas que promovam a biogênese lisossomal ou aumentem a capacidade do sistema autofágico-lisossomal têm sido investigadas como alternativas promissoras para mitigar os efeitos da doença (MANÇANO et al., 2024).

1.3. Disfunção lisossomal na Doença de Alzheimer

A integridade funcional do Sistema Nervoso Central (SNC) é especialmente suscetível à disfunção dos lisossomos. Esta condição pode resultar em comprometimento das funções neurais e gliais, desencadeando processos de neurodegeneração (MAHUL-MELLIER et al., 2020).

Os lisossomos desempenham papel vital na degradação e reciclagem de uma ampla gama de macromoléculas. Essas organelas são essenciais para processar e digerir materiais tanto dentro da célula quanto fora dela, abrangendo desde lipídios e proteínas até ácidos nucleicos e carboidratos (DE DUVE, 2005).

Foi demonstrado que os exossomos apresentam uma função fundamental como veículos de

transporte para a difusão de peptídeos neurotóxicos derivados da APP e A β para as células receptoras. Estudos confirmaram que as vesículas extracelulares (EVs) são o ambiente propício para a produção e acumulação desses peptídeos nocivos (ZHAO et al., 2023).

Os pacientes diagnosticados com DA frequentemente apresentam elevados níveis de oligômeros A β em seus cérebros. Estes oligômeros podem ser encapsulados em exossomas, que subsequentemente são internalizados por neurônios cultivados. Através deste processo, o conteúdo tóxico dos exossomas é transferido para as células receptoras (ZHENG et al., 2018).

Os exossomos foram identificados como veículos para proteínas mal dobradas, como exemplificado pelo aumento do transporte de A β 42 exógeno para dentro dos exossomos, resultando em uma maior concentração desses peptídeos nas células cultivadas (CHOI et al., 2021).

Na DA, uma disfunção endolisossomal ocorre devido ao acúmulo e disseminação de formas oligoméricas de proteínas neurotóxicas. Nesse contexto, as catepsinas emergem como componentes essenciais desempenhando um papel significativo (BOLAND et al., 2018). As catepsinas são enzimas lisossomais responsáveis pela degradação de proteínas e estão envolvidas em diversos processos celulares, incluindo a resposta imune e a degradação de proteínas mal dobradas ou agregadas, como as fibrilas de A β (HENEKA et al., 2015).

Após se ligarem aos receptores, as fibrilas de A β são fagocitadas pela microglia por meio da via endolisossomal. Dentro dos lisossomos, essas fibrilas desencadeiam a liberação da enzima catepsina B (CTSB) para o citosol. Esse processo, provocado pelas fibrilas de A β , compromete a integridade da membrana lisossomal (HENEKA et al., 2015).

A principal função dos lisossomos não é causar a destruição celular, mas sim preservar a estabilidade interna da célula e reciclar seus componentes, capacitando-se para decompor e digerir proteínas. Entretanto, quando as catepsinas são liberadas dos lisossomos, as enzimas se tornam disfuncionais e esse processo de preservação fica comprometido. Elas estão comumente ligadas a várias condições patológicas em humanos, incluindo distúrbios neurodegenerativos, como a DA (AMARAL et al., 2023).

Estudos sobre a patologia da DA destacaram um papel complexo da CTSB: ela demonstra efeito neuroprotetor ao reduzir os níveis de A β e melhorar a disfunção neuronal (BERNSTEIN; KEILHOFF, 2018). As catepsinas têm um papel vital na iniciação e mediação de vários processos de morte celular, como a apoptose e outras formas de morte celular (CATALDO; NIXON, 1990).

Por outro lado, foi observado que a CTSB se acumula em placas amiloides em cérebros humanos com DA. Essas descobertas sobre a regulação positiva de CTSB na DA foram corroboradas em modelos animais da doença, nos quais o gene CTSB foi nocauteado, evidenciando o envolvimento da CTSB nos déficits de memória associados à DA (KINDY et al., 2012).

Pesquisas recentes demonstraram que a deleção genética da catepsina D (CTSD) em camundongos leva a um acúmulo pronunciado do peptídeo β -amiloide ($A\beta$) nos lisossomos. Esses resultados indicam que, em condições normais, $A\beta$ é transportado em quantidades significativas para o compartimento lisossomal, onde a CTSD exerce um papel fundamental em sua degradação. Essa descoberta destaca a importância da CTSD na regulação dos níveis de $A\beta$, sugerindo sua participação ativa na manutenção da homeostase proteica e possivelmente na prevenção de processos neurodegenerativos associados ao acúmulo desse peptídeo (SUIRE; LEISSRING, 2021).

Estudos mostram que a CTSD processa as isoformas $A\beta$ 40 e $A\beta$ 42 de maneira distinta, podendo reduzir a carga de proteína amiloide na célula. Enquanto o $A\beta$ 40 é degradado com eficiência moderada, o $A\beta$ 42 apresenta uma forte afinidade pela CTSD, mas sua degradação ocorre de forma mais lenta. No entanto, a atividade da enzima está comprometida na doença, uma vez que está fora do compartimento lisossomal (SUIRE; LEISSRING, 2021). Além disso, quando há mutações no APP, a produção de $A\beta$ é acelerada, resultando em um aumento na formação de $A\beta$ 142, que está associada ao desenvolvimento da doença (CATALDO et al., 1995).

A relação entre a CTSD e a DA pode estar ligada ao estresse oxidativo, um fator-chave na neurodegeneração. A ativação da via p38 MAPK, induzida por esse estresse, foi associada à diminuição da CTSD em fibroblastos de pacientes com DA, sugerindo uma interação complexa entre essas vias de sinalização. Essas mudanças podem prejudicar a função do sistema lisossomal, resultando no acúmulo de substâncias não digeridas e aumentando a vulnerabilidade das células nervosas ao estresse oxidativo. No cérebro, a CTSD pode acelerar processos celulares como a endocitose e a autofagia, mas esse aumento não é uniforme, já que em fibroblastos de pacientes com DA, tanto a atividade quanto a expressão da CTSD estão reduzidas (URBANELLI et al., 2008).

A inibição da CTSD por $A\beta$ 42 pode prejudicar a degradação da proteína tau, resultando no acúmulo de fragmentos neurotóxicos de tau e favorecendo a formação dos emaranhados neurofibrilares (NFTs), estruturas associadas à degeneração neuronal (SUIRE; LEISSRING,

2021). Quando a CTSD é excessivamente expressa, pode ser liberada do lisossomo para o citosol, levando à morte celular e agravando a neurodegeneração (VIDONI et al., 2016).

Nos estágios iniciais da doença, o sistema endossômico-lisossomal é ativado para eliminar proteínas anormais, mas essa tentativa se torna disfuncional com o avanço da doença (CATALDO et al., 1995). Além disso, o envelhecimento deteriora a função lisossomal, agravando os efeitos da inibição da CTSD por A β 42. O acúmulo de A β nos lisossomos compromete sua integridade, levando ao vazamento de fragmentos neurotóxicos de tau no citosol, que estimulam a formação de NFTs e a degeneração neuronal. Dessa forma, a CTSD surge como um elo molecular importante entre a deposição de A β , a desregulação do sistema lisossomal e o avanço da tauopatia na DA, destacando seu potencial como alvo terapêutico (SUIRE; LEISSRING, 2021).

Portanto, a CTSD desempenha um papel crucial na resposta celular à presença de A β , sugerindo um papel central na defesa contra a neurodegeneração, mas também destacando a complexidade das vias de degradação celular na DA. Abordagens terapêuticas focadas na modulação da atividade da CTSD, seja por ativação direta ou pela promoção do fluxo autofágico, podem ser estratégias promissoras para o tratamento da DA (VIDONI et al., 2016).

1.4. Animais marinhos como fonte de novas moléculas

Os organismos marinhos desempenham um papel essencial nos ecossistemas, formando estruturas tridimensionais que servem de habitat para uma ampla variedade de seres vivos, desde vírus até vertebrados, e podem ser encontrados em diferentes tipos de substratos, como mineral, vegetal, animal ou artificial (MOURA, 2020).

Com uma biodiversidade que abrange 34 dos 36 filos conhecidos, o ambiente marinho tem sido uma fonte promissora na busca por novas moléculas com potencial terapêutico. Estudos indicam que mais de 13.000 moléculas bioativas foram identificadas em organismos marinhos nos últimos anos, sendo que cerca de 3.000 demonstraram atividade biológica relevante (MORENO et al., 2022).

Apesar de seu potencial, os produtos naturais marinhos foram por muito tempo negligenciados pela Indústria Farmacêutica devido à falta de tecnologia para exploração dos oceanos e às dificuldades na obtenção de matéria-prima para a produção de fármacos (ALVES,

2014). No entanto, avanços científicos e tecnológicos no século XXI permitiram grandes descobertas, especialmente em relação aos micro-organismos marinhos, cuja diversidade química e possibilidade de fermentação oferecem uma alternativa sustentável para a produção de compostos bioativos (COSTA-LOTUFO et al., 2009).

Atualmente, diversas moléculas de origem marinha estão em fases avançadas de desenvolvimento para o tratamento de doenças como câncer, infecções virais, dor, inflamação e distúrbios neurológicos (MORENO et al., 2022). Embora ainda existam poucos fármacos aprovados com origem marinha – seis pela FDA – há 24 substâncias químicas em diferentes estágios de ensaios clínicos e centenas em testes pré-clínicos. Nos últimos anos, diversas empresas foram criadas com o objetivo de explorar o potencial farmacológico desses compostos (COSTA-LOTUFO et al., 2009).

Entre os organismos marinhos estudados, algumas espécies, como *Eudendrium carneum*, possuem um papel importante na complexidade estrutural dos habitats costeiros. Como outros hidroides coloniais, essa espécie pode fornecer substrato para diferentes organismos, oferecer abrigo para pequenos invertebrados e participar das interações tróficas locais, influenciando a dinâmica das comunidades bentônicas (GROHMANN, 2006).

Em estudos prévios do grupo, foi visto que o extrato metanólico de *E. carneum* reduziu a morte celular causada pelo peptídeo beta amiloide 42. Nesse trabalho então, o extrato foi purificado e as moléculas isoladas testadas no mesmo modelo, para se identificar uma molécula ativa, além da elucidação inicial de seu mecanismo de ação, com relação à restauração do sistema de autofagia e remoção de peptídeo amiloide.

Considerando que a Doença de Alzheimer é um dos maiores desafios da medicina moderna, e a principal causa de demência em idosos e afetando milhões de pessoas em todo o mundo, são necessárias alternativas para o tratamento e melhora da qualidade de vida dos pacientes. A doença caracteriza-se por um declínio progressivo das funções cognitivas, impactando significativamente a qualidade de vida dos pacientes e de seus cuidadores, e embora os tratamentos disponíveis proporcionem alívio sintomático, eles não impedem a progressão da neurodegeneração, o que reforça a necessidade urgente de novas abordagens terapêuticas.

A obtenção de novas moléculas para o tratamento da DA é fundamental porque a fisiopatologia da doença envolve múltiplos mecanismos ainda não completamente compreendidos.

Os principais alvos terapêuticos atualmente estudados incluem a deposição do peptídeo beta-amiloide, a hiperfosforilação da proteína tau, o estresse oxidativo, a neuroinflamação e a disfunção mitocondrial. No entanto, os fármacos disponíveis no mercado apresentam eficácia limitada, não sendo capazes de interromper ou reverter o curso da doença. Isso ocorre, em parte, devido à complexidade da DA, que envolve interações entre diferentes vias biológicas.

A busca por novas moléculas se justifica pela necessidade de terapias mais eficazes e seguras, que possam atuar simultaneamente em diferentes alvos envolvidos na neurodegeneração. Além disso, muitas das opções farmacológicas atuais estão associadas a efeitos adversos significativos, o que limita sua utilização em longo prazo. Novos compostos podem oferecer alternativas com maior especificidade e menos toxicidade, melhorando a adesão ao tratamento e a qualidade de vida dos pacientes.

Outro fator crucial para o desenvolvimento de novas moléculas é a variabilidade na resposta ao tratamento entre os pacientes. Como a DA possui um caráter multifatorial, intervenções personalizadas podem ser mais eficazes. Dessa forma, a exploração de novas substâncias permite ampliar as opções terapêuticas, possibilitando abordagens mais direcionadas conforme o perfil molecular e clínico de cada paciente.

Além do impacto clínico, a descoberta de novas moléculas também é essencial para reduzir o ônus socioeconômico da doença. Com o envelhecimento da população global, a prevalência da DA tende a aumentar, gerando um custo crescente para os sistemas de saúde. O desenvolvimento de tratamentos mais eficazes poderia retardar a progressão da doença, reduzindo a necessidade de cuidados intensivos e melhorando o prognóstico dos pacientes.

Portanto, investir na obtenção de novas moléculas é essencial para superar as limitações das terapias atuais, ampliar as estratégias terapêuticas disponíveis e proporcionar avanços no tratamento da DA, beneficiando tanto os pacientes quanto a sociedade como um todo.

2. OBJETIVOS

2.1. OBJETIVO GERAL

O objetivo desse trabalho é avaliar a atividade de moléculas isoladas do *Eudendrium carneum* na inibição da morte neuronal causada pelo peptídeo amiloide 42 oligomerizado (oA β 42) e estudar os mecanismos de autofagia envolvidos.

2.2. OBJETIVOS ESPECÍFICOS

- Obter moléculas isoladas do *Eudendrium carneum* por meio de cromatografia líquida
- Selecionar moléculas ativas por meio do efeito de inibição da morte celular causada pelo oA β 42;
- Caracterizar pelo menos uma molécula ativa;
- Estudar a morfologia e funcionalidade de lisossomos após a exposição ao oA β 42 e o tratamento com as moléculas isoladas;
- Avaliar a capacidade das moléculas isoladas em remover o oA β 42 das células neuronais.
- Verificar a possível toxicidade da molécula isolada.

3. CAPÍTULO 1

O capítulo mostra o artigo que foi submetido para publicação. O artigo mostra o efeito de uma molécula isolada do hidróide *Eudendrium carneum* na inibição da morte neuronal induzida pelo peptídeo amiloide oligomerizado. Essa molécula foi caracterizada como Sarmentosídeo B.

A atividade foi relacionada à capacidade da molécula em restaurar a funcionalidade de lisossomos neuronais, prejudicados pela ação do peptídeo - isso se comprovou pela ação da marcação fluorescente da organela, inibição de mTOR e restauração da atividade de catepsinas D. Com isso, foi observada redução de agregados proteicos nos neurônios.

Além disso, a molécula não interferiu na dinâmica de cálcio do neurônio e não apresentou toxicidade, em uma avaliação *in silico*.

Sarmentoside-B removes oligomerized amyloid peptide from neurons by inhibiting mTOR and restoring lysosomal function, in *in vitro* Alzheimer's disease model

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Abstract

Background/Objectives: Alzheimer's disease (AD) is characterized by beta-amyloid (A β) plaque deposit, which impairs cellular processes, including autophagy. There are anti-bodies therapies targeting A β , but their efficacy and safety remain limited. Considering its multitarget characteristic, the development of therapies acting on other molecular targets is necessary. In this study, we evaluated the neuroprotective effects of sarmentoside B from the hydrozoan *Eudendrium carneum*, and its impact on autophagy pathways. **Methods:** E. carneum secretion was fractionated by RP-

HPLC according to its neuroprotective activity SH-SY5Y cells exposed to by A β 42, evaluated by LDH and MTT. The pure molecule, characterized by mass spectrometry, was evaluated by its in silico toxicity, and calcium dynamics. Neuronal lysosome morphology was assessed using LysoTracker probe, and cathepsin D activity was determined using synthetic substrate. Autophagy-related protein expression (mTOR, LAMP-1 and LC3b) was verified by western blotting and amyloid plaques were quantified by Thioflavin-T. Results: The steroid glycoside Sarmentoside B was identified by its neuroprotective effect and showed no toxic effect or changes in neuronal calcium or sodium channel dynamics. Sarmentoside B restored neuronal lysosomal morphology and cathepsin D activity, reversing the impairment caused by A β 42. Furthermore, sarmentoside B decreased mTOR expression, thereby activating the autophagy pathway. This restoration of lysosomal and autophagic function led to the clearance of oA β 42, as evidenced by Thioflavin-T staining, and a subsequent reduction in neuronal death. Conclusions: Sarmentoside B has neuroprotective effect against A β 42 by acting as a mTOR inhibitor, promoting autophagy-mediated amyloid clearance, mechanisms that hold therapeutical potential for AD.

Keywords: Alzheimer's disease; Eudendrium carneum; sarmentoside B; autophagy; lysosome; amyloid plaque clearance.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by synaptic loss, neuronal degeneration, and cognitive impairment [1]. Among its main pathological features are the extracellular accumulation of beta-amyloid peptide (A β) and the formation of intraneuronal aggregates of hyperphosphorylated tau protein, events that trigger cellular stress, inflammation, and neuronal death [2,3]. The formation and aggregation of A β from the abnormal processing of amyloid precursor protein (APP) is considered a central step in the disease's pathophysiology, with this peptide capable of accumulating either extracellularly or inside neurons, where its soluble oligomers exert early neurotoxic effects [1,4].

Beyond protein accumulation, dysfunction of autophagy pathways has emerged as a key factor in disease progression. Autophagy is an essential process for the degradation of damaged or misfolded/aggregated proteins, ensuring the maintenance of cellular homeostasis, especially in neurons, in which proteostasis pathways are critical [5].

The amyloid plates are encapsulated by autophagic vesicles to be addressed to lysosomes, which contain proteolytic enzymes for protein degradation. In AD, the autophagy pathway is impaired: there is accumulation of immature autophagic vacuoles and hyperactivation of the mTOR pathway, phenomena that reduce autophagic flux and reduce the clearance of A β ,

contributing to neuronal toxicity [6].

In this context, there is growing interest in new molecules capable of modulating cellular pathways related to autophagy restoring in AD [7]. To achieve that, the marine environment, with the enormous biodiversity and vast chemical variety, emerges as a strategic source for the prospecting of bioactive compounds. Our research group has isolated some molecules from marine animals with antioxidant, anti-inflammatory, beta-secretase inhibitor, anti-amyloid aggregation and that reduced neuron's death induced by amyloid peptide [9-14].

Previous studies from our group have shown that the secretion of the hydrozoan *Eudendrium carneum* reduces A β 42-induced cell death, suggesting the presence of active compounds with therapeutic potential, applied to AD. Thus, in this study, we aimed the isolation and characterization of an active molecule from *E. carneum* able to reduce the neuronal death induced by amyloid peptide, and studying the molecular pathway related to such effects, with emphasis autophagy pathway and lysosomes restoring after A β 42 exposure.

2. Materials and Methods

2.1. Samples

Specimens of *Eudendrium carneum* were collected in São Sebastião/SP, under authorization from ICMBio (93947-1), and the extract was obtained as described by Moreno et al.7. Briefly, the animal was immersed in a solution of methanol containing 0.1% acetic acid for 48 h at room temperature. The secreted content was centrifuged at 5000 $\times$ g for 10 min and the supernatant was lyophilized and stored at -20°C . This extract was subjected to fractionation by reverse-phase high-performance liquid chromatography (RP-HPLC) using a C-18 column (Phenomenex, 4.6×250 mm, 5 $\text{\AA}$). Elution was performed at a constant flow rate of 1 mL.min $^{-1}$ using a 30 min gradient with solvents A (ultrapure water containing 0.1% trifluoroacetic acid) and B (90% acetonitrile in ultrapure water containing 0.1% trifluoroacetic acid). Peaks were detected at $\lambda = 214$ nm and collected manually according to the peak profile. The obtained fractions were tested in neurons cultures (described below) and active peaks were analyzed by ESI-MS/MS mass spectrometry (IT-TOF, Shimadzu Co, Japan) for purity assessment and characterization.

For characterization, mass spectrometry analysis was performed in both positive and negative modes over a scan range of 50–1500 m/z. The main ion was fragmented by collision with

argon gas (50%), and the resulting fragments were manually analyzed to verify functional groups, as well as submitted to database searches to check for similarity with other molecules described in natural products. Raw files were converted to mzML using MSConvert (ProteoWizard 3.0) and processed through the GNPS-MassIVE public data repository for untargeted MS2 data using compound identification and molecular networking based on MS2 and spectral similarity ([//gnps.ucsd.edu](http://gnps.ucsd.edu)). Data parameters were set with a precursor ion mass tolerance of 0.5 Da, fragment ion mass tolerance of 0.2 Da, combined peaks of 6 min, and a threshold of 0.7. All public GNPS spectra curated by the natural products scientific community were utilized.

2.2. Neuronal Culture

SH-SY5Y cells (European Collection of Authenticated Cell Culture) were cultured in DMEM/F12 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin, maintained at 37 °C in a humidified atmosphere with 5% CO₂.

After growth, cells were transferred to 96-well plates at 5×10^3 cells/well. To induce the Alzheimer's disease cellular model, SH-SY5Y cells were treated with synthetic oligomerized A β 42 peptide (5 μ M, GenScript, NJ, USA) for 48 h, following the method established by the group in previous studies [12].

The molecules obtained after chromatography (lyophilized and diluted in sterile PBS) were incubated with the cells at a concentration of 10 μ g.mL⁻¹ for 24 h. After this period, neurons were observed under light microscopy for general morphological assessment and evaluated as described in the following sections.

For all experiments, three groups were considered:

- Cells without any treatment;
- Cells exposed to oA β 42;
- Cells exposed to oA β 42 and subsequently treated with the molecule.

Each analysis was performed in triplicate, in two independent experiments.

2.3. Cell Viability

The MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay was employed as a colorimetric method to assess cell viability. After treatment, the reagent was added

to the cells (0.5 mg.mL⁻¹ and after 3 h in the 37°C incubator, the formazan crystals were diluted in DMSO and the absorbance read in $\lambda = 570$ nm.

Alternatively, cell viability was assessed by quantifying LDH (lactate dehydrogenase), which evaluates plasma membrane integrity following apoptosis or necrosis. For this, 10 μ L of the cell culture medium, after treatment, was mixed with 60 μ L of reagent (LDH Cytotoxicity Assay Kit, Promega, WI, USA) in a 96-well plate. Luminescence was read after 30 min at room temperature.

The percentage of cytotoxicity was calculated considering the negative control as 100% viable cells.

2.4. Lysosome Evaluation

To assess lysosomal functionality after amyloid peptide exposure and treatment with the molecules, neurons were incubated with LysoTracker Green (Invitrogen™, MA, USA), which labels acidic organelles, such as lysosomes. Treated cells were trypsinized, incubated with the probe (25 nM), and kept at 37 °C for 30 min. Cells were analyzed by flow cytometry, acquiring 10,000 events (Guava® easyCyte, Millipore, MA, USA). An initial gate was determined with non-labeled cells.

Additionally, treated cells were immersed in lysis buffer (sodium citrate buffer, pH 4.5, containing 1 mM EDTA, 2 mM PMSF and 1 mM DTT) and submitted to ultrasonic pulses (3 pulses of 10s), followed by centrifugation at 11,180 x g for 15 min at 4 °C. The supernatant had the protein concentration determined by Bradford assay, and 10 μ g of protein were incubated with a synthetic substrate, specific for cathepsin D (Bz-Arg-Gly-Phe-Phe-Pro-4M2NA, Sigma-Aldrich), in sodium citrate buffer pH 4.0. Fluorescent values were obtained in $\lambda_{ex} = 365$ nm and $\lambda_{em} = 410$ -460 nm each 10 min, until 60 min, at 37°C.

2.5. Protein expression evaluation

Anti-LC3B (PA146286, Invitrogen™, MA, USA), Anti-LAMP-1 (MA529385, Invitrogen™, MA, USA) and anti-mTOR (PA534663, Invitrogen™, MA, USA) was used to identify autophagy-related proteins in control or treated neurons by dot blot. Ten microliters of sample (1 mg.mL⁻¹) were deposited in a nitrocellulose membrane, which was positioned in the SNAP i.d™ System (Millipore, MA, USA). BSA 1% was added for blockage and then the primary

antibody was incubated (all of them 1:1000). After wash with TBS-T, the secondary antibody was incubated (1:1000, 32460, Invitrogen) and the detection was made using the ECL Substrate (Pierce™ Fast Western Blot Kit, Thermo Scientific™).

Images were acquired by ChemiDoc™ Imaging System (Biorad, CA, USA) and spots were measured using ImageJ software (v1.54) to determine area of plots, identified automatically, in triplicate.

2.6. Molecular docking

The mTORc1 (target protein) was evaluated in two models: 3JBZ and 2FAP from PDB (Protein Data Bank), chosen based on their resolution by the X-ray diffraction method (~ 2 Å) and isolated from humans. PDB codes were individually inserted in SwissDock platform and AutoDock Vina mechanism was selected. The search space was defined as 20 x 20 x 20 Å box size and box center -5 x -35 x -39 for 3JBZ and -11 x 25 x 35 for 2FAP (in the x, y, and z axes, respectively), place defined by the analysis of Cavities, by UCSF ChimeraX v1.10, and also by the literature, considering relevant amino acid for the biological activity of the proteins.

The ligand was inserted in SwissDock by its SMILES, code provided by PubChem. Analysis of bind (location, energy and distances between atoms) were performed using UCSF ChimeraX v1.10.

2.7. Assessment of Amyloid- β 42 Clearance

To quantify A β 42 clearance, samples of the supernatant were analyzed using the ThT assay. An aliquot of 10 μ L of supernatant of the culture was incubated with 5 μ L of ThT (1 mM diluted in 50 mM PBS pH 7.4) and 5 μ L of PBS, and after 10 min of incubation at room temperature, in the dark, fluorescence was measured at $\lambda_{ex} = 365$ nm and $\lambda_{em} = 410$ -460 nm.

2.8. Calcium dynamics

SH-SY5Y cells, culture according to the method described above, were plated at 40,000 cells per well in 384-well flat clear-bottom black plates (Corning Inc., Corning, NY) and maintained at 37°C in a humidified 5% CO₂ incubator for 48 h before assaying as previously described by us [15]. Briefly, after 48 h incubation, cells were incubated with Calcium 4 dye

(Molecular Devices, Sunnyvale, CA) reconstituted in assay buffer containing (in millimolar) 140 NaCl, 11.5 glucose, 5.9 KCl, 1.4 MgCl₂, 1.2 NaH₂PO₄, 5 NaHCO₃, 1.8 CaCl₂, and 10 HEPES (pH 7.4) and incubated for 30 min at 37°C in a humidified 5% CO₂ incubator. Fluorescence responses were recorded using excitation of 470 nm and emission of 515 nm for 10 seconds to set the baseline, and then 600 seconds after incubation of EC5 (1st addition) and for a further 600 seconds after incubation of 90 mM KCl + 5 mM CaCl₂ or 50 µM veratridine (2nd addition).

2.9. In silico toxicity

The toxicity of sarmentoside B was predicted using ProTox 3.0 - Prediction Of Toxicity Of Chemicals. The structure of the molecule was drawn, according to the Pubchem, and the report containing the potential toxicity was generated, with default parameters.

2.10. Statistical Analysis

Results were processed using GraphPad Prism software (v.10) to calculate mean ± SEM from data. Data were plotted in graphs for bar chart generation and group comparison. Additionally, statistical analysis for evaluation of data normality was performed using Shapiro-Wilk test. After that, difference between groups was evaluated using one-way ANOVA followed by Tukey's post hoc test, considering $p < 0.05$.

3. Results

3.1. Fractionation of the *Eudendrium carneum* extract

Eudendrium carneum extract was submitted to a reverse-phase high-performance liquid chromatography (RP-HPLC), and it was separated into 12 distinct fractions, which were manually collected, named EC1 to EC12 (Figure 1). For the collection, only the chromatographic peaks of highest relevance and intensity were selected, since less prominent peaks, although visually detectable, were not considered for collection.

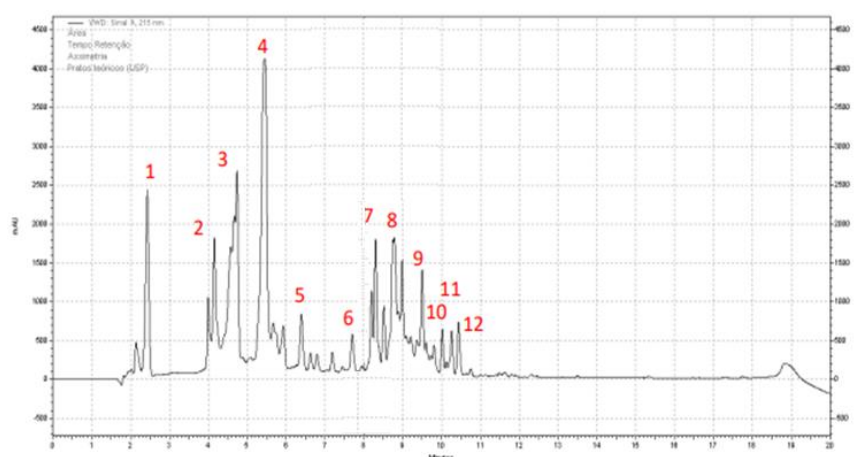


Figure 1. Chromatogram of the fractions of the methanolic extract of *Eudendrium carneum* after reverse-phase high-performance liquid chromatography (RP-HPLC). Numbers indicate the fraction collected.

3.2. Cell Viability

Twelve HPLC-fractions collected were evaluated in two cell viability assays, in an in vitro model of AD. In MTT assay, it was possible to observe that after the treatment with oA β 42, the cell viability was reduced to ~60% compared to control (Figure 2a). The incubation of EC1, EC2, EC3, EC4, EC5, EC11 and EC12, after oA β 42, increased the cell viability to ~90%, being EC5 97.7%, similar to control. This method enabled the identification of fractions that better preserved cell viability, with EC5 and EC11 standing out as those with superior performance. In contrast, fractions EC7 and EC10 showed the lowest viability indices, with values even lower than those observed in the sample treated with A β 42.

To confirm that results, lactate dehydrogenase (LDH) release assay was used with 5 fractions that showed the best performance in the MTT assay (EC1, EC2, EC4, EC5 and EC11). All fractions showed increase in cell viability after the treatment (Figure 2b).

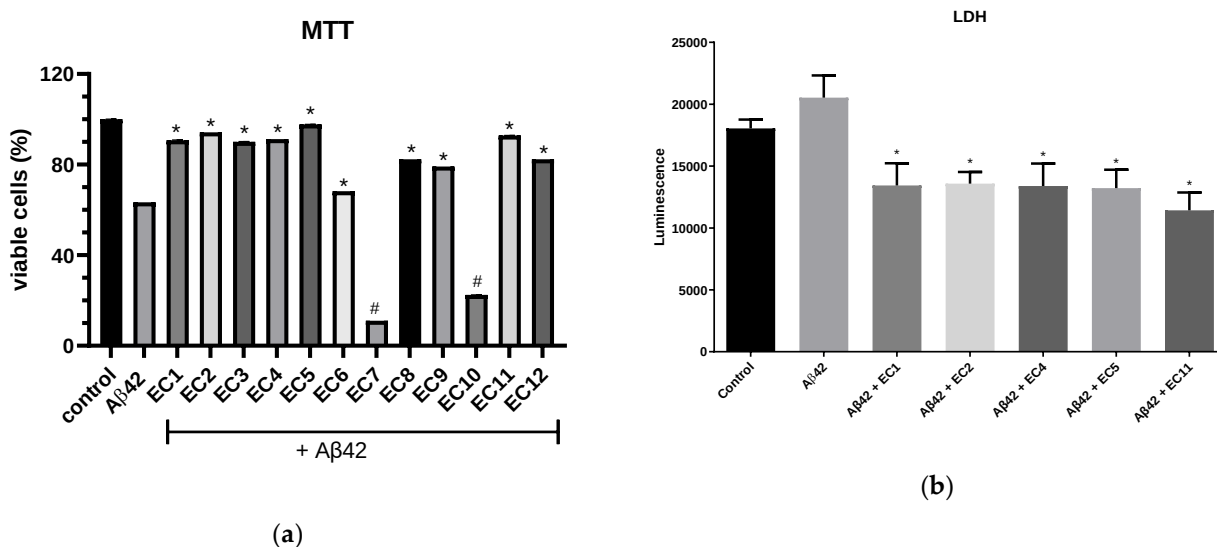


Figure 2. Viability of SH-SY5Y cells without treatment (control), Aβ42 or *Eudendrium carneum* HPLC fractions. (a) MTT assay; (b) LDH assay. Data are present as mean ± SEM. *p<0.05 vs Aβ42, showing a neuroprotective effect; #p<0.05 vs Aβ42, showing a neurotoxic effect.

3.3. Molecular Structure Analysis

According to the mass spectrometry analysis, it was possible to isolate a pure biologically active molecule, in EC5 sample, the one with best result in MTT and confirmed by LDH assay. Initial characterization was performed using mass spectrometry, in a positive mode of ionization, and the main ion (663 m/z) was selected and fragmented (Figure 3a and 3b). To further elucidate the structure, the raw data was submitted to the GNPS platform, which relies on spectral similarity networks to compare experimental spectra with those previously deposited in its database (Figure 3c). This procedure allows correlation of masses and fragmentation patterns with sarmentoside B (Figure 3d), within a pre-established tolerance range. Moreover, the fragmentation profile of EC5 was manually checked and matched with the fragmentation profile of sarmentoside B, according to the literature and databases.

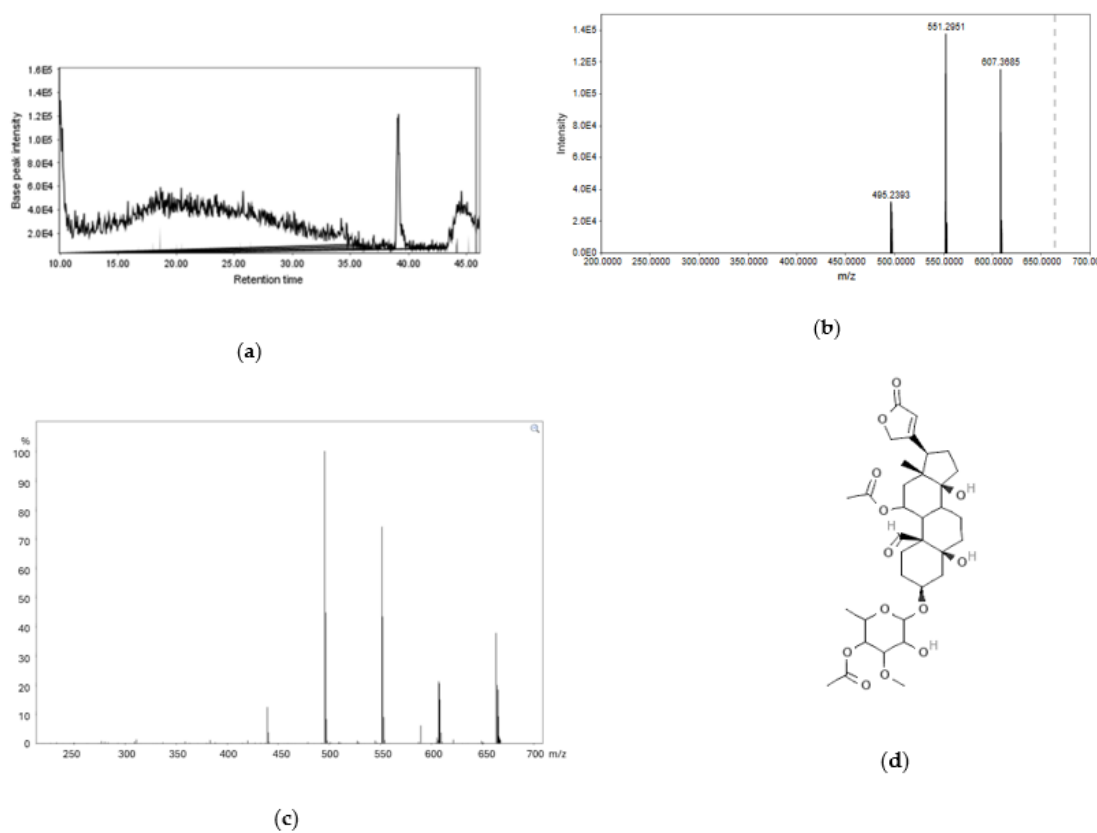


Figure 3. Characterization of EC5 active fraction. (a) Mass spectra of EC5 in a MS scan, in positive ionization mode; (b) MS/MS spectra after fragmentation of 663 m/z ion. (c) MS/MS spectra of sarmentoside B, identified by GNPS, showing the similar pattern as experimental MS/MS. (d) Chemical structure of sarmentoside B

3.4. Lysosome Evaluation and autophagy-related proteins

The lysosome evaluation was conducted using a green, fluorescent lysotracker probe and then flow cytometry. Initially, neurons without any treatment and labeling were analyzed, and the gate was determined (Figure 4A). Control neurons, labeled with the probe but without treatment, were positioned to the right from the gate determined before, indicating a lysosome labeling and consequently the intact organelle (Figure 4B). On the other hand, the treatment with 5 μ M oA β 42 reduced fluorescence to values similar to non-labeled cells, indicating disruption of the lysosome (Figure 4C). With EC5 treatment (15 μ M), lysosomes were labeled similarly to control (Figure 4D). In the quantitative analysis (percent of events in each gate R1 and R2), it is possible to see the potential protective effect of molecules regarding lysosomal integrity (Figure 4E).

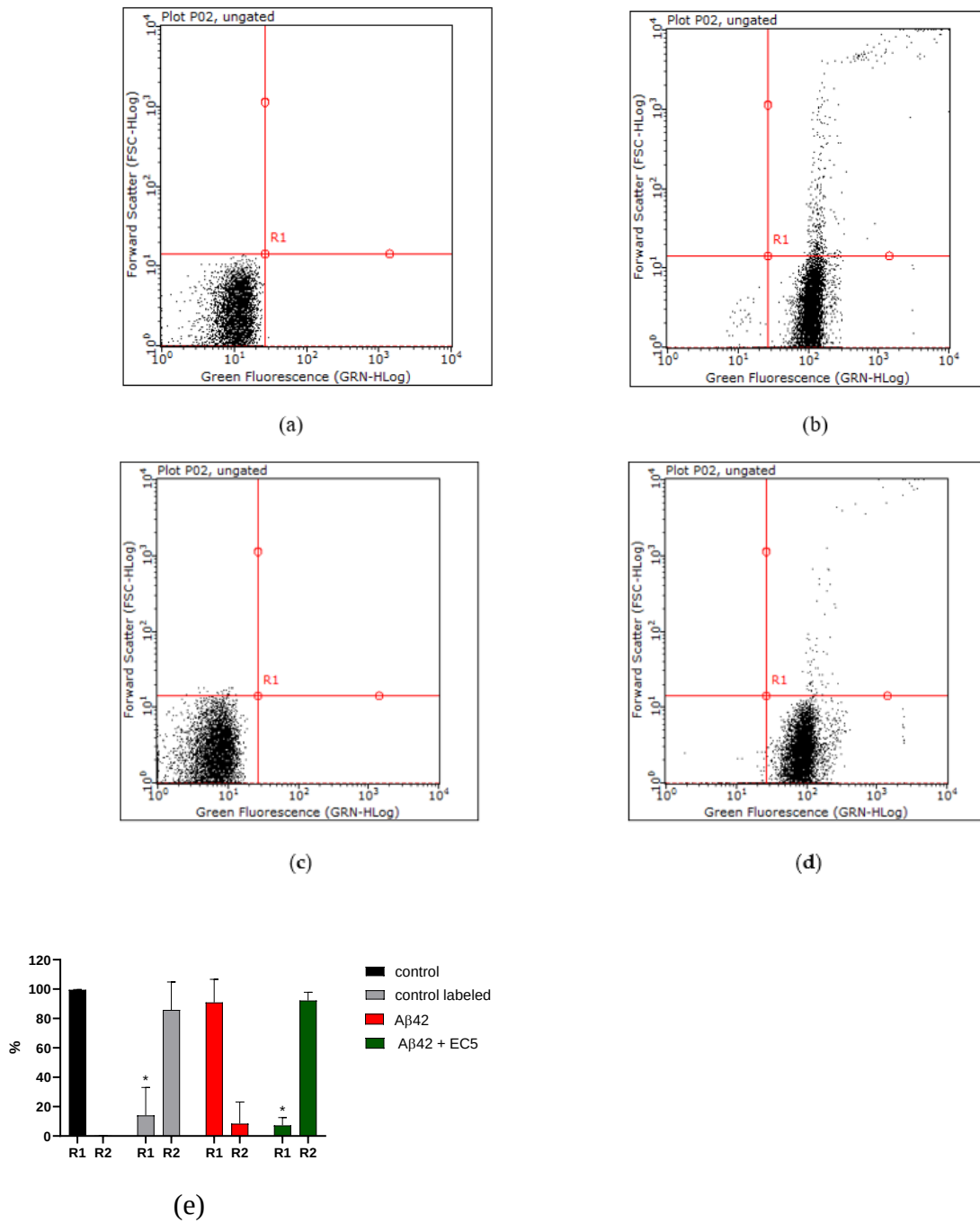


Figure 4. SH-SY5Y labeled with Lysotracker green, fluorescent probe. (a) cells without labeling; (b) Control cells, labeled and without any treatment; (c) Labeled cells treated with A β 42; (d) Labeled cells treated with A β 42 and EC5; (e) Percent of R1 (gate determined with cells without labeling) and R2 (gate for cells labeled with Lysotracker) as mean $\pm$ SEM. * $p < 0.05$ for R1 analysis vs A β 42.

Proteins related to autophagy – mTOR, LC3B and LAMP-1 – were evaluated in neurons after treatment. It was possible to verify that LC3B and LAMP-1 levels were decreased after oA β 42, compared to control (Figures 5A and 5B, respectively), although for LC3B was not statistically different (Figure 5D). The incubation of EC5 had no effect on the expression of both proteins. On the other hand, mTOR was increased after oA β 42 and its levels were reduced with EC5 treatment (Figure 5C).

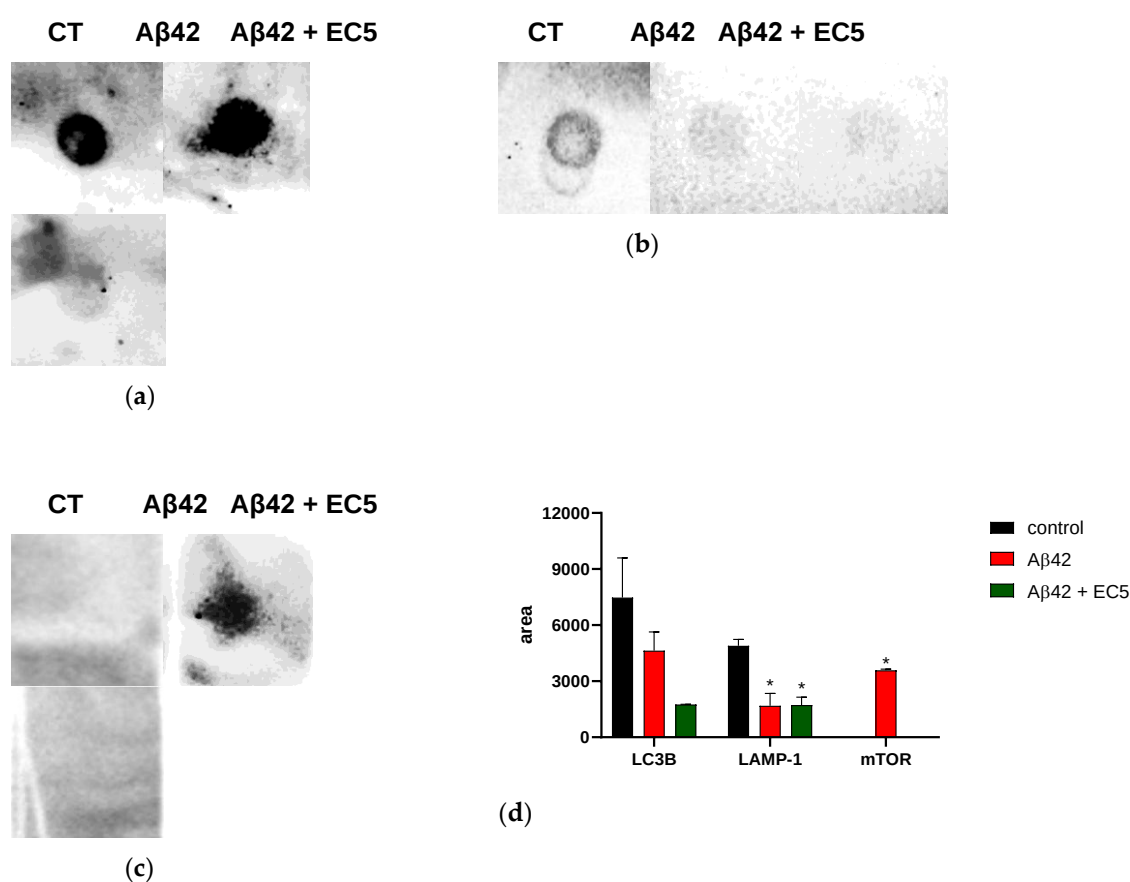


Figure 5. Dot blot of autophagy-related proteins from SH-SY5Y after treatment with A β 42 or A β 42 + EC5. (a) anti-LC3B; (b) anti-LAMP-1; (c) anti-mTOR; (d) Area of the spots presented as mean $\pm$ SEM. *p<0.05 vs control group.

To confirm that mTOR was being inhibited by sarmentoside B, molecular docking analysis was performed, using two models: 3JBZ to analyze the ATP/ADP binding site and 2FAP to

analyze known inhibitor (rapamycin) site. The molecule binds to 3JBZ with the affinity of -6.67 kcal.mol⁻¹, considering the model 1 (Table 1 and Figure 6A), and it identified interactions to Gln2167 (the key amino acid for ATP binding) and His2340, two amino acids distance from Asp2338, the active site of the protein. Regarding 2FAP, the affinity was calculated at -9.832 kcal.mol⁻¹, with the same positioning as rapamycin, and the binding in Ser 2035, near to the rapamycin amino acid interaction Glu 2032 (Table 1 and Figure 6B), indicating a mTOR inhibition.

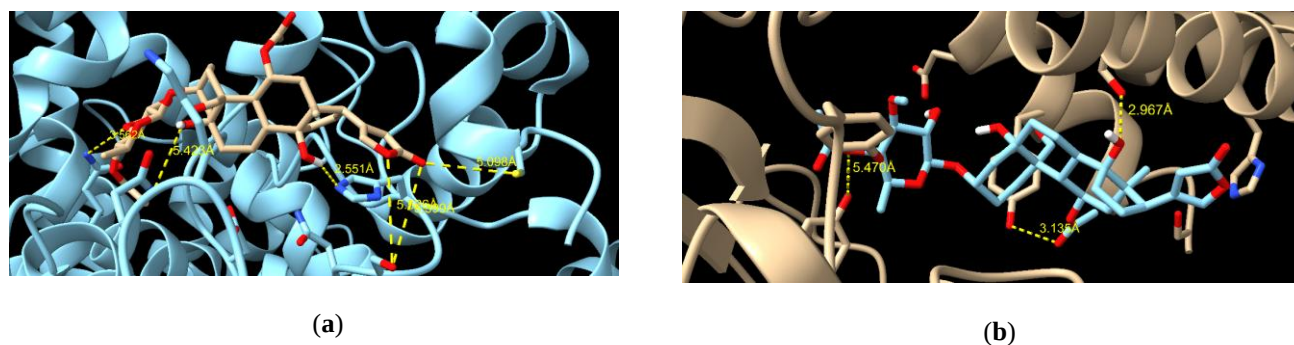


Figure 6. Molecular docking of sarmentoside B and mTORc1. Yellow dashes show the interaction and atom distance. (A) 3JBZ. (B) 2FAP

Table 1. Molecular docking of Sarmentoside B and two models of mTORc1.

Protein	Amino acid binding	Distance (Å)
3JBZ	GLN 2167	5.423
	GLN 2194	3.562
	SER 2342	5.526 and 5.399
	CYS 2546	5.098
	HIS 2340	2.551
2FAP	TYR 26	5.470
	SER 2035	2.967
	TYR 2105	3.135

3.5. Cathepsin D activity

The cathepsin D from neurons had its activity detected using a specific substrate, being the control the highest rate of fluorescence and reaction velocity increase. In contrast, cells treated with 5 μ M oA β 42 had a reduced reaction velocity, suggesting decreased cathepsin D activity and possible lysosomal impairment, consistent with the toxic and dysregulating effects of the amyloid

peptide. The group treated with oA β 42 and then EC5 (15 μ M) showed an intermediate curve, higher than oA β 42 group, indicating that the treatment was able to at least partially preserve cathepsin D catalytic activity (Figure 7).

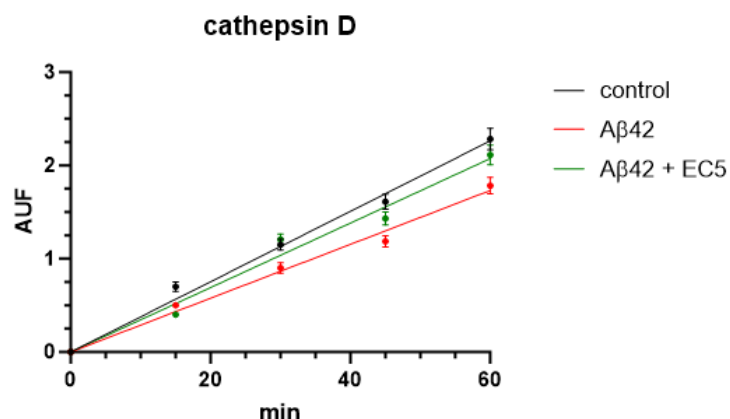


Figure 7. Catalytic activity of cathepsin D from neurons after exposure to A β 42 and A β 42 + EC5. Arbitrary units of fluorescence (AUF) over time, showing the mean $\pm$ SEM of each point

3.6. Assessment of Protein Aggregate Removal

Using Thioflavin-T (ThT), protein aggregates in neurons, after treatment with oA β 42 or oA β 42 + EC5, were quantified. Comparison between the groups revealed differences in fluorescence levels (Figure 8). It is possible to see that in neurons treated with oA β 42, there is higher number of aggregates detected, whereas treatment with EC5 resulted in a reduction of aggregates, similarly to the control. These findings indicate that EC5 was able to reduce the quantity of oA β 42 aggregates.

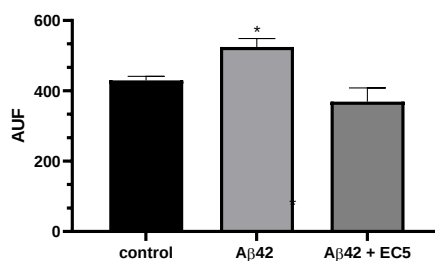


Figure 8. Arbitrary units of fluorescence (AUF) of protein aggregates from neurons by Thioflavin T labeling, in 3 experimental groups: control, A β 42 and A β 42 + EC5. * $p < 0.05$ vs control

3.7. Calcium homeostasis

In order to check if EC5 disrupts the homeostasis of calcium in neurons, the dynamic of the ion was measured by fluorescence probe after stimulation of calcium and sodium channels with KCl/CaCl₂ and veratridine, respectively. The molecule (15 μ M) did not have any effect when incubated in SH-SY5Y neurons (Figure 9A and B, 1st addition). When the agonists of channels were added, the intracellular calcium levels increased with KCl/CaCl₂ for calcium channels (Figure 9A/2nd addition) and for sodium channels (Figure 9B/2nd addition with veratridine). EC5 could not inhibit or potentialize the action of the agonists, indicating that the molecule did not have any effect of ion channels or change the calcium dynamics on neurons.

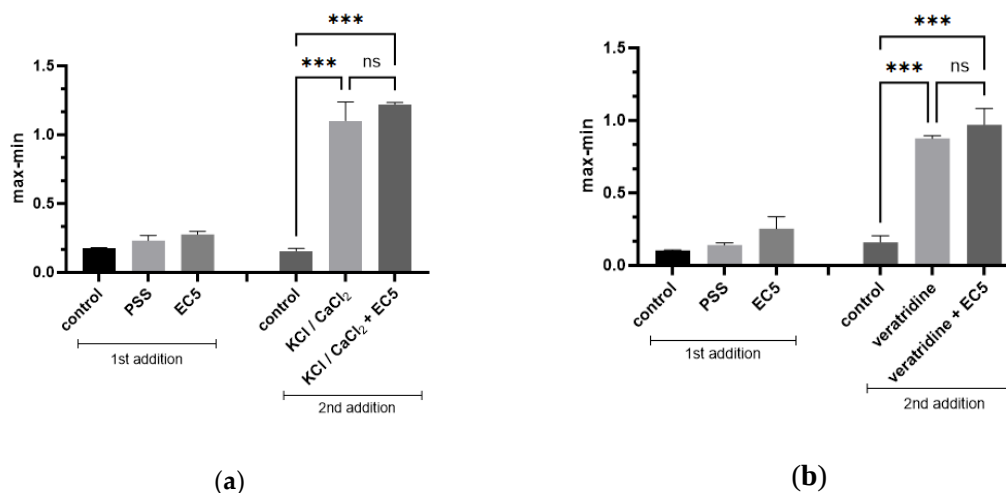


Figure 9. Maximum over minimum response (max-min) of fluorescence inside SH-SY5Y neurons, detected by calcium probe, after stimulation of (a) calcium channels, by KCl/CaCl₂, and (b) sodium channels, by veratridine, in the absence or presence of EC5. *** $p < 0.05$ vs control. ns = no significant.

3.8. Predicted toxicity

The possible toxic effects of sarmentoside B were predicted using in silico approaches. Several targets were analyzed: organ toxicity, receptors, pathways and metabolism. A possible nephrotoxic and respiratory toxicity could be predicted, but the probability is low, as well as androgen and estrogen receptors, aromatase, AChE, p53, NADH-quinone oxidoreductase and PRX (Table 2). On the other hand, 3 targets could be relevant: immunotoxicity, nutritional toxicity and Mitochondrial Membrane Potential (MMP).

Table 2. Predicted toxicity of sarmentoside B in organs and targets.

Target	Prediction	Probability
Hepatotoxicity	Inactive	0.94
Neurotoxicity	Inactive	0.92
Nephrotoxicity	Active	0.60
Respiratory toxicity	Active	0.78
Cardiotoxicity	Inactive	0.61
Carcinogenicity	Inactive	0.62
Immunotoxicity	Active	0.99
Mutagenicity	Inactive	0.89
Cytotoxicity	Inactive	0.96
BBB-barrier	Inactive	0.60
Ecotoxicity	Inactive	0.72
Clinical toxicity	Inactive	0.63
Nutritional toxicity	Active	0.98
Aryl hydrocarbon Receptor	Inactive	1
Androgen Receptor	Inactive	0.77
Androgen Receptor Ligand Binding Domain	Active	0.63
Aromatase	Active	0.83
Estrogen Receptor Alpha	Active	0.60
Estrogen Receptor Ligand Binding Domain	Inactive	0.99
Peroxisome Proliferator Activated Receptor Gamma	Inactive	0.62
Nuclear factor like 2/antioxidant responsive element	Inactive	0.93
Heat shock factor response element	Inactive	0.93
Mitochondrial Membrane Potential	Active	0.9
Phosphoprotein (Tumor Suppressor) p53	Active	0.59
ATPase family AAA domain-containing protein 5	Inactive	0.92
Thyroid hormone receptor alpha	Inactive	0.83
Thyroid hormone receptor beta	Inactive	0.95
Transthyretin	Inactive	0.62
Ryanodine receptor	Inactive	0.74
GABA receptor	Inactive	0.56
Glutamate N-methyl-D-aspartate receptor	Inactive	0.99
alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor	Inactive	1
Kainate receptor	Inactive	1
Achetylcholinesterase	Active	0.50
Constitutive androstane receptor	Inactive	0.99
Pregnane X receptor	Active	0.68
NADH-quinone oxidoreductase	Active	0.57
Voltage gated sodium channel	Inactive	0.94
Na ⁺ /I ⁻ symporter	Inactive	0.60
Cytochrome CYP1A2	Inactive	0.99
Cytochrome CYP2C19	Inactive	0.96
Cytochrome CYP2C9	Inactive	0.84
Cytochrome CYP2D6	Inactive	0.90
Cytochrome CYP3A4	Inactive	0.98
Cytochrome CYP2E1	Inactive	0.99

4. Discussion

Autophagy is an important system to maintain a proteostasis in neurons, which is impaired in AD. Thus, molecules that recover autophagy could be an alternative to remove amyloid plaques and to improve symptoms of the disease [16].

Based on previous study of the group, which identified that *E. carneum* reduced the neuron's death caused by amyloid peptide, we isolated one molecule based on bioassay-guided fractionation, being the main activity the cell death reduction, and the further study of lysosomes [6].

Two methods were used to evaluate cell viability: MTT and LDH. While MTT evaluates the metabolic activity in cell cultures through the reduction of the yellow tetrazolium salt (MTT) into insoluble purple formazan crystals by viable cells, through the action of mitochondrial dehydrogenase enzymes [17], the LDH assay evaluates cell viability by membrane integrity of cells, based on the detection of lactate dehydrogenase (LDH) enzyme released into the extracellular medium when the cell membrane is compromised, being an indirect indicator of cellular damage or death [18].

Using this approach, we identified the sarmentoside B, which is a cardenolide glycoside with the molecular formula $C_{34}H_{48}O_{13}$. Cardenolide glycosides are commonly found in plants, as chemical defenses against herbivores, and also have been found in animals, such as amphibians [19]. In Bufonidae family, bufadienolides have been used for the treatment of several diseases, including Ch'an Su, a preparation from toad's venom, used as Traditional Asian medicines [20].

The presence of multiple hydroxyl groups, methoxy groups, and esters confers physicochemical properties that influence both its bioavailability and binding affinity to molecular targets [21]. Biologically, sarmentoside B shares the characteristic mechanism of action of cardiotonic glycosides, acting as an inhibitor of Na^+/K^+ -ATPase. This inhibition results in an increase in intracellular sodium concentration and a consequent rise in calcium via the Na^+/Ca^{2+} exchanger, promoting greater contractility of myocardial fibers [22-24].

Such toxic effects could be excluded by *in silico* analysis, which did not find any cardiotoxic effects. Moreover, with calcium dynamic experiments, we could not see inhibitory or excitatory action of the molecule in calcium or sodium channels, and nor in calcium mobilization.

In addition to cardiotonic activity, the cardenolide class to which it belongs has been

reported to possess anti-inflammatory, antioxidant, and cell signaling modulatory properties, suggesting potential pharmacological applications [25]. Studies with AD models have shown important properties of cardenolide glycoside in this neurodegeneration. Thakur et al. isolated three cardenolide glycosides from plant extracts by bioassay-guided fractionation, with ability to decrease the A β 42 levels in cell line experiments [26].

Digoxin reduced TNF- α and restored choline acetyl transferase activity, enhancing rat memory and neuronal survival [27]. Ouabain improved cognitive function in FAD4T transgenic mice by TREM2 upregulation and PI3K/Akt pathway activation, with consequent anti-inflammatory activity [28]. Moreover, ouabain reduced tau pathology in mouse model and acted on autophagy pathway by enhancing the activation of transcription factor EB through inhibition of the mTOR pathway [29].

Here, we saw that sarmentoside B inhibited and /or decreased the expression of mTOR, previously increased with oA β 42 treatment, corroborating this finding. The hyperactivation of PI3K/Akt/mTOR pathway is observed in AD brain and it is associated with reduced autophagy, increasing the accumulation of protein aggregates [30]. Moreover, studies in AD brain and in AD early stages show that markers of autophagy, such as LC3, are reduced [31]. We saw in our model that LC3B and LAMP-1 were reduced, indicating that the autophagosome have not been formed, as well as the fusion of the vesicle with the lysosome was not concluded, impairing the aggregates removal. EC5 had no effect on these proteins, but only on mTOR.

The reduction of cell death caused by sarmentoside B can be explained by the lysosomal restoring, previously impaired by oligomer amyloid peptide, considering that the treatment with the molecule resulted in a lysosome correctly labeled.

Under lysosomal dysfunction in AD, a membrane permeabilization of the organelle occurs, resulting in the release of cathepsins into the cytosol. This leakage compromises essential cellular processes, promotes the accumulation of protein aggregates, and can initiate cell death pathways [32].

The green, fluorescent LysoTracker probe was applied as a fundamental method to evaluate lysosomal integrity and functionality in neurons. This probe selectively accumulates in acidic lysosomes, allowing the analysis of their quantity and activity through flow cytometry. By quantifying fluorescence intensity, it was possible to identify the protective effects of sarmentoside

B [33].

In addition to lysosome labeling, which indicates that the organelle morphology is intact, we saw that cathepsin D, the main lysosomal enzyme, had its activity restored, after being reduced by the amyloid peptide. Under physiological conditions, this protease remains confined within lysosomes, participating in the processing of intracellular components and the elimination of misfolded proteins, such as β -amyloid peptide [34]. Thus, the ability of sarmentoside B in maintaining the lysosome morphology recovered its functionality, especially regarding the proteolytic effect, able to cleave misfolding proteins.

This ability to remove misfolding proteins could be observed by ThT labeling, in which after sarmentoside B treatment the amount of protein aggregates was reduced, to similar levels to control. The ThT has specific affinity for β -sheet-rich structures, which are characteristic of amyloid aggregates. When the dye binds to these deposits, a significant increase in fluorescence occurs, and allows the assessment of aggregate formation. Therefore, the restoring of lysosome in both morphology and functionality, with cathepsin D with catalytic activity, could result in elimination of the amyloid plaque [35].

Although promising, these results were obtained using *in vitro* experiments, and additional assays should be performed in *in vivo* studies to confirm the efficacy of sarmentoside B in removing amyloid plaque by lysosomal restoring, in a complex system. Moreover, in the animal model it would be possible to confirm the safety of the molecule and the pharmacokinetics profile.

5. Conclusions

Sarmentoside B is able to restore lysosomal morphology and function, as well as autophagy pathway, and consequently remove amyloid plaque from neurons, the main cause of AD. Therefore, this study contributes to the advance on the search for new therapies capable of acting on processes such as autophagy, offering new perspectives for the treatment of Alzheimer's disease.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/doi/s1, Figure S1: Dot blot of proteins extracted from SH-SY5Y>

after treatment with A β 42 or A β 42 + EC5. (a) anti-LC3B; (b) anti-LAMP-1; (c) anti-mTOR. Yellow arrows show the control (CT), A β 42 and A β 42 + EC5 groups.

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Abbreviations

The following abbreviations are used in this manuscript:

A β	Amyloid beta-peptide
AD	Alzheimer's Disease
APP	Amyloid precursor protein
EC	<i>Eudendrium carneum</i>

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4. CONCLUSÃO

A partir da secreção do hidroide marinho *Eudendrium carneum* foi possível obter uma molécula neuroprotetora para o peptídeo oligomerizado amiloide 42, em um modelo celular de doença de Alzheimer.

A molécula ativa foi caracterizada como sarmentosideo B, que demonstrou atividade restauradora da autofagia, na restauração da morfologia lisossomal, inibição da proteína mTOR e aumento da atividade da catepsina D. Todos esses efeitos resultaram na capacidade de remoção do peptídeo amiloide, e consequente inibição da morte neuronal.

Assim, a molécula tem potencial para ser um protótipo e no futuro desenvolvida como um novo medicamento para ser utilizado no tratamento da doença de Alzheimer, ainda sem cura.

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