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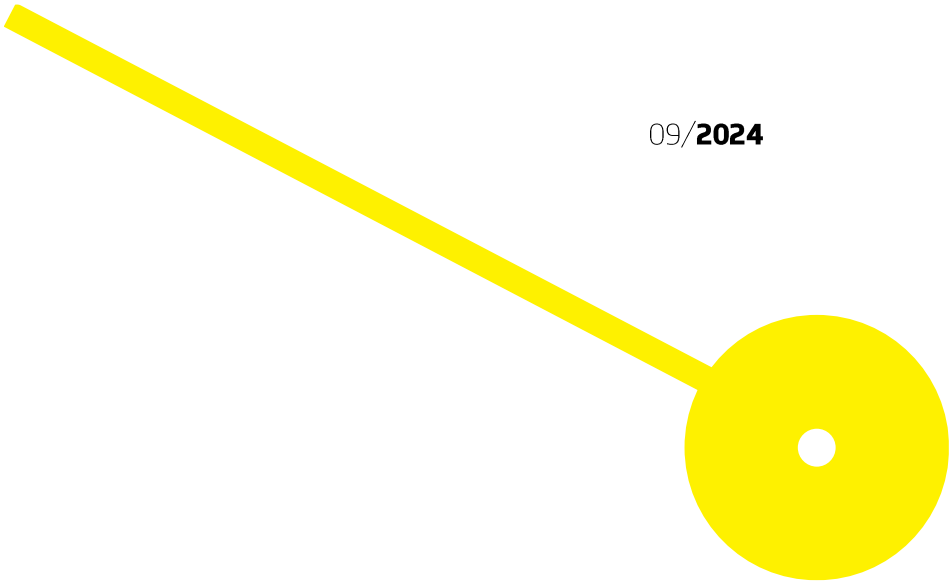
A new approach for tackling neurodegenerative diseases: screening marine Actinomycetota for Alzheimer therapies

Bárbara Salgado



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A new approach for tackling neurodegenerative diseases: screening marine *Actinomycetota* for Alzheimer therapies

Autor

Bárbara Salgado

Orientador(es)

Professora Doutora Maria de Fátima Carvalho/ CIIMAR/ICBAS

Professora Doutora Clara Grosso/ GRAQ/REQUIMTE

Professor Doutor Ricardo Ferraz/ ESS

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Resumo

A doença de Alzheimer (DA) mantém-se como a principal causa de demência, contribuindo para 60% a 80% dos casos. No entanto, ainda não foi encontrado um tratamento efetivo para esta doença neurodegenerativa.

As bactérias do filo Actinomycetota, conhecidas como actinobactérias, são um grupo de bactérias gram-positivas com um potencial biotecnológico bastante promissor, sendo responsáveis pela produção de um elevado número de antibióticos e outras moléculas clinicamente importantes. Enquanto as actinobactérias terrestres se encontram extensivamente exploradas, os ambientes marinhos ainda são pouco investigados em termos destes recursos microbianos valiosos.

Neste trabalho, foram obtidos extratos de várias estirpes de actinobactérias previamente isoladas de macroalgas marinhas e amostras de mar profundo. Estes extratos foram avaliados quanto ao seu potencial anti-AD, testando enzimas envolvidas na patogénese da doença de Alzheimer, nomeadamente a acetilcolinesterase (AChE) e a butirilcolinesterase (BuChE). A percentagem de inibição destas enzimas foi avaliada recorrendo a um protocolo adaptado do método colorimétrico de Ellman. Após a realização dos ensaios, um grupo selecionado de estirpes de actinobactérias demonstrou algum grau de inibição tanto contra a AChE como contra a BuChE (acima de 30%) numa concentração de 2 mg/mL. Para além da inibição da AChE e BuChE, foi também avaliado o potencial inibidor dos extratos contra uma outra enzima, a tirosinase, demonstrando uma elevada percentagem de inibição desta enzima (acima de 60%). A atividade antioxidante foi estudada contra o radical anião superóxido, onde apenas a estirpe S_113_5 mostrou algum grau de eliminação do radical (53.7 %). Por fim, foram testados os efeitos citotóxicos destes extratos na linhagem de células de neuroblastoma SH-SY5Y e fibroblastos 3T3-L1, sendo possível observar que houve citotoxicidade nas células de neuroblastoma, mas não nos fibroblastos, o que sugere um efeito seletivo que não prejudica as células saudáveis. Esses resultados levantaram a hipótese de um potencial efeito anticancerígeno pelo que extratos foram testados na linha celular de adenocarcinoma de colón RKO. Os resultados mostraram algum nível de citotoxicidade em RKO, indicando uma potencial atividade anticancerígena.

Tendo em conta os resultados obtidos, este estudo mostra que as actinobactérias podem ser uma fonte de novas terapias para a prevenção ou tratamento da doença de Alzheimer.

Palavras-chave: Actinobactérias, Doença de Alzheimer, Macroalgas, Mar profundo, Acetilcolinesterase, Butirilcolinesterase, Tirosinase, Citotoxicidade

Abstract

Alzheimer's disease remains as the most common cause of dementia, accounting for an estimated 60% to 80% of total cases. However, effective treatment for this neurodegenerative disease has yet to be found.

Bacteria from the phylum Actinomycetota, known as actinobacteria, are a group of gram-positive bacteria with highly promising biotechnological potential, being responsible for the production of a large number of antibiotics and other clinically important molecules. Terrestrial Actinomycetota are extensively explored, but marine environments are yet poorly investigated in terms of these valuable microbials resources.

In this work, the crude extracts from various strains of actinobacteria previously isolated from marine macroalgae and deep-sea samples were obtained. These extracts were screened for their anti-Alzheimer potential by testing enzymes involved in the pathogenesis of Alzheimer's disease, namely acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) (1,2). The percentage of inhibition of these enzymes was evaluated by performing an adapted protocol of Ellman's colorimetric method (3). After performing the assays above, a selected group of the actinobacteria strains demonstrated some degree of inhibition of AChE and BuChE at a concentration of 2 mg/mL in each well. Besides AChE and BuChE inhibition, their inhibitory potential against tyrosinase was also evaluated, showing that the extracts had a high percentage of inhibition of this enzyme (above 60%). The antioxidant activity was also assessed against superoxide anion radical, and only one strain (S_113_5) showed some degree of scavenging (53.7%). Lastly, their cytotoxic effects were tested on both neuroblastoma cells (SH-SY5Y), and healthy fibroblasts (3T3-L1) and being possible to see some cytotoxicity on the neuroblastoma cell line and not on the fibroblasts, suggesting a selective effect that doesn't harm the healthy cells. These results suggested a putative anticancer potential and additionally extracts were tested in the cancer cell line RKO. Results indicate that all extracts showed some level of cytotoxicity against RKO cells, indicating potential anti-cancer activity.

Taking into account the results obtained, this study shows that actinobacteria can be a source of new therapies for tackling Alzheimer's disease.

Keywords: Actinomycetota, Alzheimer's disease, Macroalgae, Deep-sea, Acetylcholinesterase, Butyrylcholinesterase, Tyrosinase

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List of Abbreviations

3T3 – L1 – Embryonic mouse fibroblast cell line

ACh – Acetylcholine

AChE – Acetylcholinesterase

Aβ – beta amyloid

AD – Alzheimer's Disease

AMPAr – α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

ATCI – Acetylthiocholine Iodide

BTCI – Butyrylthiocholine Iodide

BuChE – Butyrylcholinesterase

CNS – Central Nervous System

COX-2 – cyclooxygenase-2

DMSO – Dimethyl sulfoxide

EAATs – Excitatory amino acid transporters

L-DOPA – Levodopa

MAO – Monoamine oxidase

MAPT – Microtubule-associated protein tau

mGluR2 – Metabotropic glutamate receptor 2

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NADH – Nicotinamide adenine dinucleotide

NBT – Nitro Blue Tetrazolium Chloride

ND – Neurodegenerative diseases

NLRP3 – NLR family pyrin domain containing 3

NMDARs – N-Methyl-D-aspartate receptors

Nrf2 – Nuclear factor E2-related factor 2

PMS – Phenazine methosulfate

RKO – Human colorectal cancer cell line

ROS – Reactive Oxygen Species

SH-SY5Y – Human neuroblastoma cell line

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1. Introduction

Neurodegenerative diseases (NDs) are progressive, incurable disorders characterized by the degeneration and loss of neurons. They include conditions such as Alzheimer's disease (AD), Parkinson's disease (PD) and multiple sclerosis (MS), affecting millions of people worldwide. With increasing life expectancy, the prevalence of these diseases is projected to rise to an estimated 130 million people affected by these diseases by 2050 (4).

Actinomycetota is a phylum of Gram-positive bacteria with remarkable biotechnological potential, with a high number of antibiotics and other clinically important molecules being derived from these microorganisms. Recent studies have started to investigate the potential of Actinomycetota-derived compounds in the treatment of NDs, such as AD and PD (5–7), highlighting the importance of further research into their therapeutic applications.

1.1. Phylum Actinomycetota

Actinomycetota represent a gram-positive group of bacteria, being one of the largest bacterial taxonomic units (8). These bacteria contain a GC-rich linear genome and are known to produce a wide range of different secondary metabolites that can have varied uses, like antibiotics, anti-cancer, antiviral, anti-inflammatory compounds, among others (9).

These bacteria have a wide range of morphological structures, like unicellular cocci or rods (10). Actinomycetota grow by a combination of tip extension and branching of the hyphae, and, like filamentous fungi, some of these bacteria produce a mycelium, and many reproduce by sporulation. Nonetheless this similarity to fungi, Actinomycetota cells have a chromosome that is organized in a prokaryotic nucleoid and a peptidoglycan cell wall, making them susceptible to antibacterial agents. Most Actinomycetota are aerobic, and are nutritionally classified as heterotrophic or chemoautotrophic (11,12).

Their ecological versality allows them to thrive in various environments, ranging from terrestrial to aquatic habitats. The ability to adapt to diverse environmental conditions is attributed to their genetic and metabolic flexibility. Actinomycetota play a crucial role in nutrient cycling, decomposing organic matter and contributing to soil fertility. Besides this, they often form symbiotic relationships with plants and animals, enhancing their ecological significance.

Although several Actinomycetota can be found in terrestrial ecosystems, recently, there has been more interest in exploring them in marine environments (11). Marine Actinomycetota, in particular, have gathered significant attention due to their unique adaptations and biosynthetic capabilities.

1.1.1. Marine Actinomycetota

Actinomycetota are widely distributed in the marine environments. These microorganisms have been found to establish symbiotic relationships with many marine organisms, like sponges, sediments, corals and seawater (8). Marine Actinomycetota exhibit unique adaptations that enable them to survive in harsh conditions, such as high salinity, pressure, and varying temperatures. They have been attracting the attention of the scientific community due to their robust biosynthetic potential, more particularly in producing secondary metabolites with a broad structural diversity and significant commercial importance (9). The study of these bacteria in marine environments has revealed a wealth of novel compounds with potential applications in medicine, agriculture, and industry. For instance, marine-derived Actinomycetota have been found to produce potent antibiotics, anticancer agents, and enzymes that are stable under extreme conditions (8,12).

1.1.2. Bioactive compounds produced by Actinomycetota

Actinomycetota are responsible for the production of two-thirds of the naturally derived antibiotics and drugs that have useful properties in medicine, such as antibacterial, anticancer, antitumor, antifungal, cytotoxic, antiviral, anti-inflammatory, and immunosuppressive drugs (8,9). These secondary metabolites have profound implications for biotechnology, medicine and industry (9). Notably, within Actinomycetota, the genus *Streptomyces* is the largest producer of antibiotics, contributing significantly to the strains isolated from soil (11).

The secondary metabolites produced by Actinomycetota are structurally diverse and often complex, reflecting the biosynthetic pathways within these microorganisms. Among antibiotics, the most well-known are streptomycin, tetracycline, and erythromycin, which have revolutionized the treatment of bacterial infections. In addition to antibiotics, Actinomycetota produce anticancer agents like doxorubicin and bleomycin, which are widely used in chemotherapy (5,12).

1.2. Alzheimer's Disease

Alzheimer's disease is a progressive brain disease caused by the damage to nerve cells in the brain (13). Even though the leading risk factor for AD is aging, genetic risk factors play the second prominent role (14). Amongst the different brain changes that occur in this NDs, there are two underlying pathological hallmarks: the accumulation of the protein fragment β-amyloid (Aβ) outside neurons and the accumulation of an abnormal form of the protein tau – tau angles – inside the neuron/intracellular neurofibrillary tangles (NFTs) (13,15). The accumulation of Aβ plaques may damage neurons by interfering in communication made at synapses (13). On the other side, NFTs are formed by the accumulation of the hyperphosphorylated-tau protein (15).

Additionally, this disease is characterized by acetylcholine (ACh) depletion and oxidative stress (1,16).

Another early pathological hallmark is a dysfunction in the blood-brain barrier (BBB). This is characterized by barrier leakage and associated with cognitive decline (17).

1.2.1. Pathological characteristics of Alzheimer's disease

As were mentioned in the previous section, in both AD and other NDs, two key pathological hallmarks are found: the accumulation of Aβ plaques outside neurons and neurofibrillary tangles (NFTs) inside the neurons. However, other characteristics also play a role in AD, such as unbalance of the cholinergic system, oxidative stress, amongst others.

1.2.1.1. Amyloid-β

Aβ plaques arise from the breakdown of a protein called amyloid precursor protein (APP) found in neuronal membranes. The enzyme responsible for the cleavage of APP - beta-secretase 1 (BACE1) – ends up leading to the production of Aβ peptides that eventually aggregate into plaques (18). In AD, this process becomes deregulated, leading to the overproduction and accumulation of insoluble Aβ peptides, particularly Aβ42, which is more prone to aggregation than Aβ40. These peptides cluster together to form amyloid plaques, one of the primary pathological hallmarks of AD.

The presence of Aβ plaques disrupts neuronal function and triggers a cascade of neurodegenerative processes, including synaptic damage, mitochondrial dysfunction, and neuroinflammation (19).

1.2.1.2. Tau pathology

The tau protein is a healthy component of neurons, crucial for their structure and function. However, in neurodegenerative diseases, tau becomes abnormally hyperphosphorylated, causing it to misfold and clump together into harmful NFTs. These tangles disrupt vital processes within neurons, leading to cell dysfunction (13,18,20). Tau NFTs correlate better with neuronal loss and symptoms in AD. Tau is involved in various physiological processes, including microtubule stabilization, myelination, axonal transport, and neuronal functions. Tauopathies, a group of neurodegenerative diseases, also feature tau pathology distinct from AD.

Mutations in the microtubule-associated protein tau (MAPT) gene, which encodes tau, are linked to inherited forms of frontotemporal dementia (FTD) and parkinsonism, characterized by tau deposits but not Aβ. Tau has six isoforms in the adult brain, with different isoforms predominating in various tauopathies. Hyperphosphorylation impairs tau's interaction with microtubes.

Tau aggregates differently across diseases but follows a consistent pattern within the same tauopathy. Aggregation of tau begins with Aβ oligomers inducing tau oligomerization, suggesting a common interface for aggregation. Despite being mainly intracellular, tau is also found extracellularly and can spread between brain regions, promoting aggregation. In AD, tau deposition follows a predictable pattern, progressing from the entorhinal cortex to other brain regions, corresponding to Braak stages of AD progression, with symptoms appearing as the disease advances (21).

1.2.1.3. Blood-Brain Barrier

The Blood-brain barrier (BBB), normally a high selective gatekeeper that protects the brain from harmful substances in the bloodstream, can become compromised in neurodegenerative states. This breakdown allows communication between the brain and blood. Interestingly, exosomes, small cellular messengers released by brain cells, have been shown to cross the weakened BBB (17,22). These exosomes have the potential to carry both abnormal tau and Aβ into the bloodstream, raising questions about their role in disease progression and communication between brain cells (13,17,20).

1.2.1.4. Oxidative Stress

Oxidative stress is central to AD, linking Aβ aggregation, tau hyperphosphorylation, neuroinflammation, and metal ion dysregulation. It results from an imbalance between

reactive oxygen species (ROS) production and antioxidant defences, leading to neuronal damage. Aβ peptides and metal ions produce ROS, causing mitochondrial dysfunction and promoting Aβ and tau aggregation. Oxidative stress also activates glial cells, releasing pro-inflammatory cytokines and further ROS, creating a harmful feedback loop (23).

1.2.1.5. Neuroinflammation

Neuroinflammation corresponds to an inflammatory response centralized to the brain and spinal cord. Neuroinflammation response involves all cells present in Central nervous system (CNS) such as glial cells, astrocytes, and neurons. Microglia provide primary immune surveillance in the CNS and release cytokines and chemokines along with macrophage like activity. Involved in these responses are the pro-inflammatory cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6), TNF-α and various chemokines (CXCL-1, CCL2, CCL5), nitric oxide, and ROS (24).

In AD patients, their brains have shown to have an accelerated level of pro-inflammatory cytokines like IL-6 and TNF-α, along with direct neurotoxic effects, microglia and astrocyte activation. Cytokines and chemokines act through the stimulation of the chemotaxis in the brain and cause neuronal loss (24).

Microglia play a crucial role in neuroinflammation: Aβ can bind different microglial receptors, resulting in the production of inflammatory cytokines and chemokines, and also oxygen free radicals, nitric oxide, and TNF-α. The NLR family pyrin domain containing 3 (NLRP3) inflammasome is a recently found cytoplasmic protein complex involved in neuroinflammation and innate immune response. Recent studies have shown that Aβ induced NLRP3 activation in both microglia and astrocytes, resulting in the production of caspase 1 and inducing the release of cytokines IL1β and IL-18, which in turn results in irreversible damage (25).

1.2.1.6. Cholinergic System

Another cause leading to AD is the decrease of neurotransmitter acetylcholine (ACh) in synaptic clefts inside the brain. ACh deficit is partly due to changes in the activity of different isoforms of the enzyme acetylcholinesterase (AChE) (26,27), which is responsible for its hydrolysis. Because of this, some of the potential treatments for AD relies on inhibiting the AChE enzyme, having been developed many AChEIs (AChE inhibitors), both natural and synthetic, in order to counteract the progression of this disease. Apart from AChE, butyrylcholinesterase (BuChE) is another enzyme that participates in the breakdown of

ACh, being the main cholinesterase in liver, glia, neurons and in tangles and neurotic plaques. However, between the two enzymes, BuChE has a lower affinity to ACh than AChE (2). Some cholinesterase inhibitors are more selective for AChE, while others target BuChE or exhibit dual inhibition of both enzymes with varying levels of affinity (28).

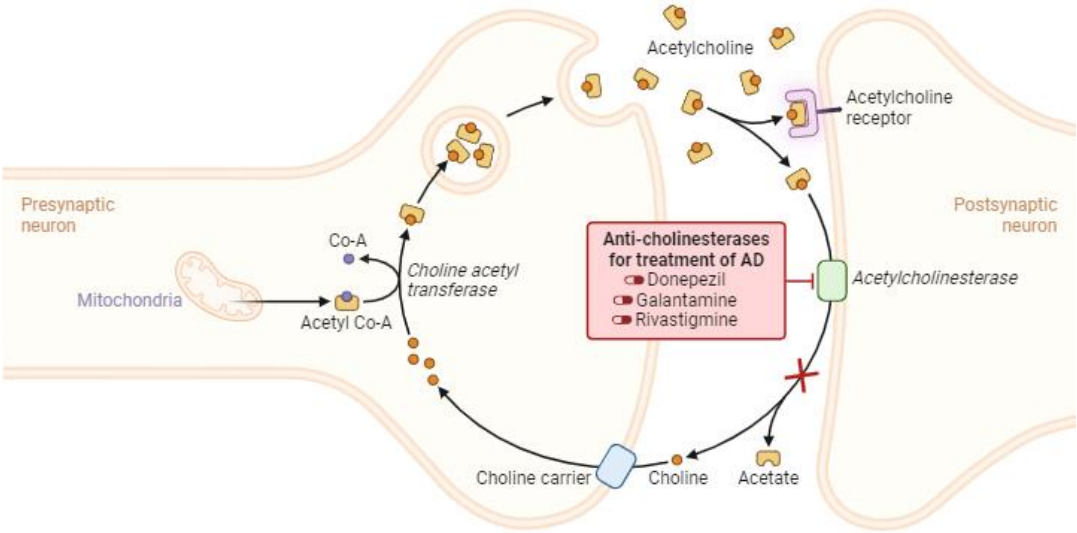

Figure 1 - Anti-cholinesterase Mechanism of Action in AD (29,30). From Biorender.com.

1.2.1.7. Tyrosinase

Tyrosinase is implicated in AD through its role in catalysing the conversion of L-tyrosine to Levodopa (L-DOPA), a precursor to neurotransmitters such as dopamine (31). The enzyme's activity – that is regulated by 14-3-3 proteins – leads to the production of dopaquinone, which can cause neuronal damage and contribute to AD's pathology by inhibiting dopamine and glutamate locomotion and impairing mitochondrial respiration (31). Moreover, tyrosinase inhibitors can reduce oxidative stress and inflammation (32). These inhibitors work through various mechanisms, including reducing dopaquinone and H_2O_2 levels, and scavenging O-dopaquinone (31,32).

1.2.1.8. Glutamate

Glutamate is the most abundant neurotransmitter in the CNS, with its receptors being present on more than 90% of neurons and 40% of synapses. Glutamate plays a crucial role in synaptic stability and plasticity. In AD patients, there is a dysregulation of the glutamatergic system throughout the tripartite synapse (pre and postsynaptic neurons along with glial cells). This dysregulation influenced by protein accumulation such as Aβ and hyperphosphorylated tau, leads to neuronal excitability and glutamatergic excitotoxicity

early in AD, contributing to cognitive dysfunction. Overactivation of N-Methyl-D-aspartate receptors (NMDAr) disrupts memory processes, while later stages show decreased glutamate levels due to neuronal loss. Altered glutamate signaling involves changes in multiple receptors and transporters, including increased metabotropic glutamate receptor 2 (mGluR2) expression and biphasic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAr) and NMDAr subunit regulation. Additionally, disruptions in mgluR5 and downregulation of glial excitatory amino acid transporters (EAATs), along with reduced GABAergic neurons, further imbalance the excitatory and inhibitory signaling, exacerbating glutamate dysregulation throughout AD progression (33).

1.2.2. Risk Factors

There are 3 principal risk factors that influence the development of AD: age, genetics and family history, with age being the greatest of these factors.

Amongst the many genes shown to be involved in this pathology, APOE-e4 has the biggest impact. This gene is connected to the transportation of cholesterol in the bloodstream and having the e4 form of APOE increases the risk of developing AD in comparison of having the e3 or e2 forms. It does not, however, guarantee the development of the disease (34).

Apart from these, there are some risk factors that can be modifiable, such as cardiovascular health factors, hypertension, obesity, exposure to pollution, psychosocial factors, and the variability of blood pressure and high lipid levels. Preventive strategies should focus on managing these risk factors through lifestyle changes (35).

1.2.3. Treatment of Alzheimer's disease

The drug development for this disease focus on different targets such as neurotransmitter systems, Aβ pathology, neuroinflammation, tau pathology, and cholesterol metabolism (17). As for now, 8 drug treatments are approved by the FDA, in which 2 of these change the underlying biology of AD - monoclonal antibodies aducanumab and lecanemab – by helping remove plaques and a form of Aβ called protofibrils that plays a role in the development of Aβ plaques; 5 treat cognitive symptoms by increasing the amount of neurotransmitters in the brain – donepezil, rivastigmine, galantamine, memantine, and memantine combined with donepezil; and one – brexpiprazole – has been approved to treat agitation (34). From the drugs that are focused on the neurotransmitter systems, donepezil (36), rivastigmine

(also able to inhibit BuChE) (37), and galantamine (38) are cholinesterase inhibitors. The drug treatment combining memantine with donepezil (39) mixes AChE inhibition with the antagonism of the NMDARs, whose excessive activation has been associated with neuronal loss (40).

1.3. Potential of Actinomycetota in combating Alzheimer's disease

Recent studies have expanded our understanding of Actinomycetota, revealing their potential in various medical fields. Notably, beyond their well-established applications, these bacteria have shown promise in neuroprotection and possible interventions in NDs, such as AD. NDs are characterized by progressive neuronal loss and the exploitation of Actinomycetota as neuroprotective agents opens new options for developing novel therapeutic strategies (10, 35). Actinomycetota produce a variety of bioactive compounds that can exhibit neuroprotective properties. These compounds can modulate oxidative stress, inflammation and apoptosis, which are key pathological processes in neurodegenerative diseases. For example, some Actinomycetota metabolites have been shown to inhibit the enzymes BACE1 and AChE, and the aggregation of Aβ and tau proteins, which are characteristic features of AD (41,42). Furthermore, the anti-inflammatory properties of Actinomycetota-derived compounds may mitigate neuroinflammation, a common feature of many neurodegenerative diseases (24).

The potential of secondary metabolites produced by Actinomycetota to fight AD has been more and more explored, as some recent studies show (Table 1). A large number of the compounds produced by these microorganisms has the capacity to inhibit the enzymes AChE, BuChE, tyrosinase and BACE-1; while others have antioxidant, anti-inflammatory, anti-amyloid or anti-tau hyperphosphorylation effects. N98-1272 A, B and C, from the strain *Streptomyces bannaensis* sp. Nov. N98-1272, which selectively inhibited AChE (43). Anhydroexfoliamycin, a compound from a *Streptomyces* strain, strongly reduced tau phosphorylation in vitro, at the concentration of 0.1 μM. Additionally, it decreased the levels of ROS, thereby reducing oxidative stress. This compound was also able to inhibit BACE1 in vitro (36, 40). A polyketide from the *Streptomyces* strain KS-619-1 demonstrated inhibition of BACE1, with IC_{50} values for BACE1 and Aβ aggregation of 0.48 and 1.1 μM, respectively, indicating that it could be a lead compound for the development of therapeutic agents for AD (45). The compounds Streptocyclinones A and B, produced by *Streptomyces* sp. CA-

237531, reduced oxidative stress-induced injury, protected mitochondrial function, and activated the pathway crucial for antioxidant and detoxifying enzyme regulation – nuclear factor E2-related factor 2 (Nrf2) pathway. These compounds were also able to modulate neuroinflammation, by increasing anti-inflammatory cytokines production, and to reduce the hyperphosphorylation of the tau protein. Additionally, Streptocyclinone B was found to inhibit BACE1 (42). Several other compounds have shown similar effects. Caniferolide A, a macrolide from *Streptomyces caniferus* (46), stands out for its multifaceted properties, exhibiting antioxidant, anti-inflammatory, and anti-amyloid effects. Moreover, Caniferolide A demonstrates anti-tau hyperphosphorylation and inhibits BACE1. Homoprejadomycin, produced by *Streptomyces tendae* MCCC 1A01534 (47), and certain secondary metabolites from *Streptomyces antibioticus* DSM 40234 (48) showed similar properties, with the former exhibiting anti-tau hyperphosphorylation effects (47) and the latter inhibiting the formation of stress-induced Aβ in neurons (48).

Exopolysaccharides (49), a group of molecules produced by *Streptomyces* sp. NRCG4, exhibit AChE inhibitory potential, alongside with anti-inflammatory and antioxidant properties. These molecules demonstrated a significant inhibition of AChE, which helps increasing cholinergic transmission in the brain, and of tyrosinase, that can prevent neurotoxic effects associated with dopamine oxidation. Additionally, it exhibited strong antioxidant properties such as metal chelation and ROS scavenging, which can protect neuronal cells from oxidative stress. Its selective inhibition of cyclooxygenase-2 (COX-2) – an enzyme that plays a vital role in inflammation and is responsible for the production of prostaglandins, a class of lipid compounds that mediate acute and chronic inflammation (50) - demonstrates anti-inflammatory properties. This inhibition may help reduce the production of inflammatory cytokines, potentially mitigating neuroinflammation linked to AD.

An aminopeptidase from *Streptomyces* sp. KK565 inhibited the formation of fibrils and protected Aβ-induced neurotoxicity (51). Bonnevillamides D and E, isolated from *Streptomyces* sp. UTZ13, were shown to be able to reverse fibril formation by inducing the monomerization of Aβ aggregates (52).

Rhizolutin, a compound isolated from *Streptomyces* sp. WON17, was able to dissociate Aβ plaques and tau tangles, and it was tested in vivo with APP/PS1 double transgenic mice, demonstrating that it could substantially decrease apoptosis induced by Aβ plaques and the

inflammation in neuronal and glial cells (53). Sharing the same novel carbon scaffold of Rhizolutin, Collinolactone, isolated from *Streptomyces Gö* 40/10, has shown to have neuroprotective effects, displaying protection against intracellular oxidative stress (54).

Table 1 – Anti-Alzheimer potential of secondary metabolites produced by Actinomycetota.

Microorganism	Compound/Extract	Activity	Reference
***Streptomyces* sp.**	Anhydroexfoliamycin	Antioxidant. BACE 1 inhibition	41,45
***Streptomyces* sp. NRCG4**	Exopolysaccharide	Anti-inflammatory AChE inhibition Antioxidant Tyrosinase inhibition	50
***Streptomyces* sp. CA-237531**	Streptocyclinones A and B	Antioxidant Anti-inflammatory Anti-Aβ effects BACE1 inhibition Anti-tau hyperphosphorylation	42

***Streptomyces* sp. RD 063311**	Polyketide KS-619-1	BACE1 inhibition Inhibition of Aβ aggregation	46
Streptomyces caniferus	Caniferolide A	Antioxidant Anti-inflammatory Anti- Aβ effects Anti-tauhyperphosphorylation BACE1 inhibition	47
***Streptomyces* sp. KK565**	*Streptomyces* sp. KK565 aminopeptidase	Inhibition of Aβ aggregation Inhibition of fibril formation	52
***Streptomyces* sp UTMC 1334**	Pyrrole derivatives	AChE inhibition Antioxidant	54
***Streptomyces tendae* MCCC 1A01534**	Homoprejadomycin	Anti-tau hyperphosphorylation	48

Streptomyces **sp. CNQ-031**	5,7-dihydroxy-2-isopropyl-4H-chromen-4-one; 5,7-dihydroxy-2-(1-methylpropyl)-4H-chromen-4-one; 1-methoxyphenazine	MAOs inhibition AChE inhibition BuChE inhibition BACE-1 inhibition	55
Streptomyces **sp. CNQ-027**	Piloquinone	MAOs inhibition	56
Streptomyces Gö **40/10**	Collinolactone	Neuroprotection from intracellular oxidative stress	58
Streptomyces **sp. WON17**	Rhizolutin	Disaggregation of Aβ plaques Disaggregation of tau tangles Reduction of apoptosis and inflammation	57
Streptomyces **sp. UTZ13**	Bonnevillamides D and E	Reverse fibril formation by inducing the monomerization of Aβ aggregates	53
Streptomyces bannaensis **N98-1272**	N98-1272 A, B and C	AChE inhibition BuChE inhibition	43
Streptomyces **sp. LB173**	Geranylphenazinediol	AChE inhibition Antimicrobial effect	44

1.4. Aim and outline of this thesis

Given the current lack of effective treatments for AD, the pursuit of new therapeutic interventions has become critical. This thesis aimed to explore the anti-AD potential of Actinomycetota strains previously isolated from marine macroalgae and deep-sea samples, exploring relevant bioactive properties in the context of this disease. Organic extracts of selected Actinomycetota extracts were prepared and screened for their AChE, BuChE, and tyrosinase inhibitory potential, as well as their antioxidant activity against physiological important ROS and RNS, and their cytotoxic effects on neuroblastoma cells and normal fibroblasts SH-SY5Y and 3T3-L1 respectively. Ultimately, the findings of this work can benefit the growing field of AD therapies, offering new hope for effective treatments.

2. Materials and methods

2.1. Actinomycetota strains selected for the study

A collection of 20 Actinomycetota strains previously isolated from macroalgae and deep-sea samples collected in Portugal was used in order to achieve the goals of this work (see Table 2).

Amongst these strains, DS4_3 has already been published (59).

Table 2 - Actinomycetota strains used to obtain the extracts tested in this study

Number	STRAIN	ISOLATION ORIGIN	CLOSEST TAXONOMIC ID (16S)
1	CC-R183	Macroalgae (*Chondrus crispus*)	*Micromonospora maritima*
2	CT-F150	Macroalgae (*Codium tomentosum*)	*Microbacterium aerolatum*
3	CC-R25	Macroalgae (*Chondrus crispus*)	*Streptomyces hydrogenans*
4	CC-F69	Macroalgae (*Chondrus crispus*)	*Kitasatospora albolonga*
5	CT-F6	Macroalgae (*Codium tomentosum*)	*Streptomyces violascens*
6	CT-F87	Macroalgae (*Codium tomentosum*)	*Gordonia terrae*
7	CT-R47	Macroalgae (*Codium tomentosum*)	*Nocardiopsis prasina*
8	CT-R48	Macroalgae (*Codium tomentosum*)	*Nocardiopsis prasina*
9	CT-R82	Macroalgae (*Codium tomentosum*)	*Streptomyces violascens*
10	CT-R87	Macroalgae (*Codium tomentosum*)	*Streptomyces violaceus*
11	C_002 5-1.1	Madeira – Coral (Deep-sea)	*Nocardiopsis lucentensis*
12	C_003 1.11	Madeira – Coral (Deep-sea)	*Streptomyces sp.*
13	C_003 1.3	Madeira – Coral (Deep-sea)	*Streptomyces sp.*
14	DS4_3	Azores – Sediment (Deep-sea)	*Rhodococcus cerastii*
15	S_113 5	Azores – Sponge (Deep-sea)	*Nocardiopsis lucentensis*
16	C_003 5.1.7.	Madeira – Coral (Deep-sea)	*Streptomyces sp.*
17	SED_058 9	Madeira – Sediment (Deep-sea)	*Rhodococcus cercidiphylli*
18	SED_044 11	Azores – Sediment (Deep-sea)	*Gordonia sputi*
19	SED_313 29 (B)	Azores – Sediment (Deep-sea)	*Micromonospora sp.*
20	C_003 5.2.3	Madeira - Coral (Deep-sea)	*Microbacterium aerolatum*

2.1. Preparation of crude extracts from Actinomycetota cultures

Each isolate (shown in Figures 2A to 2D) was grown in 250 mL Erlenmeyer flasks containing 50 mL M1 medium (5 g of agar, 0.5 g of peptone, 1 g of yeast extract and 2.5 g of soluble starch), 50 mL RH medium (5 g of agar, 2.5 g of raffinose, 0.25 g of L-histidine, 0.25 g of KH_2PO_4, 0.125 g of $MgSO_4 \cdot 7H_2O$, and 0.0025 g of $FeSO_4 \cdot 7H_2O$) or 50 mL ISP2 medium (2 g of agar, 0.4 g of yeast extract, 1 g of malt extract, and 0.4 g of glucose) (Figures 3A and 3B) and incubated for 1–2 weeks. After this period, 1.5 g of Amberlite XAD-16 resin was added to each flask, and the cultures were incubated for an additional week. The cultures were first centrifuged to separate the biomass and resin, which were then extracted twice using a methanol/acetone solution at a 1:1 ratio (v/v). The organic layer of the extracts was then dried using a rotary evaporator.

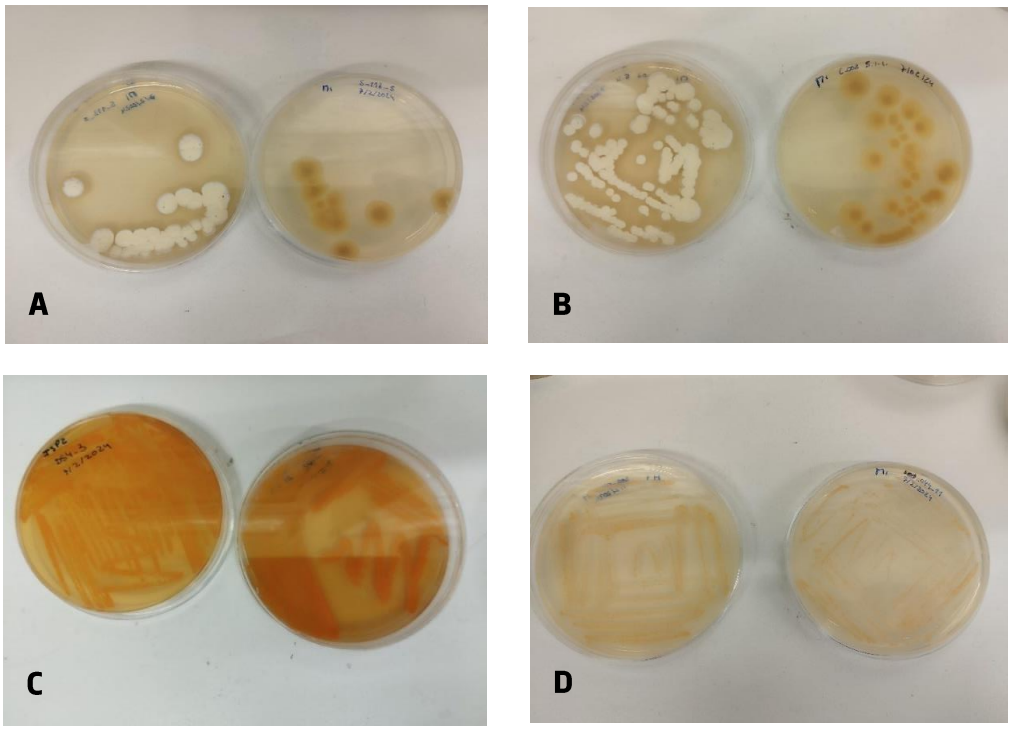

Figure 2 - Cultures of Actinomycetota prepared: 2A – Strain S_113 5, 2B – Strain C_002 5.1.1, 2C – Strain DS4_3, and 2D – Strain SED_044 11.

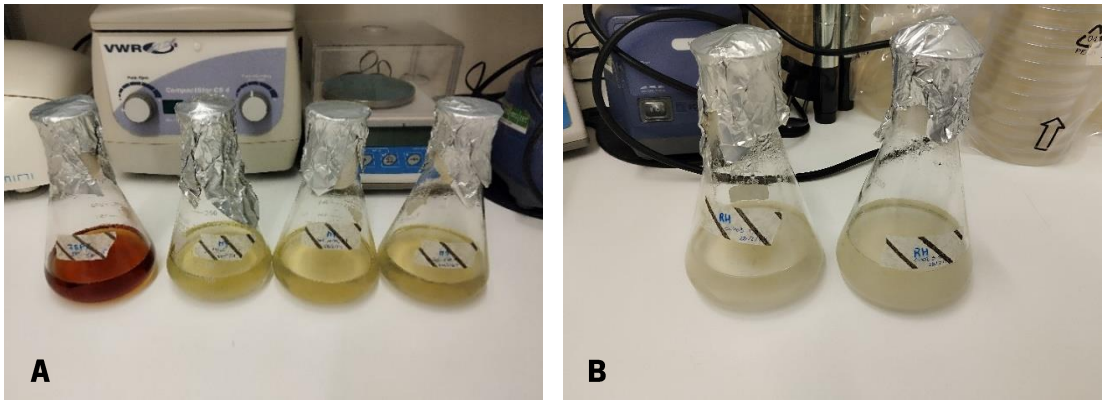

Figure 3 – Isolates and their respective medium.

2.2. AChE and BuChE Inhibition assay

The inhibition assay of AChE and BuChE was based on Ellman's protocol (60), with some modifications (61,62). In each well of the 96-well microplate, 25 µL of sample (extracts dissolved in buffer A -50 mM Tris-HCl, pH 8 - with 10% of methanol); 125 µL of 3 mM DTNB solution in buffer C (50 mM Tris-HCl, pH 8, containing 0.1 M NaCl and 0.02 M MgCl2.6H20); 50 µL of buffer B (50 mM Tris-HCl, pH 8, containing 0.1% of bovine serum albumin); 25 µL of 15 mM ATCI (for AChE inhibition) or BTCI (for BuChE inhibition) water solution; and 25 µL of enzyme (AChE or BuChE, at 0.44 U and 0.40 U, respectively) were added (Figure 4). The negative controls contained all the mentioned above, with the samples being replaced by 25 µL of buffer A and the blanks were prepared by substituting the enzyme by buffer B.

The absorbance was then read at 405 nm, at 37°C, for 2 minutes and the values for the inhibition percentage were calculated according to the following formula:

$$\%\ inhibition = \frac{(slope_{control-blank\ of\ control}) - (slope_{sample-blank\ of\ sample})}{(slope_{control-blank\ of\ control})} \times 100$$

After the reading, a yellow-coloured product was obtained, as seen in Figure 5.

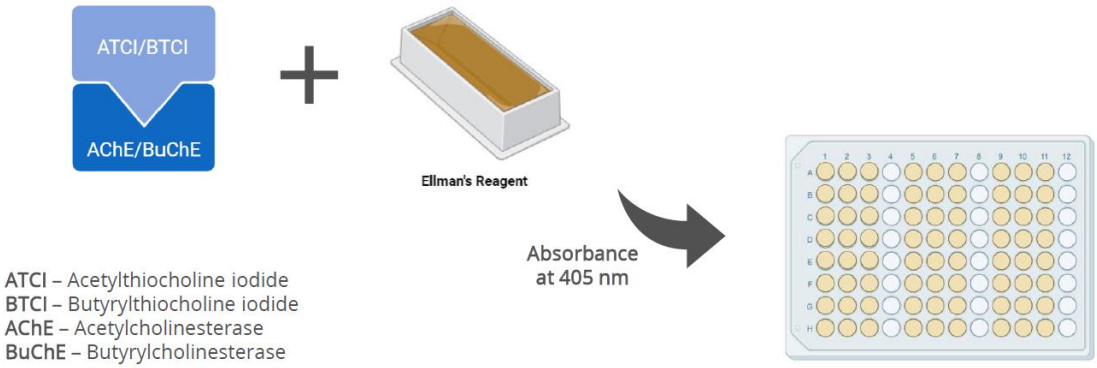

Figure 4 – Scheme of the AChE and BuChE assays

Data was reported as IC_{50} values (mg/mL).

To decide which organic solvent should be used to dissolve crude Actinomycetota extracts, different organic solvents (DMSO), methanol and ethanol) were tested at concentrations of 1%, 10%, 20%, 50% and 100%. The AChE and BuChE assay were then tested using sample with 10% of methanol (see Appendices 2 and 3).

Crude Actinomycetota extracts were then dissolved in buffer A with 10% methanol, at a concentration of 2 mg/mL (in each well). After testing these extracts, 6 were selected to continue the studies. Serial dilutions of 150 µL were performed so that a Dose-Response curve could be made.

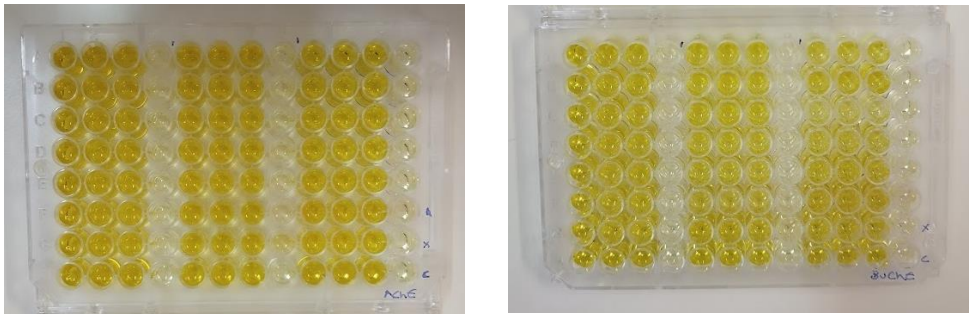

Figure 5 – Microplates after the reading of AChE (A) and BuChE (B) assays.

2.3. Tyrosinase Inhibition Assay

The inhibition assay of tyrosinase was adapted from Masuda protocol (63). A phosphate buffer (1/15 M) with pH 6.8 was prepared, alongside with the solution of L-DOPA (2.5 mM) in the same buffer. A 46 U/mL solution of tyrosinase was prepared using the same buffer and then adjusted to achieve an absorbance value of 0.2. Using a 96-well microplate, in

each well, 40 µL of sample, 80 µL of phosphate buffer and 40 µL of tyrosinase were added and incubated for 10 minutes at room temperature. After this period, 40 µL of L-DOPA were added, except on the blanks, where instead were added 40 µL of buffer. Negative controls were prepared by replacing samples by buffer. After 10 minutes, the absorbance was then read at 475 nm at room temperature. The scheme from Figure 6 illustrates this procedure. The percentage of inhibition of tyrosinase was calculated using the formula:

$$\%\ inhibition = \frac{(Abs_{control-blank\ of\ control}) - (Abs_{sample-blank\ of\ sample})}{(Abs_{control-blank\ of\ control})} \times 100$$

Results were expressed as IC_{50} values (mg/mL).

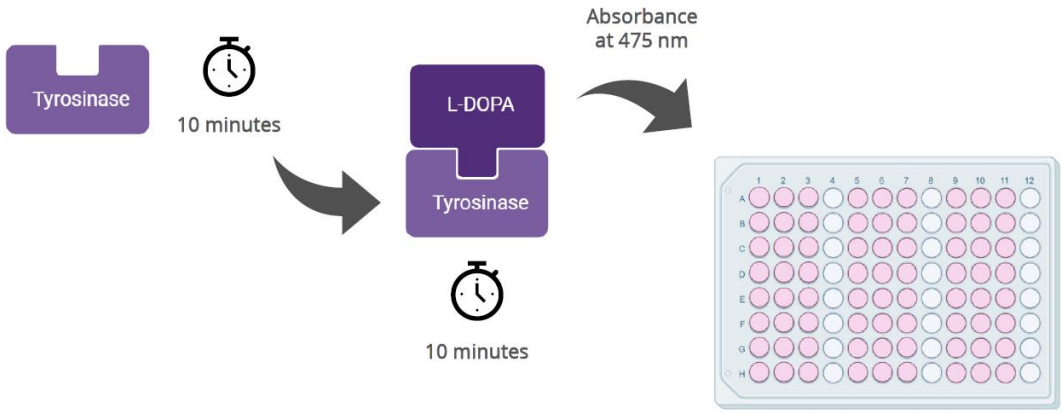

Figure 6 - Scheme of the Tyrosinase assay

After testing the influence of different organic solvents (DMSO, methanol and ethanol at concentrations of 1%, 10%, 20%, 50% and 100%), the selected solvent to dissolve the samples was 5% DMSO (see Appendix 4).

The 6 selected Actinomycetota extracts were tested for their tyrosinase inhibition. The extracts were dissolved in phosphate buffer with 5% DMSO, at a concentration of 2 mg/mL (in each well). To assess the tyrosinase inhibition capacity in different concentrations, serial dilutions were performed with these extracts. The visual representation of the microplate with the samples can be seen in Figure 7.

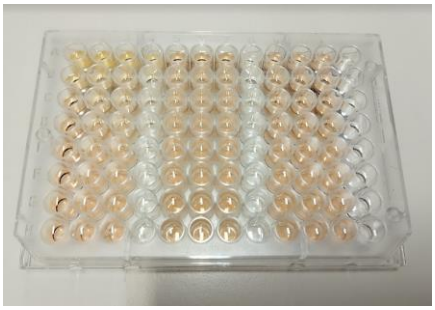

Figure 7 – Microplate after the reading of the tyrosinase assay.

2.4. Antiradical activity assay

Superoxide anion radical was generated by the NADH (nicotinamide adenine dinucleotide + hydrogen)/PMS (phenazine methosulfate) system. This radical reacts with nitroblue tetrazolium chloride (NBT), reducing it to formazan, a blue compound that has a maximum absorption at 560 nm. In the presence of a radical scavenger, formazan is not produced.

Six selected Actinomycetota extracts were tested for their antioxidant activity, based on the scavenging of the superoxide radical. The extracts were dissolved in buffer phosphate with 10% methanol, at a concentration of 2 mg/mL in each well. In each well, 50 µL of samples dissolved in phosphate buffer with 10% of methanol, 50 µL of NADH (in phosphate buffer), 150 µL of NBT (in phosphate buffer), and 50 µL of PMS (in phosphate buffer) were added (Figures 8 and 9). The assay was then conducted at room temperature, for two minutes at 560 nm.

In order to obtain dose-response curves, serial dilutions were performed from these extracts and tested in the bioassay.

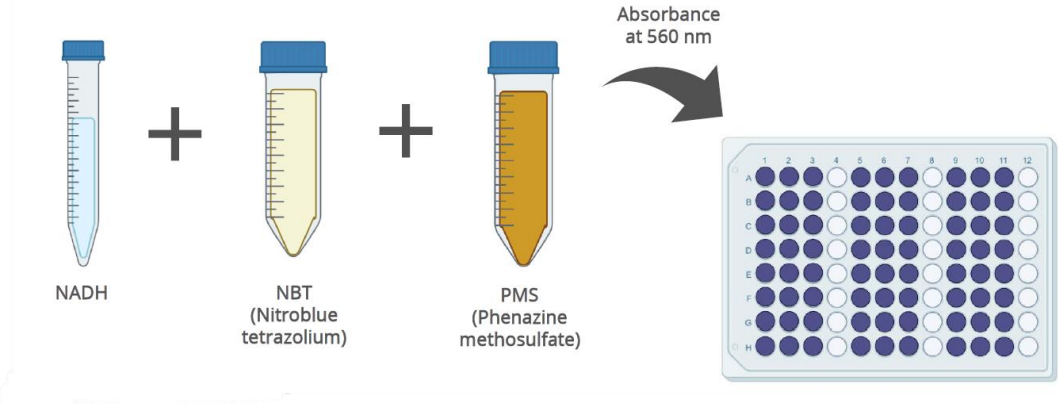

Figure 8 – Scheme of the antioxidant activity assay

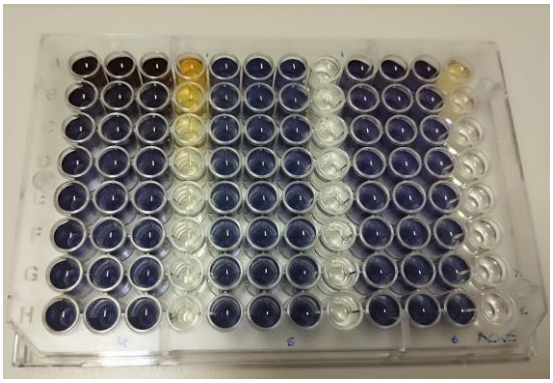

Figure 9 – Microplate reading after the superoxide scavenging assay.

2.5. Cell lines and culture

Cells were cultured in DMEM Glutamax medium (Dulbecco's Modified Eagle Medium with Glutamine – Gibco, Germany) supplemented with 10% (v/v) fetal bovine serum (Gibco, USA), 0.1% Amphotericin B (Gibco, Germany), and 1% penicillin-streptomycin (Pen-Strep 100 IU/ml and 10 mg/ml, respectively) (Gibco, Germany). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO2. The culture medium was replaced every two days. When the cells hit 80-90% confluence a cell passage was performed. Brefly the previous medium was removed and cells were washed with 2 mL of warm phosphate-buffered saline (PBS) (Gibco, Germany). Then cells were detached form the flasks by adding 1 mL of TrypLE express enzyme (1x) (Gibco, Denmark) and incubated for 3-5 mins. After incubation, 4 mL of medium was added to inactivate the enzyme. The cell suspension was then transferred to a Falcon tube and centrifuged for 5 min at 1200 rpm. The medium was removed, and the pellet was resuspended in 1 mL of fresh medium. 50-100 μL of cell suspension were transferred to new culture flasks containing medium (25 cm2 with 4mL or 75 cm2 with 10mL) and incubated as previously described. For cell counting, 20 μL of the cell suspension was mixed with 20 μL of trypan blue. The cell concentration was determined using a Neubauer Chamber and the Trypan Blue dye, which interacts with cells that have a damaged membrane, leaving the viable cells colorless.

2.6. Cytotoxicity – MTT assay

To test the cytotoxic effect of the Actinomycetota extracts, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. For the assay cells SH-SY5Y, 3T3-L1 and RKO, were seeded in 96-well culture plates, in quadruplicates and at a density of 1,5 x 10^5 cells/mL, 3,3 x 10^4 cells/mL and 3,6 x 10^4 cells/mL (see Appendix 5),

respectively. density of 1,5 x 10^5 cells/mL, 3,3 x 10^4 cells/mL and 3,6 x 10^4 cells/mL (see Appendix 5), respectively. After 24 h of incubation to allow cell adhesion, the medium was removed and substituted with new medium with extracts at a concentration of 100 µg/mL. Since extracts were dissolved in DMSO the negative control consisted in medium with 1% DMSO. For the positive control, a solution of medium with 20% DMSO was added (Figure 2).

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	2	3	4	5	6				C-	C-	C+
B	1	2	3	4	5	6				C-	C-	C+
C	1	2	3	4	5	6				C-	C-	C+
D	1	2	3	4	5	6				C-	C-	C+
E												
F												
G												
H												

Figure 10 – Microplate scheme of MTT assay. Numbers 1 to 6 correspond to the Actinomycetota extracts, C- represents the negative control (1% DMSO) and C+ represents the positive control (20%DMSO).

After 24h and 48h of incubation, 20 µL of a 1 mg/mL MTT solution was added and incubated for an additional 3 hours. After incubation the medium was removed and 100 µL of DMSO were added (Figure 11). Absorbance was measured at 562 nm using a UK EZ Read 800 Plus microplate reader running Galapagos Expert (version 1.1.2.0) software. Cell viability was calculated as the percentage of MTT reduction compared with the negative control by considering 100% viability in the negative control. Three independent experiments were performed.

$$Cell\ viability\ (\%) = \frac{Abs_{fraction}}{X_{abs\ negative\ control}} \times 100$$

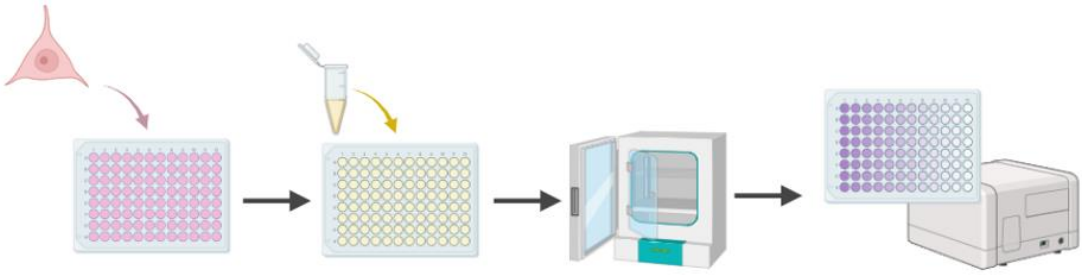

Figure 11 - Scheme of the MTT assay.

2.7. Statistical Analysis

Statistical analysis was conducted with GraphPad PRISM software (GraphPad® software, CA, USA) (Version 8 for Windows). The data were expressed as mean ± SD. Normality and variance homogeneity were evaluated using Kolmogorov–Smirnov and Levene's tests. Cytotoxicity data underwent a two-way ANOVA followed by Tukey's HSD multiple comparisons test.

3. Results and Discussion

3.1. AChE and BuChE inhibition

As it was mentioned before, the enzymes AChE and BuChE have an important role in AD pathology, since they are part of the cholinergic system by participating in the hydrolysis of ACh. As the levels of ACh in AD tend to decrease, inhibiting the breakdown of this neurotransmitter through the inhibition of the activity of cholinesterase enzymes is a crucial treatment for this NDs. Currently, there are three cholinesterase inhibitors in the market: donepezil (36), rivastigmine (also able to inhibit BuChE) (37), and galantamine (39) . Therefore, finding Actinomycetota extracts with some inhibitory potential over these enzymes could be of significant importance.

The procedure used was an adaptation of the colorimetric method by Ellman, which is a simple, accurate, and fieldable method. Nonetheless, this method still has some drawbacks, such as spontaneous hydrolysis of thioesters at high concentration and some colour interferences (64). Ellman's method is based on the measurement of the release of

thiocholine during the process of ACh hydrolysis. The number of free thiocholine molecules is quantified spectrophotometrically by the reaction with 5,5-bisdithionitrobenzoic acid (DTNB). This reaction leads to a yellow product – 5-thio-2-nitrobenzoate anion (TNB^{2-}) – with an absorption maximum at 405-412 nm (59).

Twenty Actinomycetota extracts were tested for their potential to inhibit AChE and BuChE at a concentration of 2 mg/mL, and we selected the extracts that showed a higher potential to inhibit AChE and BuChE - SED_044 11, SED_058 9, C_003 1.3, DS4_3, C_002 5.1.1, and S_113 5 - as seen in colour in Figures 12A and 12B.

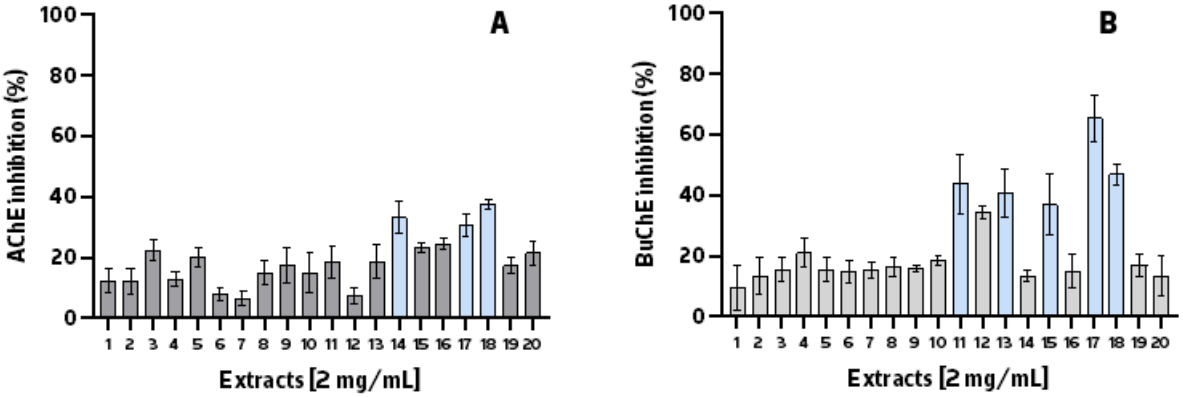

Figure 12 - AChE (A) and BuChE (B) percentage of inhibition of the 20 Actinomycetota extracts tested in this study. Values are expressed as mean ± SD, in triplicate.

These extracts showed some degree of inhibition of AChE, as seen in Figure 13A, with the higher percentage registered for extract DS4_3 with 38% with a standard deviation of ±2.5%. For the inhibition of BuChE (Figure 13B), the percentage was higher (all above 30%) except for DS4_3, with the highest being from SED_58 9 at 71.7% ±10.861%.

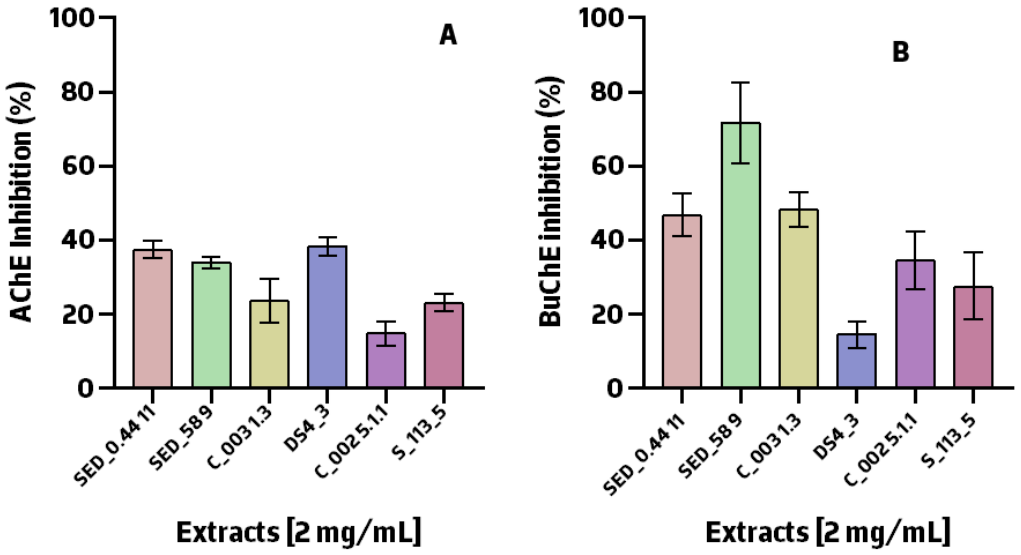

Figure 13 – Percentage of inhibition of AChE (A) and BuChE (B) for the 6 selected Actinomycetota extracts. Values are expressed as mean ± SD, in triplicate.

In the dose-response curves of the 6 selected extracts, both for AChE and BuChE, it can be seen that the percentage of inhibition increases as the concentration of the extracts increase as well (Figure 14). This demonstrates a concentration-dependent inhibition by the extracts against both enzymes.

For AChE inhibition (Figures 14A and 14B), the percentage starts increasing at the concentration of 0.5 mg/mL, with the value increasing in a similar way with all of the 6 extracts.

For BuChE inhibition (Figures 14C and 14D), there were some extracts with a higher percentage of inhibition, namely SED_058.9, SED_0.44 11 and C_003 1.3.

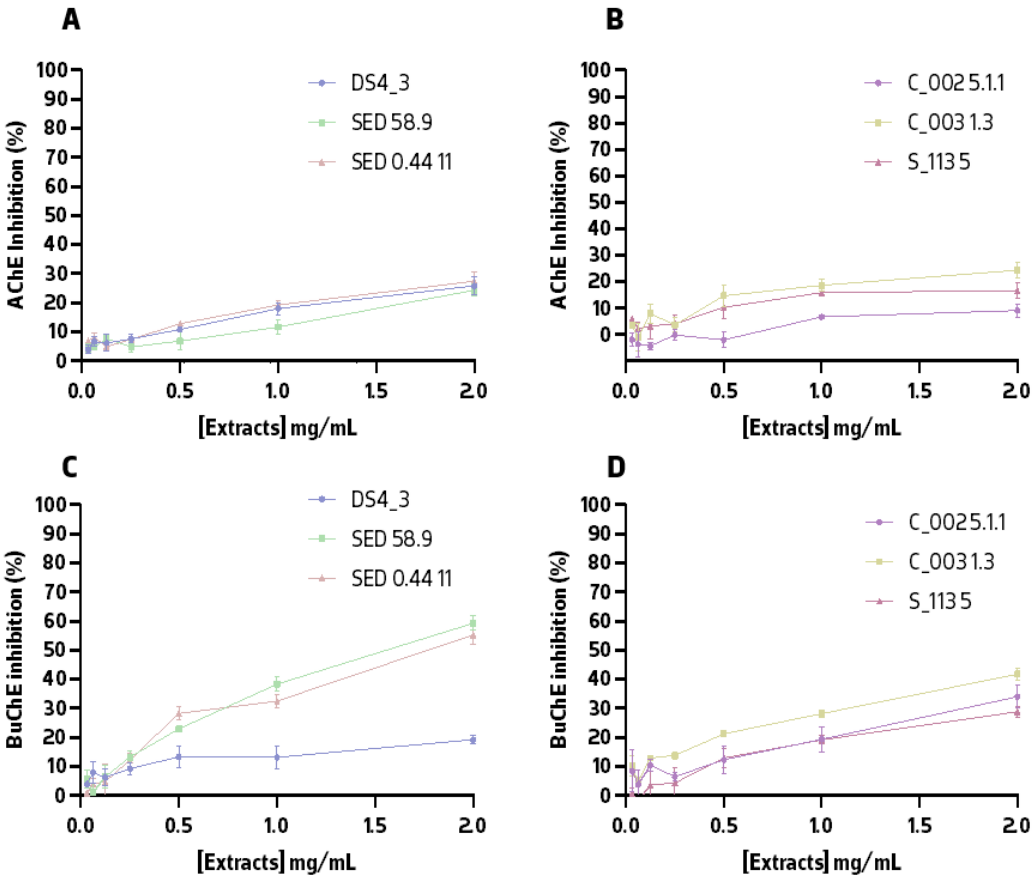

Figure 14 – Dose-Response curves, at different concentrations, of the inhibition of AChE (A,B) and BuChE (C,D). Values are expressed as mean ± SD, in triplicate.

According to Vinutha et al. (67), a potent percentage of AChE inhibition is above 50%; a moderate one is between 30–50%; and a low inhibition corresponds to percentages below 30%. Taking into consideration the drugs currently available for the treatment of AD, a good selectivity of the compounds for the brain cholinergic system corresponds to a percentage of inhibition above 50%. Rivastigmine, one of the treatment drug available, at a concentration of 12 mg/day, has an AChE inhibition percentage of 61,7% and BuChE inhibition of 61,8%, being considered potent (28).

The extracts with the most interesting results were SED_0.44 11 from the strain *Gordonia sputi*, SED_58 9 from *Rhodococcus cercidiphylli*, C_003 1.3 from *Streptomyces* sp., and DS4_3 from *Rhodococcus cerastii*. *Gordonia* is a genus that has shown diverse bioactive

potential, with several strains exhibiting antimicrobial and cytotoxic effects (68). The *Rhodococcus* genus has recently been reported to produce novel siderophores and new antibiotics (69). Amongst these different Actinomycetota genus, *Streptomyces* remains as the biggest producer of secondary metabolites with antifungal, anticancer, antibacterial, antioxidant activities, and with the potential to inhibit enzymes related to AD pathology, such as AChE and BuChE (70,71). Although not as many as terrestrial, some Actinomycetota from marine environments have shown to have inhibition potential against AChE, such as marine derived *Streptomyces* sp. UTMC 1334 (55).

The AChE and BuChE inhibitory assay showed that these Actinomycetota extracts have some degree of enzyme–inhibitory properties, relatively to AChE, and a high inhibitory percentage of BuChE. Nevertheless, fractionating these extracts in a HPLC and checking their IC_{50} value will be important to perform in the future. These findings, alongside other published results from previous research, are very important in the AD treatment field, since these two enzymes are connected to the cholinergic system which is affected by this disease.

3.2. Tyrosinase inhibition

Tyrosinase plays an active role in numerous NDs, including AD, since this enzyme can damage the neurons by producing dopamine quinones that oxidize with L-DOPA and release a reactive compound named dopaquinone. High levels of this enzyme can cause neurotoxicity and inflammatory reactions (31,32).

The tyrosinase inhibitory activity of the selected 6 Actinomycetota extracts was evaluated by using the substrate L-DOPA.

The Actinomycetota extracts, mainly SED_0.44 11 from *Gordonia sputi* (with a tyrosinase inhibition of 65,5%), SED_58 9 from *Rhodococcus cercidiphylli* (tyrosinase inhibition of 79,6%), DS4_3 from *Rhodococcus cerastii* (tyrosinase inhibition of 80,5%) and S_113_5 from *Nocardiopsis lucentensis* (tyrosinase inhibition of 83%), showed a significant inhibition of tyrosinase at a concentration of 2 mg/mL, as seen on Figure 15.

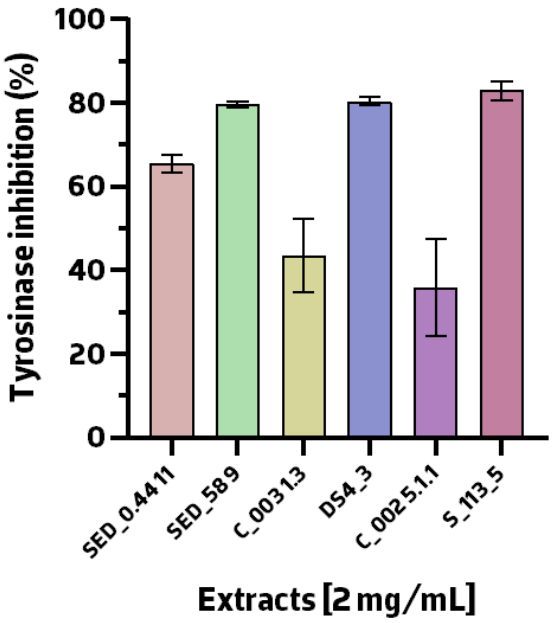

Figure 15 - Percentage of inhibition of tyrosinase for the 6 selected Actinomycetota extracts. Values are expressed as mean ± SD, in triplicate.

In the dose-response curves of the six extracts, we can see that the percentage of inhibition increases as the concentration of the extracts increase as well (Figure 16). This demonstrates a concentration-dependent inhibition of the enzyme by the extracts.

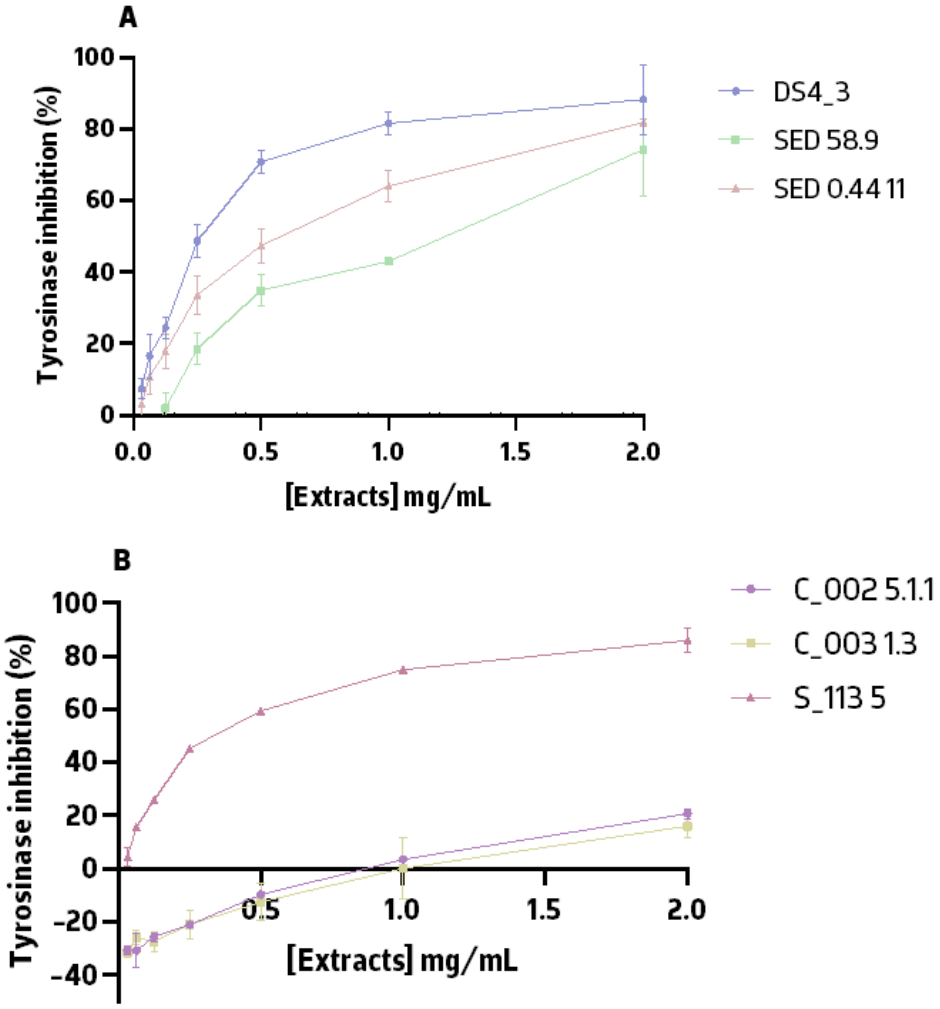

Figure 16 – Dose-Response curves, at different concentrations, of the inhibition of tyrosinase. Values are expressed as mean ± SD, in triplicate.

During the last few years, more and more research has been performed to discover potential novel tyrosinase inhibitors. Baber et al. (72) conducted a comprehensive review of tyrosinase inhibitors reported in the period between 2003 and 2023, and found that there was a compound named trichostatin A from *Streptomyces* sp. CA-129531 (72,73). An exopolysaccharide from *Streptomyces* sp. NRCG4 also showed to have potential against tyrosinase, with the highest percentage of inhibition being 98.59% at a concentration of 400 μg/mL, with an IC_{50} of 17.63 μg/mL (49). Although the genus *Streptomyces* is the most frequently cited genus in the literature for tyrosinase inhibitors (73), in our study, the extracts with the highest inhibitory activity were from the genera *Gordonia*, *Rhodococcus* and *Nocardiopsis,* which have no prior literature associated with this activity. Therefore, it

is important to explore deeper these extracts, namely, to evaluate their IC_{50} values and compare them with potent tyrosinase inhibitors such as kojic acid and analyse the chemical composition of these Actinomycetota fractions with mass spectrometry. Moreover, this assay demonstrated that these extracts have a high inhibitory potential against tyrosinase.

3.3. Antioxidant activity

As mentioned earlier, oxidative stress is one of the causes of the pathogenesis of AD, having a crucial role in the activity of AD, progressive damage of cellular components, and the activation of programmed cell death mechanisms (74,75). Oxidative stress is a condition where elevated levels of ROS can cause damage to the cell organelles (76). Donepezil, memantine, and rivastigmine (3 out of 8 FDA approved treatment drugs for AD), have antioxidant properties to counteract the oxidative stress (76).

The superoxide scavenging assay is based on NBT reduction velocity. The superoxide radical is produced by the NADH/PMS system, in which after the NADH reduction, the reduced PMS reacts with O_2 and produces the superoxide radical $O_2^{\bullet -}$. After this, the superoxide reacts with NBT and reduces it to formazan, a blue compound. Any molecule that scavenges this radical will lead to a decrease of NBT's reduction velocity (77,78).

As seen in Figure 17, the extract with the most interesting results is S_113_5 from *Nocardiopsis lucentensis*, with a percentage of superoxide scavenging of 53.7% at 2 mg/mL.

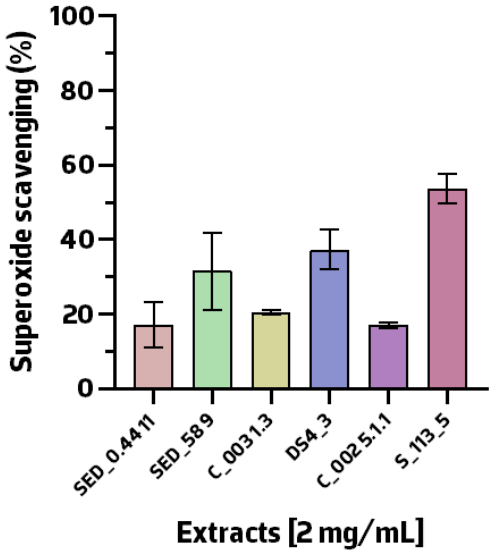

Figure 17 – Percentage of superoxide scavenging of the Actinomycetota extracts. Values are expressed as mean ± SD of triplicates.

Interestingly, the Actinomycetota species *Nocardiopsis lucentensis* has shown antioxidant potential, alongside anticancer, antimicrobial, and antibiofilm properties in some studies (79–81).

In the dose-response curves of all extracts (Figure 18), the superoxide scavenging percentage does not increase with increasing concentration of the extracts. This demonstrates that it does not exist a concentration-dependent relation between the antioxidant activity and the Actinomycetota extracts. This variation in results across different concentrations may be attributed to the relatively low superoxide scavenging of the extracts. The highest level of scavenging was observed at the highest tested concentration of 2 mg/mL.

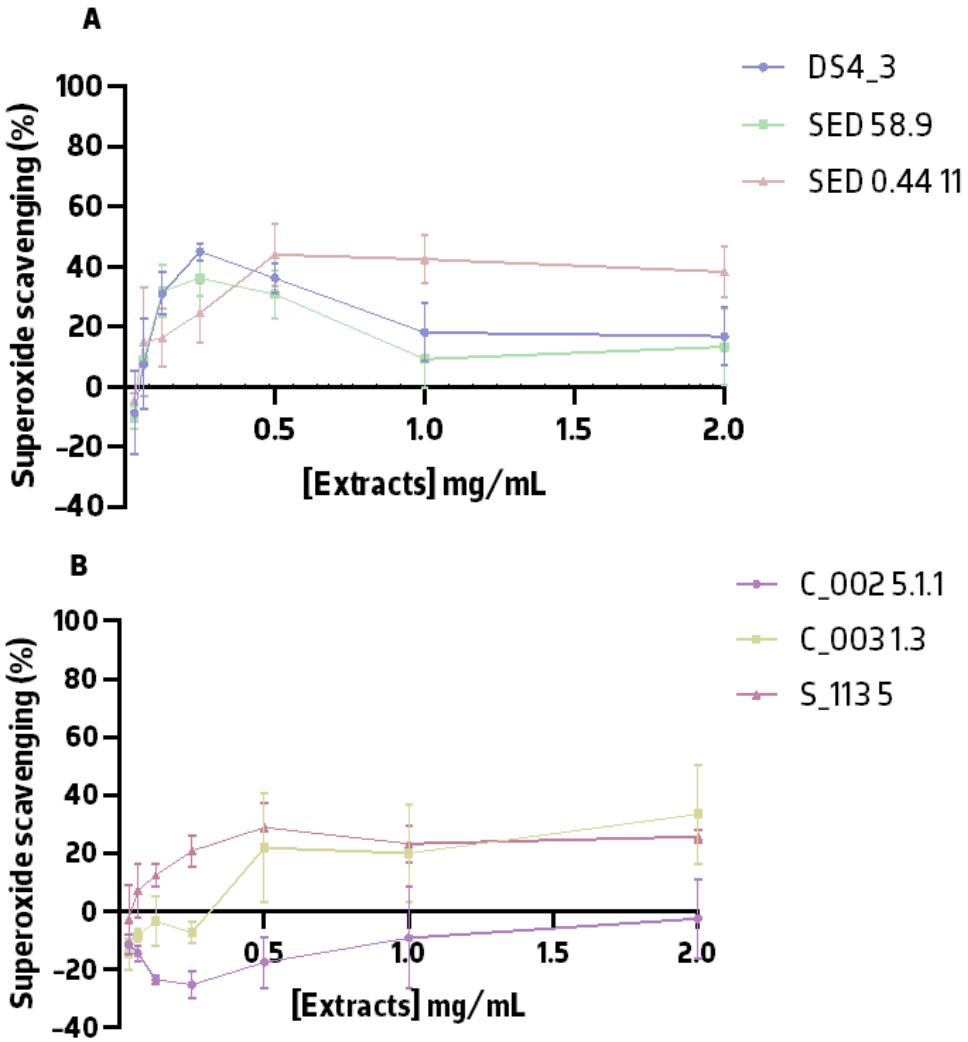

Figure 18 – Dose-Response curves, at different concentrations, of the superoxide scavenging. Values are expressed as mean ± SD, in triplicate.

Taking these results into consideration, further assays on the extract S_113 5 could be of major interest.

3.4. Cytotoxicity

Cytotoxicity is a crucial factor in assessing the biological effects of compounds in *in-vitro* studies. Cytotoxicity and cell viability assays are based on numerous cell functions and the viability levels of cells are good indicators of cell health (82). The MTT assay determines cell viability through determination of mitochondrial function of cells by measuring activity of mitochondrial dehydrogenases. This method is simple, safe, has a high reproducibility, and is used to determine both cell viability and cytotoxicity. However, it needs an organic solvent such as DMSO since it is insoluble in water (82).

The human neuroblastoma cell line SH-SY5Y has been widely used as an *in vitro* model for neurodegenerative diseases. This is due to the fact that its non-differentiated form resembles immature catecholaminergic neurons, as it exhibits a correct neurite structure and expresses immature neuronal markers (78, 79). The 3TE-L1 cell line consists of a fibroblast cell line from disaggregated Swiss albino mouse embryos. Recently, some questions on how fibroblasts could be connected to neurodegeneration have emerged and some studies suggest that fibroblasts play active roles in the central nervous system development, neuroinflammation, aging, and injury (85,86). Another cell line that was used was the human carcinoma RKO cell line from the colon, not as a principal characteristic in AD, but more to know if these extracts could also have as an anti-cancer potential. The cells were exposed with the different extracts of Actinomycetota for 24 and 48 hours. If the cell viability was below 70%, then the extract was considered toxic. The findings below show cell viability percentages compared to the negative control (1% DMSO), where 100% viability is indicated. As a comparison, the positive control included 20% DMSO, known for its cytotoxic effects, leading to cell death.

The results of SH-SY5Y cells viability can be seen in Figure 19. Amongst the 6 extracts, two showed a higher toxicity, with a cell viability below 50% at 48 h, namely extracts C_003 1.3 and DS4_3. The rest of the extracts had a cell viability above 70%, showing no sign of toxicity. Contrary to the previous results, on the 3T3-L1 cell line (Figure 20), none of the extracts showed a high level of toxicity. Considering the cytotoxicity towards the

neuroblastoma cell line SH-SY5Y and no cytotoxic effects against the normal fibroblasts 3T3-L1, it was decided to extend the evaluation of cytotoxicity of the extracts to another cancer cell line, in this case the colon adenocarcinoma cell line RKO. Regarding the RKO cell line, most of the extracts were deemed toxic, since the cell viability was below 70%, as seen in Figure 21. These results could be meaningful as an anticancer property. The values and respective standard deviation are listed below in Tables 3 to 5.

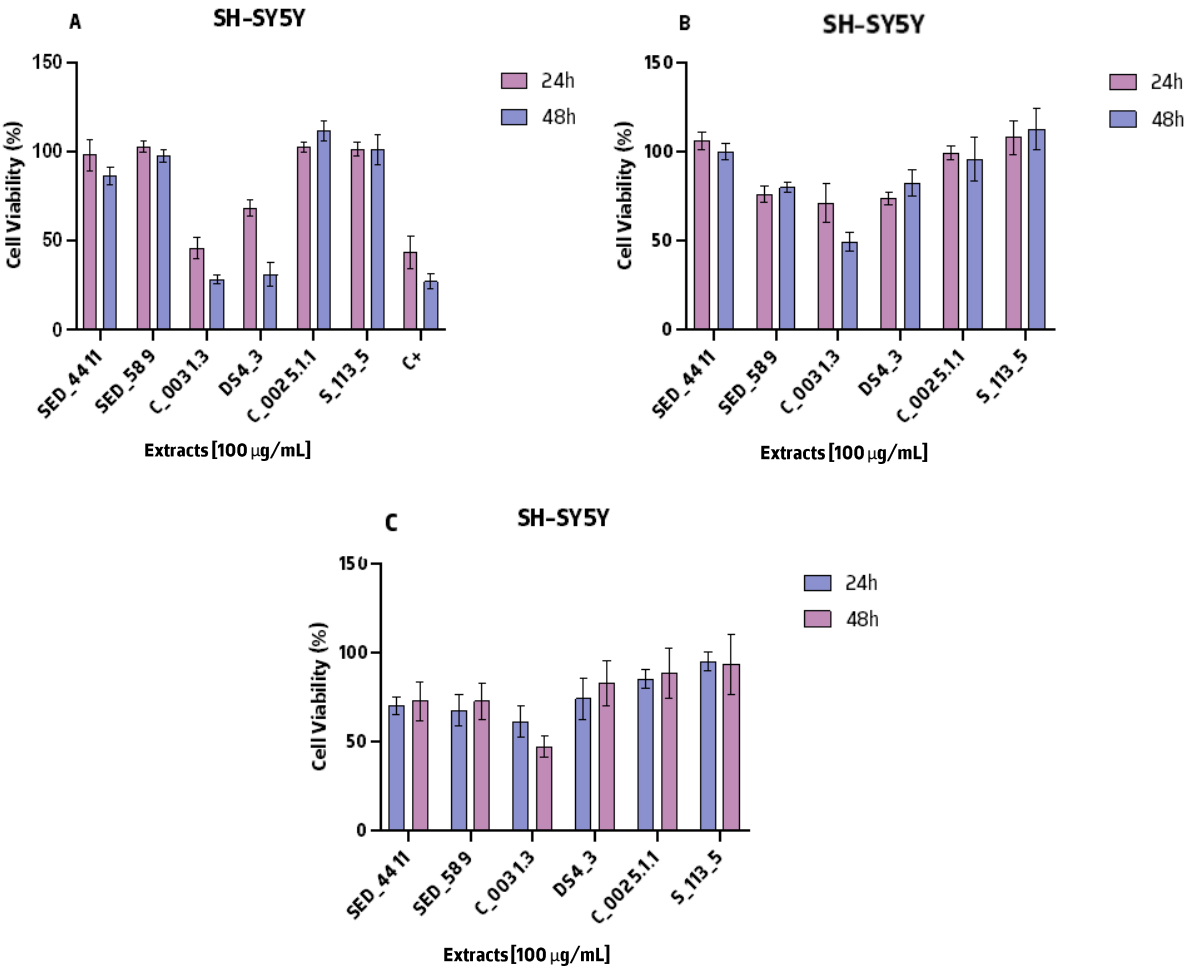

Figure 19 - Viability of the human neuroblastoma SH-SY5Y cell line after exposure to the 6 selected Actinomycetota extracts. Three independent experiments were performed (A, B, C)

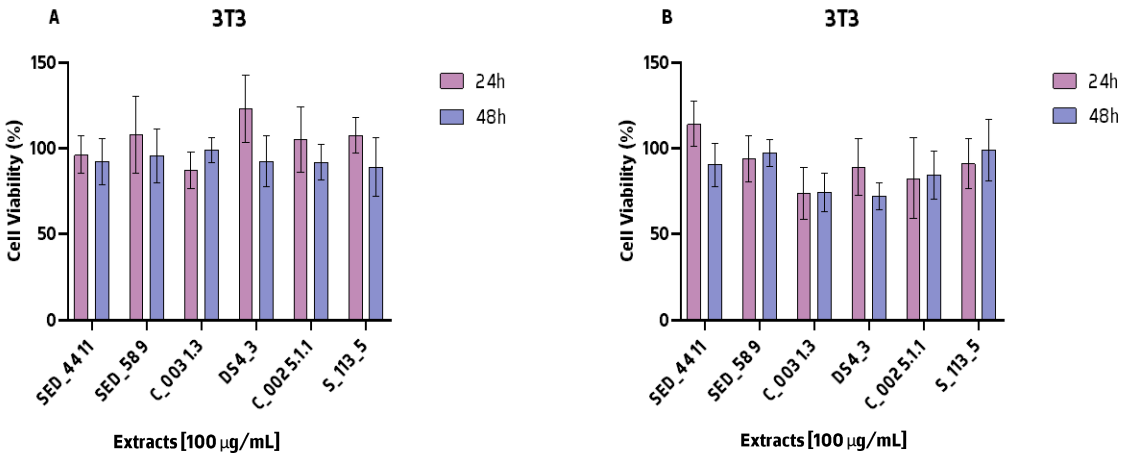

Figure 20 – Viability of Swiss albino mice fibroblast 3T3-L1 cell line after exposure to the 6 selected Actinomycetota extracts. Two independent experiments were performed (A and B).

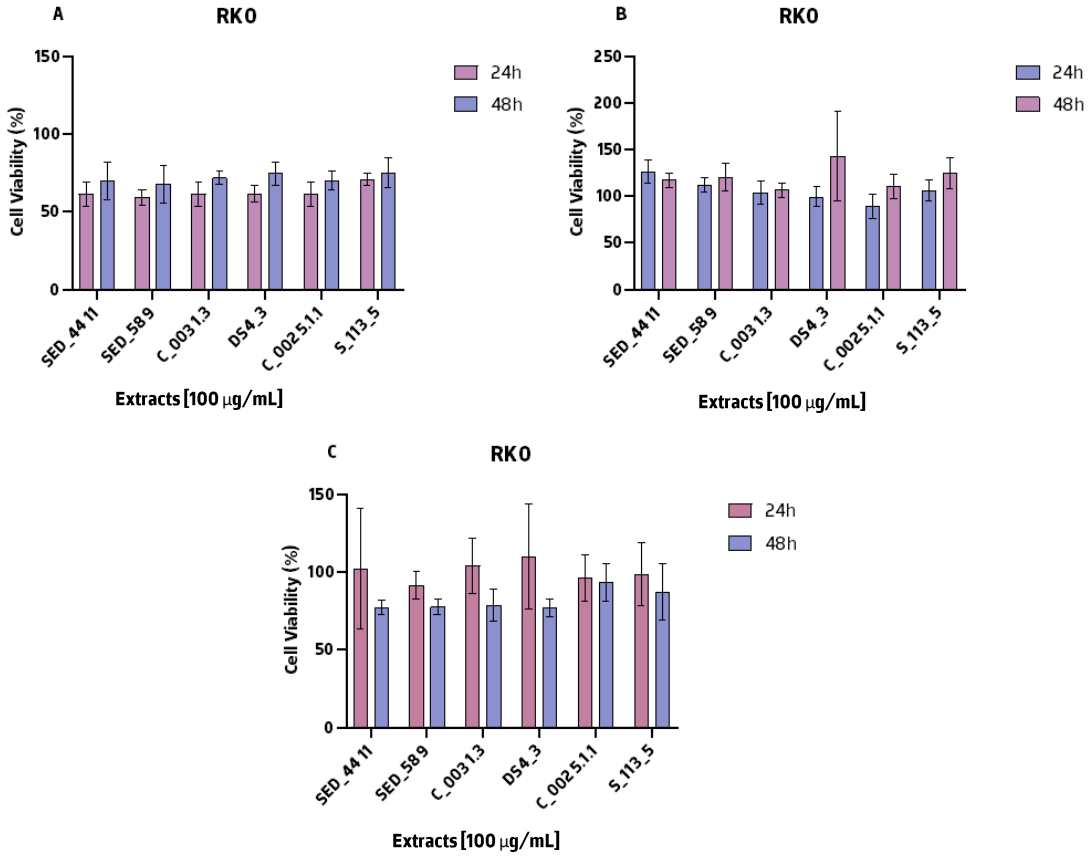

Figure 21 – Viability of the human colon carcinoma RKO cell line after exposure to the 6 selected Actinomycetota extracts. Three independent experiments were performed (A, B and C).

Two extracts – C_003 1.3 and DS4_3 – showed selective toxicity towards the cell lines SH-SY5Y and RKO, but not towards 3T3-L1 cell lines. The toxicity observed in the SH-SY5Y neuroblastoma cell line suggests potential neurotoxicity of the Actinomycetota extracts. This effect might be linked to the disruption of specific signalling pathways involved in cell proliferation, which are often dysregulated in neurodegenerative diseases, such as AD. On the other hand, the lack of toxicity towards 3T3-L1 cells, a model of healthy cells, demonstrates the safety of these extracts, since they can be effective without harming healthy and non-neuronal cells. It is important, however, to note that the independent experiments involving cytotoxicity still need the respective statistic treatment. Also dose response assays to confirm cytotoxic results must be performed.

Future research is still necessary to further explain these findings and its clinical and therapeutic potential in AD's treatment.

Table 3 - Cell viability (%) between 24h and 48h of each extract, in SH-SY5Y cell line, with the respective standard deviation (±SD). SH-SY5Y A corresponds to the values of Figure 19A, SH-SY5Y B corresponds to the values of Figure 19B, and SH-SY5Y C corresponds to the values of Figure 19C.

Extracts	SH-SY5Y A		SH-SY5Y B	
	24h	48h	24h	48h
SED_44 11	98.1±8.8	86.4±4.8	106.3±5.1	100.4±4.7
SED_58 9	102.6±3.2	97.3±3.4	76.3±4.6	80.1±2.8
C_003 1.3	45.7±5.8	28.1±2.5	71.5±11.2	49.6±5.2
DS4_3	68.4±4.8	30.9±6.7	74.1±3.6	82.5±7.5
C_002 5.1.1	102.4±3.0	111.5±5.9	99.6±3.9	96.1±12.6
S_113_5	101.3±3.8	100.8±8.4	108.3±9.5	113.0±12.0

Extracts	SH-SY5Y C	
	24h	48h
SED_44 11	70.5±5.1	73.1±10.9
SED_58 9	68.0±8.8	73.0±10.1
C_003 1.3	61.6±9.0	47.3±6.0
DS4_3	74.4±11.8	83.3±12.8
C_002 5.1.1	85.4±5.5	89.0±14.1
S_113_5	95.2±5.2	93.7±16.6

Table 4 –. Cell viability (%) between 24h and 48h of each extract, in 3T3-L1 cell line, with the respective standard deviation (±SD). 3T3-L1 A corresponds to the values of Figure 20 and 3T3-L1 B corresponds to the values of Figure 20B.

Extracts	3T3-L1 A		3T3-L1 B	
	24h	48h	24h	48h
SED_44 11	96.4±10.9	92.5±13.5	114.4±13.2	90.5±12.4
SED_58 9	108.2±22.4	95.9±15.8	94.1±13.5	97.3±8.0
C_003 1.3	87.4±10.5	99.3±7.4	74.0±15.1	74.5±11.4
DS4_3	123.2±19.5	92.4±14.8	89.2±16.4	72.1±7.9
C_002 5.1.1	105.3±19.1	92.1±10.5	82.7±23.5	84.6±14.2
S_113_5	107.9±10.4	89.3±17.4	91.2±14.8	99.4±18.0

Table 5 – Cell viability (%) between 24h and 48h of each extract, in RKO cell line, with the respective standard deviation (±SD). RKO A corresponds to the values of Figure 21A, RKO B corresponds to the values of Figure 21B, and RKO C corresponds to the values of Figure 21C.

Extracts	RKO A		RKO B	
	24h	48h	24h	48h
SED_44 11	61.5±7.7	69.9±12.3	126.5±12.5	117.2±7.5
SED_58 9	59.3±4.9	67.8±12.2	112.0±7.8	120.7±14.7
C_003 1.3	61.5±7.9	71.8±4.2	104.1±12.5	106.6±7.7
DS4_3	61.4±5.3	74.7±7.5	99.3±10.6	142.9±47.8
C_002 5.1.1	61.3±7.7	70.2±6.0	88.9±13.2	110.6±12.9
S_113_5	70.9±4.2	75.1±9.6	106.0±11.1	124.7±16.2

Extracts	RKO C	
	24h	48h
SED_44 11	102.1±47.8	77.1±4.5
SED_58 9	91.5±8.8	77.6±4.9
C_003 1.3	104.0±17.9	78.7±10.3
DS4_3	110.0±33.9	77.0±5.6
C_002 5.1.1	96.2±15.0	93.2±11.8
S_113_5	98.7±20.0	87.2±18.4

4. Conclusion

With AD affecting an increasing percentage of the population, there has been a growing urgency to discover novel therapeutic solutions. In this work, the anti-AD potential of marine Actinomycetota, renowned for their prolific production of secondary metabolites, was explored, as they can play a crucial role in advancing the development of new treatments for AD.

Twenty extracts of Actinomycetota strains were evaluated for their inhibitory potential against the enzymes of the cholinergic system (AChE and BuChE). The extract DS4_3 exhibited moderate inhibition of AChE, while the extract SED_58 9 showed significant inhibition of BuChE. In addition to their cholinergic activity, these extracts were assessed for their ability to inhibit tyrosinase. The extracts SED_0.44 11, SED_58 9, DS4_3, and S_113_5 demonstrated notable inhibition of tyrosinase.

Furthermore, antioxidant activity was observed, with S_113_5 showing the highest level of superoxide scavenging. However, further studies did not reveal a clear concentration-dependent relationship between the extracts and superoxide activity.

The cytotoxicity of the Actinomycetota extracts was evaluated on the neuroblastoma cell model SH-SY5Y, widely used in AD studies, and in normal 3T3-L1 fibroblasts. No toxicity was observed for the 3T3-L1 cell line, but some extracts, namely C_003 1.3 and DS4_3 were shown to have some cytotoxicity on the neuroblastoma cell line, suggesting a selective effect on cancer cells without harming the healthy cells. These results suggested a putative anticancer potential and additionally extracts were tested in the cancer cell line RKO. Results indicate that all extracts showed some level of cytotoxicity against RKO cells, indicating potential anti-cancer activity.

While these findings are promising, further studies are crucial to fully understand the therapeutic potential of these extracts. Investigating their capacity as BACE-1 inhibitors could clarify whether they can target Aβ production, a key feature in AD pathology. Moreover, exploring additional properties, such as anti-inflammatory, could enhance their potential application.

In conclusion, Actinomycetota demonstrated promising potential against Alzheimer's disease, particularly through mechanisms involving enzyme inhibition and antioxidant

activity. Further research is essential to explore the therapeutic potential of Actinomycetota more comprehensively. Future studies should focus on assessing the detailed composition of the extracts to identify the active compounds responsible for the observed bioactivities. In addition, conducting further bioassays, such as testing other key enzymes involved in AD, exploring their effects on neuronal cell viability, and investigating their potential to reduce glutamate-induced toxicity, would deepen our understanding of their mechanisms. Exploring additional Actinomycetota strains, beyond those tested, may also reveal new bioactive compounds with anti-Alzheimer properties. These steps will provide valuable insights into their potential for developing novel therapies targeting AD.

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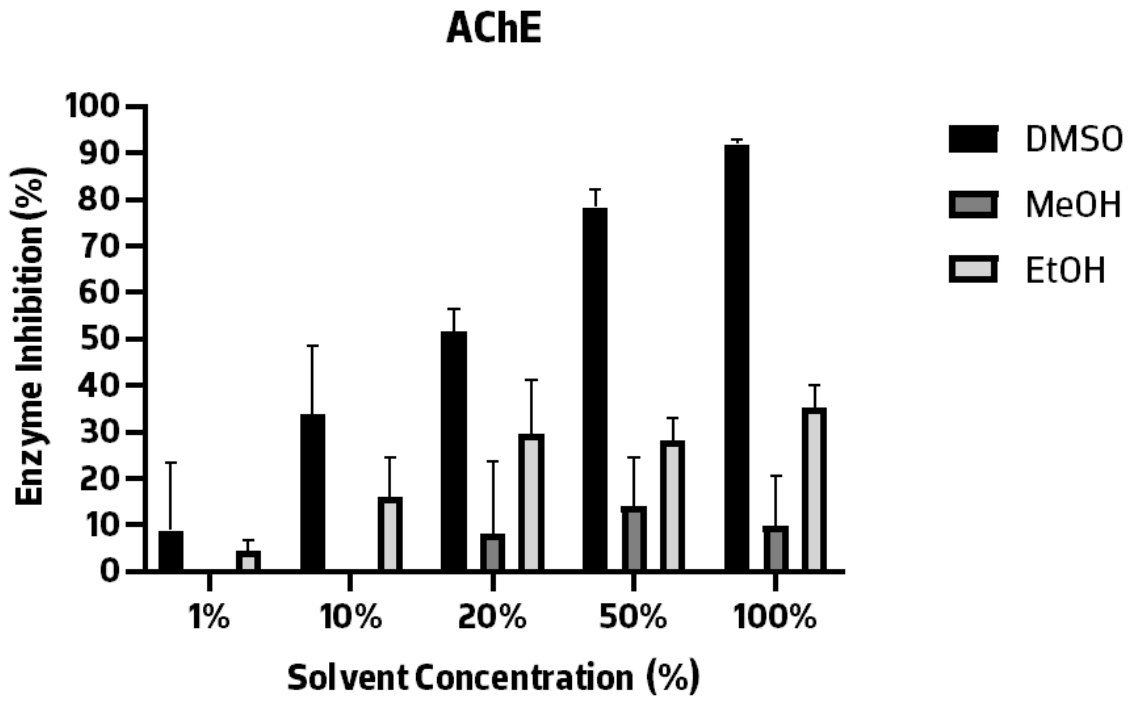
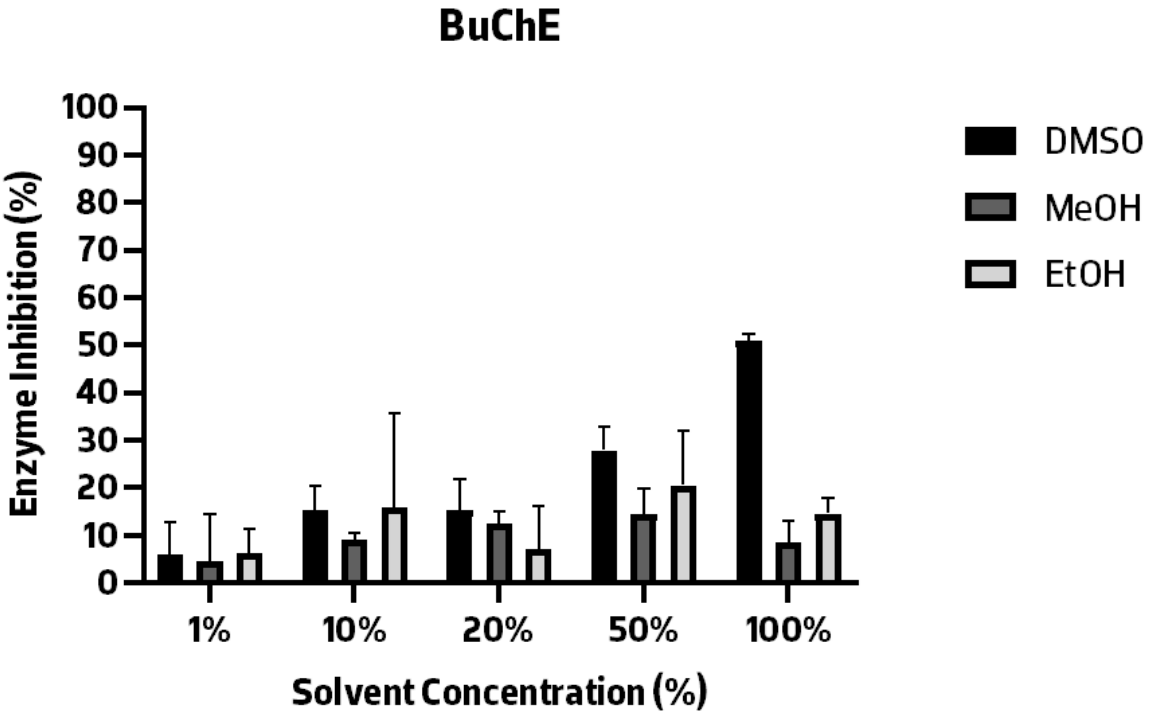
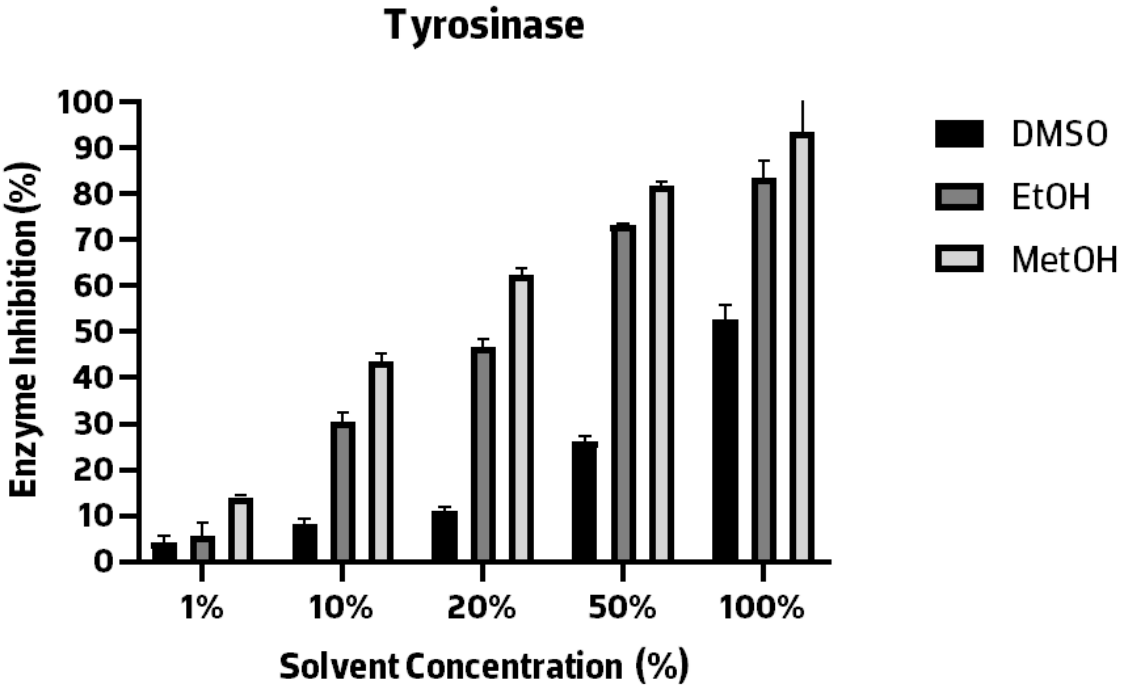
Appendices



Appendix 1 – Selection of organic solvent for AChE inhibition assay.



Appendix 2 – Selection of organic solvent for BuChE inhibition assay.



Appendix 3 – Selection of organic solvent for tyrosinase inhibition assay.

Cell Line	SH-SY5Y	3T3-L1	RKO
Density (cell/mL)	$1,5 \times 10^5$	$3,3 \times 10^4$	$3,6 \times 10^4$
Passage number	1	32	1

Appendix 4 – Density and passage of the 3T3, SH-SY5Y and RKO cell lines.

Abstract for the 17th edition of Investigação Jovem da Universidade do Porto (IJUP)

A new approach for tackling neurodegenerative diseases: screening marine Actinobacteria for Alzheimer therapies

Bárbara Salgado[1,2,3]; Clara Grosso[2]; Inês Ribeiro[3]; Rosário Martins[1,3]; Maria de Fátima Carvalho[3,4]

[1]Escola Superior de Saúde, Politécnico do Porto; [2]REQUIMTE/LAQV, Instituto Superior de Engenharia do Porto; [3]CIIMAR – Interdisciplinary Centre of Marine and Environmental Research, University of Porto, [4]ICBAS – School of Medicine and Biomedical Sciences

Background & Aim:

Alzheimer's disease remains as the most common cause of dementia, accounting for an estimated 60% to 80% of cases [1]. However, effective treatment for this neurodegenerative disease has yet to be found.

Actinobacteria are gram-positive bacteria, highly promising in terms of biotechnological potential, with the major part of naturally derived antibiotics and other clinically important molecules being isolated from these microorganisms [3]. Terrestrial actinobacteria are extensively explored, but marine environments are yet poorly investigated in terms of these valuable microbials resources [2].

In this work, the crude extracts of several actinobacterial strains previously isolated from marine macroalgae and deep-sea samples were screened in order to investigate possible bioactive metabolites with anti-Alzheimer potential.

Methods:

Anti-Alzheimer activity was investigated by screening the potential of actinobacterial extracts to inhibit enzymes involved in the pathogenesis of Alzheimer's disease, namely acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) [4], [5] . The percentage of inhibition of these enzymes was evaluated by performing an adapted protocol of Ellman's colorimetric method using 96-welled microplates [6].

Results:

After performing the assays above, a selected group of the actinobacterial strains demonstrated some degree of inhibition in both AChE and BuChE (above 30%) at a concentration of 2 mg/mL in each well.

Conclusions:

This study shows that actinobacteria can be a source of new therapies for tackling Alzheimer's disease. One of the crucial aspects of how these bacteria could be beneficial to treat Alzheimer's disease is related to cholinesterase inhibition, as some of the actinobacterial strains showed some degree of inhibition of this enzyme. Nonetheless, further research is needed, mainly the evaluation of the cytotoxic effect of the extracts in neuroblastoma cells.

Keywords: Actinobacteria, Alzheimer's disease, Potential therapy, Cholinesterase inhibition

A new approach for tackling neurodegenerative diseases: screening marine Actinobacteria for Alzheimer therapies

BÁRBARA SALGADO[1,2,3], CLARA GROSSO[2], INÊS RIBEIRO[3], ROSÁRIO MARTINS[1,3], RICARDO FERRAZ[1] & MARIA DE FÁTIMA CARVALHO[3,4]

1. Escola Superior de Saúde, Politécnico do Porto, Porto, Portugal; 2. REQUIMTE/LAQV, Instituto Superior de Engenharia do Porto, Porto, Portugal; 3. CIIMAR – Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Porto, Portugal; 4. ICBAS – School of Medicine and Biomedical Sciences, University of Porto, Porto, Portugal.

Alzheimer's disease (AD) remains as the most common cause of dementia, accounting for an estimated 60% to 80% of cases ('2023 Alzheimer's Disease Facts and Figures' 2023). However, effective treatment for this neurodegenerative disease has yet to be found. Actinobacteria are gram-positive bacteria, highly promising in terms of biotechnological potential, with a major part of naturally derived antibiotics being isolated from these microorganisms (Jose, Maharshi, and Jha 2021). Marine environments are still poorly investigated in terms of these valuable microbials resources (Girão, Ribeiro, and Carvalho 2022). In this work, the crude extracts of several actinobacterial strains previously isolated from marine macroalgae and deep-sea samples were screened to investigate possible bioactive metabolites with anti-Alzheimer potential.

Anti-Alzheimer activity was investigated by screening the potential of actinobacterial extracts to inhibit enzymes involved in the pathogenesis of AD, namely acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) (Vecchio et al. 2021; Zhou and Huang 2022). The percentage of inhibition of these enzymes was evaluated by performing an adapted protocol of Ellman's colorimetric method using 96-welled microplates (Ingkaninan et al. 2003). After performing the assays above, a selected group of the actinobacterial strains demonstrated some degree of inhibition in both AChE and BuChE (above 30%) at a concentration of 2 mg/mL in each well.

This study shows that actinobacteria can be a source of new therapies for tackling AD. One of the crucial aspects of how these bacteria could be beneficial to treat AD is related to cholinesterase inhibition. Nonetheless, further research is needed, mainly the evaluation of the cytotoxic effect of the extracts in neuroblastoma cells.

Keywords: Actinobacteria, alzheimer's disease, potential therapy, cholinesterase inhibition.