ESCOLA
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# Alzheimer's Disease: Assessing the Therapeutic Potential of Marine Cyanobacteria

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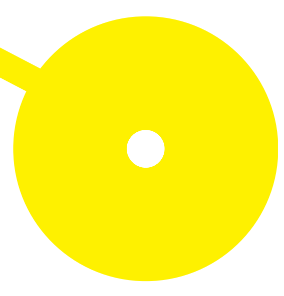
09/**2023**



# Alzheimer's Disease: Assessing the Therapeutic Potential of Marine Cyanobacteria

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Dissertation presented to fulfill the requirements to obtain a Master's degree in Health Biochemistry – branch in Clinical and Metabolic Biochemistry from the Escola Superior de Saúde do Instituto Politécnico do Porto.

**Funding**

This work was funded by the European Regional Development Fund (ERDF) through the Regional Operational Program North 2020, within the scope of Project GreenHealth - Digital strategies in biological assets to improve well-being and promote green health, Norte-01-0145-FEDER-000042.

**Agradecimentos**

I would like to express my profound appreciation to my advisor, Professor Rosário Martins, who graciously provided her knowledge and expertise. Her patience and constructive feedback have played a pivotal role in shaping the trajectory of this project. I extend my appreciation to Researcher Mariana Reis.

I thankfully appreciate Researcher Clara Grosso for her assistance and inspiration.

I owe a deep sense of gratitude to Master Ana Margarida Silva for her patience and supportive presence.

I gratefully acknowledge CIIMAR for welcoming me, particularly the BBE group, whose influence greatly enriched my work. Additionally, I express my appreciation to PRIHU for generously providing the necessary facilities and equipment for the completion of this project.

I would be remiss in not mentioning the teachers, colleagues, and those who, knowingly or unknowingly, impacted my academic journey.

Lastly, I am sincerely grateful to my beloved family, notably my mother, father, and grandparents, for their unwavering belief in my abilities. I would also like to express my gratitude to my boyfriend and my feline companion for their optimism and encouragement.

**Resumo**

A doença de Alzheimer (DA) é uma doença neurodegenerativa crónica e de natureza multifatorial, que afeta mais de 50 milhões de pessoas em todo o mundo e é a causa mais comum de demência entre os idosos. Devido à sua crescente prevalência e consequências devastadoras para a vida dos afetados, esforços contínuos têm sido feitos no sentido de compreender melhor as causas biológicas desta doença e descobrir novas abordagens terapêuticas que possam dar uma resposta adequada a esta condição neurodegenerativa. As opções terapêuticas disponíveis podem apenas aliviar os sintomas e, por vezes, causar efeitos colaterais adicionais. As cianobactérias são uma fonte rica de metabolitos secundários interessantes e compostos bioativos com propriedades benéficas e uma variedade de aplicações. Desta forma, este estudo pretendeu avaliar o potencial terapêutico de 96 frações de cianobactérias, obtidas por fracionamento em HPLC de extratos metanólicos, com base na sua citotoxicidade nas linhas celulares SH-SY5Y (neuroblastoma humano), 3T3L1 (fibroblastos) e hCMEC/D3 (células endoteliais representativas da barreira hemato-encefálica), e na atividade inibitória da enzima acetilcolinesterase (AChE). A citotoxicidade nas linhas celulares foi avaliada pelo ensaio de viabilidade celular baseado na redução do 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). A inibição da AChE foi avaliada pelo teste de Ellman. Quatro frações, no ensaio MTT, apresentaram citotoxicidade nas linhas celulares de neuroblastoma, fibroblastos e células endoteliais. As frações das estirpes de cianobactérias *Oxynema acuminatum* LEGE 06072 B e C, *Synechocystis salina* LEGE 06155 A e B, e *Cyanobium sp.* LEGE 07175 E,F e G, demonstraram os resultados mais promissores em relação à atividade de inibição da AChE com percentagens de inibição de 38,37%; 43,03%; 32,17%; 30,75%; 36,91%, 34,65% e 34,12%, respetivamente, não tendo apresentado efeitos citotóxicos para as linhas celulares SH-SY5Y, 3T3-L1 e hCMEC/D3. Estas são também as estirpes de cianobactérias que revelaram melhores rendimentos de extração, de 18,250%, 17,804% e 21,047%, respectivamente. De acordo com os resultados, as cianobactérias representam uma promessa significativa para novas abordagens terapêuticas para a DA, no entanto mais estudos são necessários para aprofundar a compreensão do potencial terapêutico destes microrganismos.

**Palavras-chave:** Doença de Alzheimer; acetilcolinesterase; citotoxicidade; cianobactérias; produtos naturais.

**Abstract**

Alzheimer's disease (AD) is a chronic neurodegenerative disease with a multifactorial nature, that affects more than 50 million people worldwide and is the most common cause of dementia among older adults. Due to its crescent prevalence and devastating life consequences, continuous efforts have been made to better understand AD's biological causes and discover novel therapeutics that can give a proper therapeutic answer to this neurodegenerative condition. Available therapeutic options can only ameliorate symptoms and sometimes cause additional side effects. Cyanobacteria are a rich source of interesting secondary metabolites and bioactive compounds with beneficial properties and an array of different applications. In this light, we aim to assess the therapeutic potential of 96 fractions of cyanobacterial methanolic extracts based on cytotoxicity in the human neuroblastoma SH-SY5Y cell line, in the fibroblasts 3T3L1 cell line, and in the brain microvascular endothelial hCMEC/D3 cell line, and acetylcholinesterase (AChE) inhibition activity. Four fractions, in the MTT assay, showed cytotoxicity on neuroblastoma cell lines, fibroblasts, and endothelial cells. The fractions of the cyanobacteria strains *Oxynema acuminatum* LEGE 06072 B and C, *Synechocystis salina* LEGE 06155 A and B, and *Cyanobium* sp. LEGE 07175 E, F, and G, demonstrated the most promising results in relation to AChE inhibition activity with inhibition percentages of 38.37%; 43.03%; 32.17%; 30.75%; 36.91%, 34.65% and 34.12%, respectively, showing no cytotoxic effects on SH-SY5Y, 3T3-L1 and hCMEC/D3 cell lines. These are also the cyanobacteria strains that revealed the best extraction yields, of 18.250%, 17.804%, and 21.047%, respectively. According to the results, cyanobacteria represent significant promise for new therapeutic approaches for AD, however, more studies are needed to deepen the understanding of the therapeutic potential of these microorganisms.

**Keywords:** Alzheimer's disease; acetylcholinesterase; cytotoxicity; cyanobacteria; natural products.

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**List of Abbreviations**

Aβ - Beta Amyloid

ACH – amyloid cascade hypothesis

AChE – Acetylcholinesterase

ACh – Acetylcholine

AD – Alzheimer's disease

BACE1 – Beta-site amyloid precursor protein cleaving enzyme 1

BBE – Blue Biotechnology and Ecotoxicology Laboratory at CIIMAR

BChE – Butyrylcholinesterase

CIIMAR – Interdisciplinary Center of Marine and Environmental Research

EOAD – Early-onset Alzheimer's disease

GABA – Gamma-aminobutyric acid

hCMEC/D3 – Human brain vessel endothelial cell line

HPLC – High pressure liquid chromatography

LOAD – Late-onset Alzheimer's disease

LPS – Lipopolysaccharides

MAP – Microtubule-associated protein

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

nbM – nucleus basalis of Meyner

ROS – Reactive oxygen species

SH-SY5Y – Human neuroblastoma cell line

3T3L1 – mice Fibroblasts cell line

NDs – Neurodegenerative diseases

CNS – central nervous system

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## 1. Introdução

Neurodegenerative diseases (NDs) encompass a collection of conditions characterized by the gradual degeneration and malfunction of neurons in the central nervous system (CNS)(1). These conditions are typically long-lasting and incapacitating, resulting in the progressive decline of cognitive abilities and, in conditions such as Alzheimer's disease (AD) (2), lateral amyotrophic sclerosis (LAS) (3), Parkinson's disease (PD) (4), spinal muscular atrophy (5) and prion diseases (6), an impairment of motor abilities.

NDs are linked to the aggregation of misfolded proteins including amyloid-$\beta$ (A$\beta$), tau, $\alpha$-synuclein, TAR DNA-binding protein 43, and prion proteins (7), dysfunction in synaptic (8) and specific large-scale neuronal networks (9), abnormal aggregates of cytoskeletal proteins such as neuronal intermediate filament (10) as well as structural and functional tubulin dysfunctions (11), oxidative damage and mitochondrial dysfunction (12), DNA unstable mutations (13) and RNA dysfunction, specifically, normal splicing disruption, interference with RNA-protein interactions, and generation of toxic RNA species (14), inflammation (15), and the selective death of neuronal cells (16). Although the exact basis for NDs is not completely understood, it is thought that an association of environmental, genetic, and lifestyle factors plays a role in their progression (1).

Neurological diseases pose a significant disability, burden worldwide, impacting around 50 million of the global population (17). Furthermore, the number of affected individuals has risen significantly over the past three decades. Since the majority of nations lack sufficient resources for neurological healthcare, the increasing prevalence of chronic neurodegenerative conditions among the growing elderly population, presents a considerable challenge in delivering accessible and sustainable neurological care that effectively addresses global needs (18).

The treatment options currently available for NDs primarily focus on relieving symptoms rather than directly targeting the underlying cause of the disease (19). While disease-modifying therapies aim to slow down or stop the progression of the disease, they have not yet been able to completely cure the neurodegenerative process (20). The limited progress in effectively treating NDs can be attributed to various factors, including the complexity of chronic inflammatory and degenerative diseases, which may require combination therapies to effectively target multiple pathways. Challenges in delivering neurotrophic factors, and the invasiveness of the delivery route, as well as the occurrence of side effects, further complicate treatment (21). Difficulties in addressing widespread neuronal loss and the restrictions of medication in crossing the blood-

brain barrier (BBB) add complexity to the search for treatment (22). Therefore, ongoing research for novel therapies is necessary to provide a suitable solution to these devastating conditions.

### 1.1. Alzheimer's disease

Alzheimer's disease (AD) is a persistent ND of a complex nature that affects a population of nearly 50 million individuals globally, thus establishing itself as the most prevalent cause of dementia amongst the elderly (1). The incidence rates escalate from 1% in patients aged between 65 and 69 years to approximately 30% in individuals surpassing the age of 85(23). AD's gradual, yet irreversible advancement, impairs memory, cognition, and ultimately hinders the execution of daily tasks, necessitating constant care.

This ND is characterized by specific changes in the brain tissue, referred to as histopathological hallmarks, that include the accumulation of Aβ in the form of senile plaques as well as the formation of neurofibrillary tangles (NFTs) mainly composed of tau protein (24). Aβ is a peptide that is normally produced in the brain, but in AD, it accumulates outside of the neurons. On the other hand, tau is a protein that is normally involved in stabilizing the structure of neurons, but in AD, it becomes hyperphosphorylated, which causes tau protein misfolding and the formation of NFTs that accumulate inside the neurons (25).

This complex disorder is caused by a combination of environmental and genetic factors. Sporadic AD (SAD) is typically linked to environmental elements, whereas familial AD (FAD) arises from inherited genetic mutations that disrupt the synthesis of Aβ. FAD and SAD differ in terms of when they manifest, with FAD usually occurring at an earlier stage compared to SAD (26). AD can be categorized based on the age when it manifests and genetic predisposition: early-onset AD (EOAD) and late-onset AD (LOAD), with the age of 65 serving as the dividing point. Approximately 5% of AD cases are classified as EOAD, whereas the remaining 95% are classed as LOAD. This suggests that the vast majority of AD cases are caused by aging combined with a multifaceted interaction of genetic and environmental factors (27).

#### 1.1.1. Diagnosis

AD is diagnosed through clinical evaluation, examination of medical history, and administration of cognitive tests. Biomarkers play a crucial role in the identification of this condition. Important biomarkers include Aβ proteins A1-42, A1-40, tau protein (t-tau, total tau), and phosphorylated tau protein (p-tau, phosphorylated tau 181), which can be found in the cerebrospinal fluid. The

detection of these biomarkers can occur years before the manifestation of clinical symptoms, aiding in the early diagnosis and implementation of an optimal treatment plan to slow down the progression of the disease (28).

Emerging methods for detecting AD at an earlier stage are being developed, including the expanded use of positron emission tomography (PET) to identify the buildup of amyloid- (A) and hyperphosphorylated tau, magnetic resonance imaging to identify changes in the columnar architecture of the cortex and the use of cerebrospinal fluid and serum biomarkers (29).

### 1.1.2. Clinical stages

AD's clinical stages are classified into pre-clinical or pre-symptomatic stage, moderate stage and late stage. The initial differential diagnosis encompasses a thorough exploration of the patient medical history, a comprehensive evaluation of the patient's neurological status, as well as cognitive and functional assessments aimed at assessing memory, executive functioning, and behaviour. The detection and diagnosis of AD during its early stages are of paramount importance to maximize the prognosis and well-being of both patients and their families (30).

The pre-clinical stage is characterized by mild memory loss and early pathological changes in the cortex and hippocampus with no functional impairment of daily activities. This stage can last for several years or more. In the mild or early stages of AD, patients begin to experience several symptoms, including difficulty with everyday activities, memory and concentration loss, place and time disorientation, mood changes, and the onset of depression (31). The moderate stage, which is characterized by the disease's progression to areas of the cerebral cortex, results in increased memory loss with difficulty identifying relatives and friends, a lack of impulse control, and difficulties with reading, writing, and speaking. Severe AD, or late-stage AD, occurs when the disease has progressed to the entire cortex area with significant neuritic plaques and neurofibrillary tangle accumulation, resulting in progressive functional and cognitive impairment. In this stage, patients lose all ability to recognize their family and may become bedridden and have difficulty swallowing and urinating, which ultimately leads to the patient's death (31).

Due to AD's complexity and multifactorial nature, several hypotheses have been formulated to better comprehend the ultimate mechanism that fuels the onset and progression of this disorder and its neurodegenerative nature. These hypotheses orient both AD understanding throughout time and the search for novel therapeutics that can ameliorate symptoms or stop the progression of this disease (32).

#### 1.1.3.1. Amyloid cascade hypothesis

Amyloid precursor protein (APP) is a membrane glycoprotein with a large extracellular glycosylated N-terminus, a single transmembrane domain, and a small cytoplasmic C-terminus that plays an important role in a variety of important biological activities, including neuronal development, signaling, intracellular transport, and other aspects of neuronal homeostasis (33). In AD, amyloid plaque formation occurs through Aβ peptide secretion, which originates through the sequential cleavage of APP(34) .

Aβ formation occurs through APP proteolytic cleavage. To initiate this process, $\beta$-secretase cleaves APP at the NH2-terminal, releasing a soluble fragment, $\beta$APPs, and C99, a COOH-terminal fragment that remains membrane-bound. C99 can be further cleaved by one or more $\gamma$-secretases, leading to the release and secretion of Aβ. In an alternate metabolic pathway, $\beta$-secretase cleaves within the Aβ sequence, excluding Aβ formation (35).

The amyloid cascade hypothesis (ACH) proposes that the neurodegenerative progression characterized by synaptic dysfunction, synaptic loss, and eventual neuronal death is activated by an increase in A$\beta$ production (36).

#### 1.1.3.2. Tau hypothesis

As aforementioned, one of the hallmarks of AD is the presence of NFT, which are mainly made of tau protein.

Tau, encoded on chromosome 17q21, is a highly soluble microtubule-associated protein (MAP) that occurs mainly in the axons of the CNS and is a naturally unfolded protein with a large number of structural conformations and biochemical modifications. Tau has a biological function of stabilizing microtubules in neurons and other cells, playing a role in cell differentiation and polarization (37), and helping regulate anterograde transport by kinesin and neurotransmitter release (38).

In the adult human brain, six isoforms of tau are expressed, and generated through alternative splicing of the MAPT gene. Tau's hyperphosphorylation is caused by mutations that alter its function and isoform expression, and all six are present in an often hyperphosphorylated state in paired helical filaments in AD (39).

According to the tau hypothesis, tau's excessive or abnormal phosphorylation causes microtubule depolymerization, neuronal synaptic dysfunction, and the formation of NFT (38).

#### 1.1.3.3. Cholinergic hypothesis

Acetylcholine (ACh) is an endogenous neurotransmitter used by all the cholinergic neurons with numerous functions in the body, modulating essential neural activities such as attention, learning, memory, stress response, wakefulness and sleep, and sensory information (40). It is present in the CNS, autonomic nervous system, and somatic nervous system at the neuromuscular junction and is released by preganglionic nerves, postganglionic parasympathetic nerves, and some postganglionic sympathetic nerves (41).

Choline acetyltransferase (ChAT) enables the synthesis of ACh by converting choline and acetyl co-enzyme within the cytoplasm of cholinergic neurons. After being released in the synaptic cleft, this neurochemical can activate both muscarinic and nicotinic cholinergic receptors. Once in the synaptic cleft, ACh can rapidly be inactivated by acetylcholinesterase (AChE), a cholinergic enzyme especially found in muscles and nerves, at postsynaptic neuromuscular junctions (41).

The cholinergic hypothesis (CH) was built on the discovery of reduced ChAT activity in AD patients brains, depleted presynaptic cholinergic markers in the cerebral cortex and the observance of severe cholinergic neuronal degeneration in the nucleus basalis of Meynert (nbM) in the brains of those with AD and advanced age (42). The nbM, and more specifically, the Ch4 group in the basal forebrain is the single most significant cholinergic input source to the cortex (43).

In the last 40 years, a fair amount of research has been made in order to better comprehend the relationship between cholinergic and cognitive loss, and despite de fact that some theories around this hypothesis have been refuted while others gain space in the search for cholinergic therapies for AD, essentially, the CH suggests that cholinergic impairment due to cholinergic neuronal loss in the brain contributes significantly to the deterioration of cognition observed in those with advanced age and AD pathology (44).

#### 1.1.3.4. Glutaminergic hypothesis

Glutamate is the most abundant excitatory neurotransmitter in the CNS of mammals. It is found extensively distributed in the CNS, being almost entirely located intracellularly (45). This non-essential amino acid is important in memory, learning, cognition, and motor behavior, and is the immediate precursor of the inhibitory neurotransmitter $\gamma$-aminobutyric acid (GABA) and glutathione, as well as an intermediary metabolism essential component, a protein building block, an energy substrate and, paradoxically, a potent neurotoxin, present at the intersection between multiple metabolic pathways (46).

In AD, glutamatergic dysfunction seems to be related to a variety of mechanisms such as Aβ binding to glutamate receptors, receptor overactivation caused by tau tethering to intrinsic cytoskeletal proteins, and the internalization of glutamate transporters resulting in glutamate accumulation in synaptic and extrasynaptic areas. Although further research is still necessary to clarify the process of Aβ -associated glutamate excitotoxicity involving the dysfunction of calcium-permeable glutamate receptors in the neuron, Aβ has been implicated in the inhibition of glutamate uptake in the synaptic cleft, overstimulation of N-methyl-D-aspartate (NMDA) receptors, the subsequent disruption of calcium-dependent intracellular pathways, and eventual neuronal death (47).

The glutaminergic hypothesis (GH) centers around the critical contribution of inappropriate stimulation of the glutamate receptors on synaptic function deterioration. NMDA receptors, closely related to memory and learning, are involved in synaptic transmission and plasticity, and the receptors hyperexcitation results in excitotoxicity mediated by increased Ca2+ influx, which impacts synaptic signaling and function (48).

#### 1.1.3.5. Inflammation and oxidative stress hypothesis

Inflammation is a non-specific immune response to harmful stimuli. In normal conditions, inflammation removes the injurious stimuli and initiates damaged tissue repair and healing. However, the process of inflammation demands a cellular adaptive response to stress that associates itself with some degree of function laesa (49). When acute inflammation progresses to chronic inflammation, the ongoing damage can ultimately lead to the development of chronic diseases and severe organ dysfunction due to tissue architecture loss, even after regeneration, and replacement by non-functional fibrous tissue (50). In the brain, inflammation can occur in response to a variety of aggressions, such as stroke, bacterial infections and traumatic brain damage (51).

Chronic inflammation in the CNS is characterized by microglia and astrocyte activation, pro-inflammatory cytokines and nitric oxide secretion, an increase in reactive oxygen species (ROS)levels, and cytotoxic neuronal effects, typically observed in AD. In the brain with AD, the connection between the development of inflammatory processes and the disease pathogenesis has been widely demonstrated (52) .

The inflammation hypothesis of AD suggests that neuroinflammation begins early in the development of the disease and plays a fundamental role in its progression, starting before Aβ

plaque formation and tau hyperphosphorylation (53). In this way, we approach neuroinflammation as directly linked to neuronal loss and cognitive decline.

#### 1.1.4. Treatment

Due to its high prevalence and devastating life consequences, enormous efforts have been made by the scientific community in the identification of the underlying mechanisms of AD and the discovery of novel therapeutics that can halt its progression. There are currently only five drugs approved by the FDA for AD treatment, namely rivastigmine, galantamine, donepezil, memantine, and memantine in combination with donepezil (54). However, none of these options can reverse, stop, or even slow down the progress of the disease processes that lead to neuron damage and loss and, consequently, result in a fatal outcome. These approved drugs, include AChE inhibitors (galantamine, donepezil, and rivastigmine) and NMDA receptor antagonists (memantine) and their combinations, along with the additional challenges that AD research presents, such as a lack of reasonable animal models, research tools, and the well-known complexity of the human brain's, result in marginal benefits, temporary symptomatic relief, and severe side effects that expose the need for a better understanding of these mechanisms and the development of novel therapies (55).

### 1.2. Cyanobacteria

Cyanobacteria are photoautotrophic organisms that live in soil, marine and freshwater environments, plants, rocks (endolithic), animals (endosymbiotic relationship), and also in extreme environments such as desert and ice. Cyanobacteria's tremendous ecological heterogeneity and ongoing competition for nutrients require a successful adaptation to different conditions that molded cyanobacteria's evolution throughout time (56). Under stressful conditions, these microorganisms developed particular characteristics that have given rise to several different taxa that differ in morphology as well as biochemical and physiological capabilities, with many species capable of fixing atmospheric nitrogen and strains capable of producing relevant bioactive compounds (57).

Marine cyanobacteria are known to produce an array of secondary metabolites that have been shown to be structurally diverse and, in the majority, biologically active. These biomolecules are significant subsets of natural products that are used as therapeutic agents. In this light, cyanobacteria's role as an important source of novel microbial secondary metabolites fuels significant drug discovery efforts (58).

### 1.2.1. Secondary metabolites

Cyanobacteria display a wide range of metabolic processes and serve as a valuable source of primary and secondary metabolites. Valued for the essential functions in which they are involved, primary metabolites are directly related to vital cellular processes such as cell division, growth, and reproduction. These include antioxidants, primary proteins, and lipids (59).

Secondary metabolites are generally characterized by not being directly required for the primary metabolism of an organism. Although there may be some overlap between the two definitions at times, secondary metabolites are typically unique to specific organisms and are not present in every environmental condition. These substances are produced for the purpose of protection and survival, and they include phenolic compounds, alkaloids, essential oils, toxins, steroids, and antibiotics (59,60).

Metabolites with known value produced by cyanobacteria comprise phenolic acid, essential for oxidative response repair; vitamins, with function in many metabolic pathways and cellular processes; peptides with high value for biotechnology in drug discovery; and terpenoids, compounds with a multitude of properties including anti-inflammatory, antioxidative, antimicrobial, light harvesting, and stress resistance activity (59).

### 1.2.2. Cyanobacteria against neurodegeneration

Cyanobacteria have been recognized as an interesting source of natural products and novel secondary metabolites with therapeutic neuroprotective potential towards AD and with promising therapeutic applications in drug and nutraceutical development. A few of the most prominent examples of cyanobacterial metabolites with demonstrated beneficial biological activity towards NDs include: Tasiamide B, a linear peptide isolated from the marine cyanobacterium Symploca sp. and characterized as a BACE1 inhibitor (61); Anatoxin-a(S), an alkaloid isolated from the cyanobacterium Anabaena flos-aquae that exhibits potent anticholinesterase activity and shows both AChE and BChE enzymatic inhibition (62); Nostocarboline, isolated from the freshwater cyanobacterium Nostoc 78-12A, shows a potent BChE inhibition, comparable to galantamine, an approved drug for AD's therapy (63). In the same light, cyanobacteria aqueous, acid aqueous and methanolic extracts of Calothrix sp. CCIBt 3320, Tolypothrix sp. CCIBt 3321, Phormidium cf. amoenum CCIBt 3412, Phormidium sp. CCIBt 3265, Geitlerinema splendidum CCIBt 3223 and Anabaena spiroides with demonstrated AChE inhibition ability have also been identified(64,65) . Crude extracts of the strains Nostoc sp. str. Lukesova´

27/97 and Nostoc ellipsosporum Rabenh. str. Lukesová 51/91 also exhibited acetylcholinesterase inhibitory activity (66). C-Phycocyanin from Spirulina sp., another enthralling compound, shows antioxidant, anti-inflammatory, and neuroprotective activity (67). In fact, the cyanobacteria genus Spirulina (Arthrospira platensis) is a great example of this microorganism's versatile applications and popular use throughout time as a food nutritional supplement.

## 2. Aims

The necessity for an enhanced comprehension of the underlying mechanism of AD, which manifests in the detrimental consequences it imprints upon the lives of those impacted, and its increasing prevalence in the population, renders this nefarious ND a subject matter of immense interest within the scientific community. These characteristics emphasize the need for the development of innovative therapeutic approaches that allow patients to alleviate symptoms and potentially stop or reverse the neurodegenerative process, all while causing minimal or no adverse effects.

The purpose of this study was to assess the therapeutic potential of cyanobacteria fractions towards AD. As such, the following course of action was carried out:

-Cyanobacteria strains culture under controlled conditions to produce biomass;

-Participation in the enlargement of a library of fractions utilizing a semi-automated collection system;

-Assessing the toxicity of the cyanobacterial fractions on neuroblastoma cells (SH-SY5Y), fibroblasts (3T3L1), and endothelial cells (hCMEC/D3);

-Assessing the therapeutic potential of cyanobacteria fractions towards AD through the AChE enzymatic inhibition.

## 3. Materials and Methods

### 3.1. Cyanobacteria strains

The Blue Biotechnology and Ecotoxicology (BBE) Culture Collection, LEGE-CC, at the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), comprises a diverse range of over 700 well stablished cyanobacterial strains from samples taken from diverse environments and locations (LEGE CC- https://lege.ciimar.up.pt/). Twelve cyanobacteria strains from the BBE Culture Collection LEGE-CC, were selected based on criteria such as previously identified anticancer activity, cosmetic applications, and growth capacity. Selected strains are presented at Table 1.

**Table 1.** Cyanobacteria strains included in this study (from LEGE CC- https://lege.ciimar.up.pt/).

| LEGE code | Strain ID | Microphotograph 1000x | LEGE code | Strain ID | Microphotograph 1000x |
|---|---|---|---|---|---|
| LEGE 06072 | *Oxynema acuminatum* | | LEGE 07167 | *Lusitaniella coriacea* | |
| LEGE 06099 | *Synechocystis salina* | | LEGE 07171 | *Synechococcus nidulans* | |
| LEGE 06102 | *Nodosilinea nodulosa* | | LEGE 07173 | *Cyanobium* sp. | |
| LEGE 06113 | *Cyanobium* sp. | | LEGE 07175 | *Cyanobium* sp. | |
| LEGE 06144 | unidentified filamentous *Synechococcales* | | LEGE 11396 | *Geitlerinema* sp. | |
| LEGE 06155 | *Synechocystis salina* | | VV11 | identification in process | |

### 3.2. Cyanobacteria culture

Cyanobacteria strains were cultured in Z8 medium with 25 g/L of sea salt. The preparation of the medium was initiated with the mixture of 25 g/L of sea salt on distilled water. After autoclaving, the preparation of the medium proceeded inside a laminar flow chamber, under sterile conditions, where three solutions were combined in order to obtain the appropriate Z8 culture medium. These solutions, which had been filtered through a 0.2 µm filter, included solution A (a nitrogen source), solution B (a phosphorous source), Fe-EDTA (a source of iron in a stabilized form) and micronutrients, in the concentrations elucidated in Table 2. Filter-sterilized vitamin B12 was added to media at a final concentration of 10 µg/L. For cyanobacteria culture and biomass harvest, priority was given to cyanobacteria strains with higher growth and biomass production capacity.

Due to the lengthy process of cultivation and the temporal restriction of the study, a portion of the cyanobacteria biomass was acquired from the Cyanobacteria Natural Products Library (LEGE-NPL) repository. The biomass was obtained for the purpose of assessing citotoxicity in three cell lines and AChE enzymatic inhibition assays.

**Table 2.** Reagents utilized in the culture medium preparation.

| Reagent | mL/L |
|---|---|
| Solution A | 10 mL/L |
| Solution B | 10 mL/L |
| Fe-EDTA | 10 mL/L |
| Sol. Micronutrients | 1 mL/L |

For culture, cyanobacteria were first inoculated in T25 culture flasks with a surface area of 25cm2, containing an approximate medium volume of 40 mL. After a period of 15 days, the biomass was transferred to approximately 350 mL medium, in T75 culture flasks with a surface area of 75cm2. Following an additional two weeks, the biomass was transferred to 6L balloons containing an approximate 4L culture medium volume. The biomass transfer process occurred inside the laminal flow chamber, under sterile conditions. In the cultivation room, conditions

throughout the experimental procedure were maintained at a temperature of 25°C, with light/dark cycle of 14/10h, and a light intensity of 10–30 μmol photons m-2s-1.

### 3.3. Biomass harvesting

The biomass harvesting was performed based on whether cyanobacteria exhibited a filamentous or unicellular morphology, either by filtration using a strainer and a 20μm net or by centrifugation (Thermo Scientific BIOS 16, Germany), respectively. The concentrated biomass was then rinsed with distilled water to eliminate the excess of salt. Following this procedure, the biomass was transferred to a properly labeled 50mL container, frozen, subsequently freeze-dried at -80ºC (TelstarLyoQuest), and stored at -20ºC.

### 3.4. Cyanobacteria extraction

The freeze-dried biomass was crushed using a porcelain mortar and pestle. For the extraction, 50 mL of methanol (MeOH) was added to the biomass in a flask and sonicated in the ultrasonic bath for 5 minutes.  To prevent heating, ice was utilized when the ultrasonic bath water reached 30ºC. Once the biomass settled at the bottom of the flask, the supernatant was filtered into a round bottom flask connected to a filtering vacuum system. This process of MeOH extraction of the resulted pellet was repeated two more times. The resulting extracts were combined and then concentrated in a rotary evaporator at a temperature of 30ºC ((BUCHI Interface I-300, Switzerland). Concentrated extract was filtered using a Pasteur pipette with pressed cotton to a pre-weighed glass vial. The solvent was evaporated until completetly dry. The vial was weigthed again and the extraction yield calculated by the following formula:

$$\text{Yield (\%)}=\frac{\text{Dry extract (g)}}{\text{Dry biomass (g)}}\times 100$$

### 3.5. HPLC separation

Each dry sample was resuspended in MeOH of LC-MS grade to obtain a final solution concentration of 40 mg/mL. The solution was then sonicated and filtrated through 0.2 μm syringe filters into a 2 mL High-Performance Liquid Chromatography (HPLC) glass vial.

The HPLC separation process was carried out using the Water Alliance e2695 system, in an automated fashion, using the fraction collector module of Alliance. In accordance with the

Empower Program (Alliance-HPLC) Protocol, the samples were fractionated using reverse-phase HPLC, injected at a concentration of 40 mg/mL, and separated using an ACE 10C8 50 x 10 mm HPLC column, employing a H2O:MeCN gradient. HPLC fractionation is a process used for the separation and purification of complex mixtures, based on their different chemical properties and interactions with the stationary phase and mobile phase, as they pass through the column (64). The 8 fractions, separated from each cyanobacterial methanolic extract, were collected in a 48-deep well plate, which was subsequently dried using a CentriVap Concentrator. The dried fractions were mixed with 500 μL of DMSO and then transferred into 96-deep well microplates, which served as the mother plates as illustrated in table 3 and 4. These plates were stored at -80°C, at a concentration of 5mg/mL, and the fractions were used for the MTT assay. To perform the AChE assay, the plates were dried using a CentriVap Concentrator and the fractions were resuspended in Buffer A and 10% MeOH, at the same concentration.

In order to contribute to the Cyanobacteria Natural Products Library (LEGE-NPL) repository, the cyanobacteria biomass not used in this work, and methanolic extracts of strains not included in the cytotoxicity and AChE inhibition assessment portrayed were included in the system. In the same light fractions included in Plate I were not cultured in the progress of this study but were obtained from the liquid inventory of the BBE Natural Products Library.

**Table 3.** Schematic representation of the mother plate I with the fractions.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | DMSO | 06072_A | 06102_A | 06144_A | 07167_A | 07171_A | 11396_A | DMSO | | | | |
| **B** | DMSO | 06072_B | 06102_A | 06144_B | 07167_B | 07171_B | 11396_B | DMSO | | | | |
| **C** | DMSO | 06072_C | 06102_A | 06144_C | 07167_C | 07171_C | 11396_C | DMSO | | | | |
| **D** | DMSO | 06072_D | 06102_A | 06144_D | 07167_D | 07171_D | 11396_D | DMSO | | | | |
| **E** | DMSO | 06072_E | 06102_A | 06144_E | 07167_E | 07171_E | 11396_E | DMSO | | | | |
| **F** | DMSO | 06072_F | 06102_A | 06144_F | 07167_F | 07171_F | 11396_F | DMSO | | | | |
| **G** | DMSO | 06072_G | 06102_A | 06144_G | 07167_G | 07171_G | 11396_G | DMSO | | | | |
| **H** | DMSO | 06099_H | 06102_A | 06144_H | 07167_H | 07171_H | 11396_H | DMSO | | | | |

**Table 4**. Schematic representation of the mother plate II with the fractions.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | DMSO | 6099_A | 6113_A | 6155_A | 7173_A | 7175_A | VV11_A | DMSO | | | | |
| **B** | DMSO | 6099_B | 6113_B | 6155_B | 7173_B | 7175_B | VV11_B | DMSO | | | | |
| **C** | DMSO | 6099_C | 6113_C | 6155_C | 7173_C | 7175_C | VV11_C | DMSO | | | | |
| **D** | DMSO | 6099_D | 6113_D | 6155_D | 7173_D | 7175_D | VV11_D | DMSO | | | | |
| **E** | DMSO | 6099_E | 6113_E | 6155_E | 7173_E | 7175_E | VV11_E | DMSO | | | | |
| **F** | DMSO | 6099_F | 6113_F | 6155_F | 7173_F | 7175_F | VV11_F | DMSO | | | | |
| **G** | DMSO | 6099_G | 6113_G | 6155_G | 7173_G | 7175_G | VV11_G | DMSO | | | | |
| **H** | DMSO | 6099_H | 6113_H | 6155_H | 7173_H | 7175_H | VV11_H | DMSO | | | | |

### 3.6. Cell culture

The cytotoxicity of the cyanobacteria fractions was tested against three cell lines (Table 5). The neuroblastoma cell line SH-SY5Y, which is an in vitro model commonly used in neurotoxicity studies namely in AD research (68). The hCMEC/D3 endothelial cell line derived from human cerebral microvasculature and representative of the BBB (69) and the fibroblasts cell line 3T3-L1 that has been used as representative cell of the connective tissue. Although SH-SY5Y cells and hCMEC/D3 cells are of human origin, 3T3-L1 cells are derived from mouse connective tissue. During the project it was not possible to obtain human fibroblasts and consequently the 3T3-L1 cell line was used.

Table 5. Cells lines used in this study.

| Cell line | SH-SY5Y | 3T3-L1 | hCMEC/D3 |
|---|---|---|---|
| Cell type | Neuroblastoma cells | Fibroblasts | Endothelial cells |
| Origin | American Type Culture Collection (ATCC) | American Type Culture Collection (ATCC) | Donated by Dr PO Couraud (INSERM, France). |
| Image | | | |
| Passages | Experiment 1- P36<br>Experiment 2- P37<br>Experiment 3- P38 | Experiment 1- P33<br>Experiment 2- P34<br>Experiment 3- P36 | Experiment 1- P28<br>Experiment 2- P30<br>Experiment 3- P31 |

Cells were cultured in DMEM Glutamax medium (Merck, Germany). The medium was supplemented with 10% (v/v) fetal bovine serum (FBS), an antifungic and an antibiotic, which consisted of 0.1% Amphotericin B, and 1% penicillin/streptomycin, known as Pen-Strep, at concentrations of 100 IU/ml and 10 mg/ml, respectively (Gibco, Germany). To initiate the culture, the cells were defrosted and placed in 25 $cm^2$ flasks containing 4 mL of medium. The flasks were then incubated at a temperature of 37°C, in the presence of 5% $CO_2$ and moderate humidity. The growth of the cells was monitored using an inverted contrast microscope, which allows for the observation of living cells. Under the microscope, the cells were expected to show elongation and adherence, indicating their viability. The medium was changed every two days to ensure optimal growth conditions.

### 3.7. Cell passage

The initial step of cell passage involved removing the previous medium and rinsing the cells with 2mL of pre-warmed PBS, followed by the addition of 1 mL of triplex with an incubation time of 3-5 minutes. After incubation, 4 mL of medium was added to deactivate the triplex. The cell suspension was then transferred to a falcon tube and subjected to centrifugation at 1200 RPM

for 5 minutes. The supernatant was subsequently discarded, and the pellet was resuspended in 1 mL of fresh medium.

It is crucial to perform passages to prevent the cells from growing on top of each other, as they reach confluence.

### 3.8. Cell counting

The quantification of the number of live cells was performed by the transfer of 20µL of the cell suspension, mixed with 20µL of trypan blue, a negatively charged dye that interacts specifically with cells that have a damaged cell membrane. To count and determine the cell concentration, a Neubauer Chamber was used.

To continue the cell culture, a volume of cell suspension was transferred to a fresh culture flask, measuring 25 cm2 with 5 mL of new medium, or 75 cm2 with 10 mL of medium. The cells were then incubated as previously described.

### 3.9. MTT cell viability assay

To assess the cytotoxicity of cyanobacteria fractions the cell viability assay based on the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (AppliChem, GmbH: Ottoweg, Darmstadt, Germany) was performed. The MTT assay is widely used for the measurement of cellular metabolic activity and is commonly employed in cell toxicity studies (70,71). It entails the conversion of the yellow tetrazolium salt MTT into formazan crystals through the action of NAD(P)H-dependent oxidoreductases, such as mitochondrial dehydrogenases, which are found in the mitochondria of viable cells. The formazan crystals are insoluble and can be dissolved using a detergent, such as dimethyl sulfoxide (DMSO), to produce a colored solution that can be measured spectrophotometrically (71).

For the cytotoxicity assay the cells were seeded at a density of $2x10^4$ cells/mL for SH-SY5Y, $1x10^5$ cells/mL for hCMEC/D3, and $3,3x10^4$ cells/mL for 3T3L1, in 96 wells plates (representative scheme in table 6) which were then incubated for 24 hours to promote cell adhesion. After 24 hours, the medium was replaced with a new medium containing cyanobacterial fractions at a final concentration of 50 µg/mL. For the negative control, 1% DMSO was used, while the positive control was 20% DMSO. The plates were then incubated for 24 and 48 hours. Three independent experiments were conducted, each one comprising three replicates.

For the MTT assay 20 µL of MTT in a concentration of 1 mg/mL, was added to each well, resulting in a final concentration of 200 µg/mL. The plates (Table 6) were then incubated for 3 hours at a temperature of 37°C in an environment with 5% CO2 and moderate humidity. The medium in each well was then removed, and 100 µL of DMSO was added to dissolve the formed formazan crystals, resulting in the medium acquiring a purple hue. The intensity of the purple staining was measured by reading the absorbance at a wavelength of 562, in a microplate reader (EZ Read 800 Plus)

**Table 6.** Schematic representation of the 96 wells plates for the MTT assay.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | 06072_A | 06102_A | 06144_A | 07167_A | 07171_A | 11396_A | 06072_B | 06102_B | 06144_B | 07167_B | 07171_B | 11396_B |
| **B** | 06072_A | 06102_A | 06144_A | 07167_A | 07171_A | 11396_A | 06072_B | 06102_B | 06144_B | 07167_B | 07171_B | 11396_B |
| **C** | 06072_A | 06102_A | 06144_A | 07167_A | 07171_A | 11396_A | 06072_B | 06102_B | 06144_B | 07167_B | 07171_B | 11396_B |
| **D** | 20%DMSO | 20%DMSO | 20%DMSO | 20%DMSO | 20%DMSO | 20%DMSO | 1%DMSO | 1%DMSO | 1%DMSO | 1%DMSO | 1%DMSO | 1%DMSO |
| **E** | 06072_C | 06102_C | 06144_C | 07167_C | 07171_C | 11396_C | 06072_D | 06102_D | 06144_D | 07167_D | 07171_D | 11396_D |
| **F** | 06072_C | 06102_C | 06144_C | 07167_C | 07171_C | 11396_C | 06072_D | 06102_D | 06144_D | 07167_D | 07171_D | 11396_D |
| **G** | 06072_C | 06102_C | 06144_C | 07167_C | 07171_C | 11396_C | 06072_D | 06102_D | 06144_D | 07167_D | 07171_D | 11396_D |
| **H** | 20%DMSO | 20%DMSO | 20%DMSO | 20%DMSO | 20%DMSO | 20%DMSO | 1%DMSO | 1%DMSO | 1%DMSO | 1%DMSO | 1%DMSO- | 1%DMSO |

Legend:

Positive control – 20% DMSO

Negative control – 1%DMSO

### 3.10. Acetylcholinesterase assay

The inhibition of AChE by the fractions of cyanobacteria methanolic extracts was determined through the colorimetric Ellman's method with minor modifications (72), based on the quantification of thiocoline produced from the hydrolysis of ACh by AChE. The quantification occurs spectrophotometrically through the reaction with 5,5-bisdithionitrobenzoic acid (DTNB), resulting in the formation of the yellow product 5-thio-2-nitrobenzote anion (TNB2) with maximum absorbance at 405 nm.

For this experiment, the following reagents were used to prepare the samples (tested fractions), the blanks for the samples, the negative control, and the blank for the negative control: AChE enzyme from an eel, the substrate ATCI (code A5751-5G sigma), Ellman's Reagent or DNTB (which is light-sensitive and turns yellow), Buffer B (used to dilute and stabilize the enzyme), Buffer C (used to dissolve the DNTB), and Buffer A and DMSO (for reagent and buffer preparation)

(Tables 7 and 8). The essay was conducted at a fraction concentration of 500 μg/mL. Plates schematic representation is demonstrated at Table 9.

### Table 7. Reagents utilized for the AChE inhibition assay.

| Samples | Blank of the samples | Negative control | Blank of the negative control |
|---|---|---|---|
| 25 μL of fraction | 25 μL of fraction | 25 μL of Buffer A + 1% of DMSO | 25 μL of Buffer A + 1% of DMSO |
| 125 μL of DTNB | 125 μL of DTNB | 125 μL of DTNB | 125 μL of DTNB |
| 50 μL of Buffer B | 50 μL of Buffer B | 50 μL of Buffer B | 50 μL of Buffer B |
| 25 μL of ATCI | 25 μL of ATCI | 25 μL of ATCI | 25 μL of ATCI |
| 25 μL AchE | 25 μL of Buffer B | 25 μL AchE | 25 μL Buffer B |

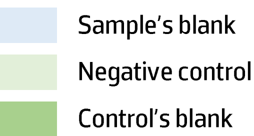
### Table 8. Buffer preparation for the AChE assay.

| | |
|---|---|
| **Buffer A**: 50 mM Tris-HCL, pH 8 | 7.88 g Tris-HCL —— 1000 mL $H_2O$ |
| **Buffer B**: 50 mM Tris-HCL, pH 8, containing 0.1% of bovine serum albumin (BSA) | 7.88 g Tris-HCL + 1 g BSA —— 1000 mL $H_2O$<br>50 μL x400 = 20000 μL= 20mL |
| **Buffer C**: 50 mM Tris-HCL, pH 8, containing 0.1 M NaCl and 0.02 M $MgCl_2.6H_2O$ | 7.88 g Tris-HCL + 5.8442 g NaCl + 2.2642 g $MgCl_2.6H_2O$ —— 1000 mL $H_2O$ |

### Table 9. Schematic representation of the AChE inhibition assay plates.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | 06072_A | 06072_A | 06072_A | SB | 06072_H | 06072_H | 06072_H | SB | 06102_G | 06102_G | 06102_G | SB |
| **B** | 06072_B | 06072_B | 06072_B | SB | 06102_A | 06102_A | 06102_A | SB | 06102_H | 06102_H | 06102_H | SB |
| **C** | 06072_C | 06072_C | 06072_C | SB | 06102_B | 06102_B | 06102_B | SB | 06144_A | 06144_A | 06144_A | SB |
| **D** | 06072_D | 06072_D | 06072_D | SB | 06102_C | 06102_C | 06102_C | SB | 06144_B | 06144_B | 06144_B | SB |
| **E** | 06072_E | 06072_E | 06072_E | SB | 06102_D | 06102_D | 06102_D | SB | 06144_C | 06144_C | 06144_C | SB |
| **F** | 06072_F | 06072_F | 06072_F | SB | 06102_E | 06102_E | 06102_E | SB | 06144_D | 06144_D | 06144_D | SB |
| **G** | 06072_G | 06072_G | 06072_G | SB | 06102_F | 06102_F | 06102_F | SB | 06144_E | 06144_E | 06144_E | SB |
| **H** | C - | C - | C - | CB | C - | C - | C - | CB | C - | C - | C - | CB |

Legend:



Sample's blank

Negative control

Control's blank

### 3.11. Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) using GraphPad Prism version 8.0.2 for Windows.

### 3.12. Extraction yield

The extraction yield refers to the quantity of the extract obtained from the raw material. Various factors have an impact on the extraction yield in different methods of extraction, including temperature, sonication time, and the pH level of the solvent used for extraction (73). Cyanobacteria extraction yield of the strains included in this study present at Table 10.

Table 10. Cyanobacteria strains utilized for the MTT and AChE assays yield.

| Strain code | Strain ID | Cultivation procedure | Freeze-dried biomass yield (g) | Organic extract (g) | Organic extract: Yield (%) |
|---|---|---|---|---|---|
| LEGE 06072 | *Oxynema acuminatum* | Freeze-dried biomass stock | 1,410 | 0,257 | 18,250 |
| LEGE 06099 | *Synechocystis salina* | Freeze-dried biomass stock | 1,007 | 0,135 | 13,381 |
| LEGE 06102 | *Nodosilinea nodulosa* | 4 L culture | 1,567 | 0,427 | 27,226 |
| LEGE 06113 | *Cyanobium* sp. | Freeze-dried biomass stock | 1,008 | 0,182 | 18,075 |
| LEGE 06144 | unidentified filamentous | 4 L culture | 3,900 | 0,761 | 19,502 |
| LEGE 06155 | *Synechocystis salina* | Freeze-dried biomass stock | 4,346 | 0,774 | 17,804 |
| LEGE 07167 | *Lusitaniella coriacea* | Freeze-dried biomass stock | 1,860 | 0,360 | 19,337 |
| LEGE 07171 | *Synechococcus nidulans* | Freeze-dried biomass stock | 3,050 | 0,774 | 25,372 |
| LEGE 07173 | *Cyanobium* sp. | Freeze-dried biomass stoc | 1,521 | 0,104 | 6,809 |
| LEGE 07175 | *Cyanobium* sp. | 4 L culture | 3,716 | 0,427 | 21,047 |
| LEGE 11396 | *Geitlerinema* sp. | - | - | - | - |
| VV11 | identification in process | Freeze-dried biomass stock | 1,001 | 0,087 | 8,719 |

### 3.13. MTT assay

The capability of the cells to reduce MTT presents an insight into the integrity and functionality of the mitochondria. Thus, the MTT assay represents a direct assessment of the celluar viability and indirect assessment of cell proliferation. The results regarding the cytotoxicity of the cyanobacteria fractions, after an incubation period of 24 and 48 hours, in the neuroblastoma,

fibroblasts, and endothelial cell lines used in the study, are presented in Figures 1, 2, and 3, respectively.

The choice of the cell lines used in this study was based in the fact that they are models widely used in AD research, particularly in evaluating the cytotoxicity of Aβ species in the presence of compounds, and the effect of compounds in terms of the BBB. In this case, it is important that the fractions are not toxic to the cell lines in question, which could compromise future work in the area.

For cytotoxicity assay, the fraction was considered cytotoxic when the percentage of cell viability was lower than 70%. To evaluate the effectiveness of the solvent and the measurement system, a positive control of 20% DMSO was used. This concentration was known to kill the cells and served as a benchmark for comparison. For the negative control, 1% DMSO was used, and its value represented the untreated cells thus determining the 100% of cell viability.

Regarding the SH-SY5Y cell line (Figure 1), a percentage of cellular viability lower than 70% was registered after an incubation period of 48 hours with 3 fraction of the LEGE 07167 namely LEGE 07167_B, LEGE 07167_C, and LEGE 07167_D, with values of 59,52%, 58,11%, and 61,91%, respectively. Percentages of cell viability above 130% were shown after a 24-hour period of incubation with the fractions LEGE 06099_E and LEGE 06113_E, with values of 146,43% and 130,85%, and after 48 hours of incubation with the fractions LEGE 07175_B and LEGE 07175_D, with cell viability values of 131,88% and 138,71, respectively. These results indicate that the fraction might have compounds that promote cellular growth.

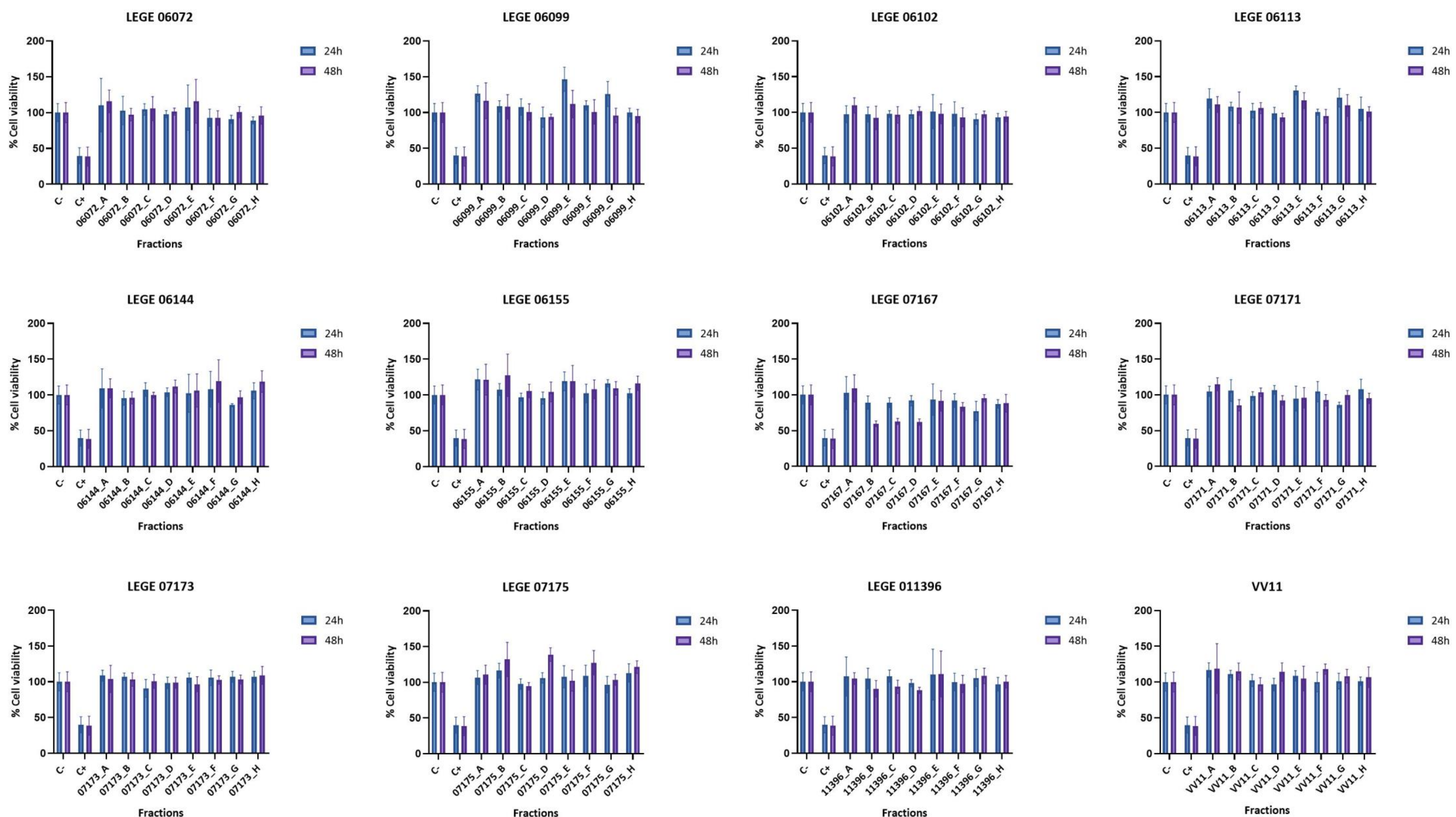



Figure 1 – Cell viability percentages after 24 and 48 hours of incubation period with the cyanobacteria fractions in the neuroblastoma cell line SH-SY5Y, and controls.

# 3T3-L1

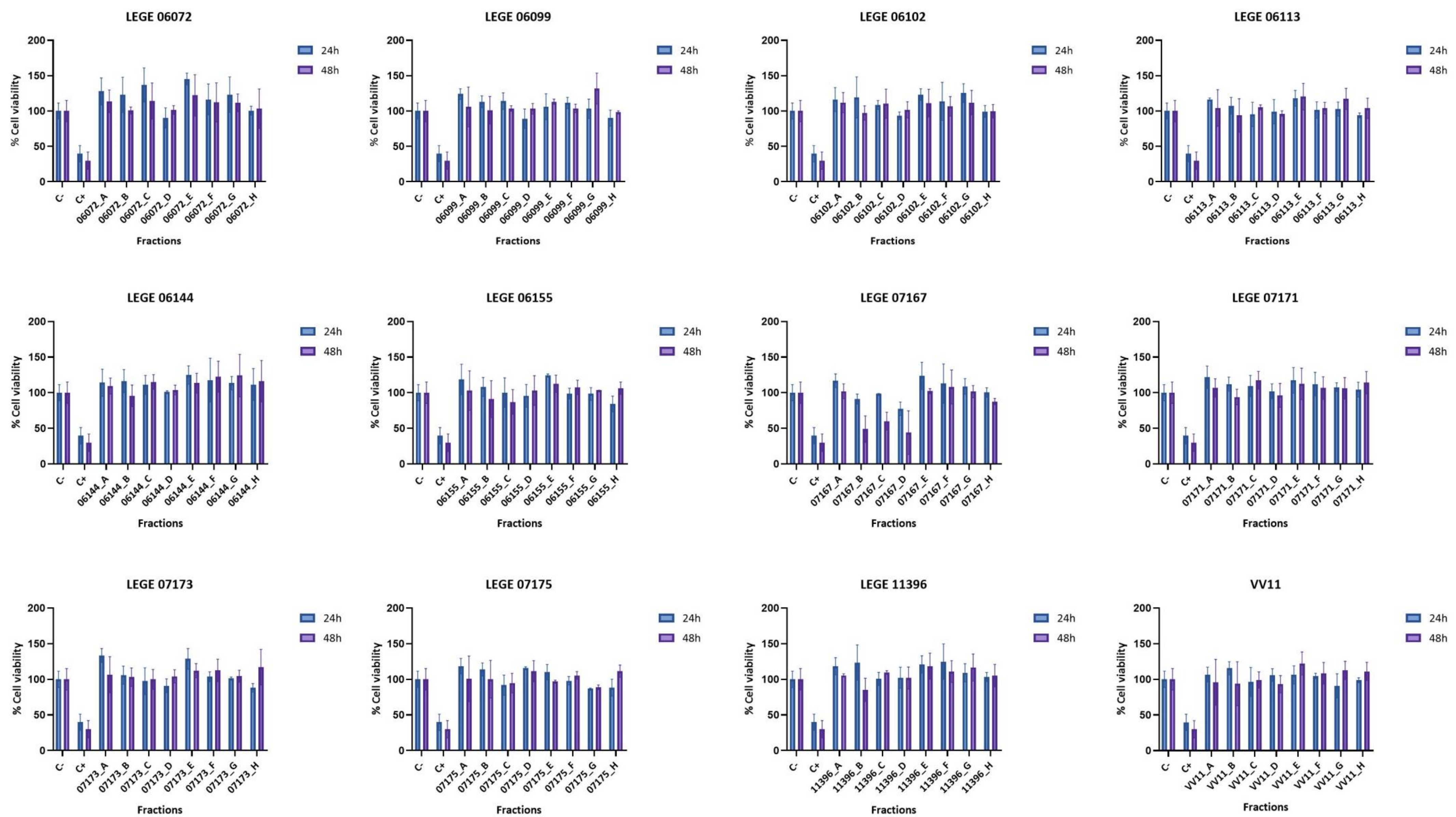



**Figure 2** – Cell viability percentages after 24 and 48 hours of incubation period with the cyanobacteria fractions in the fibroblast cell line 3T3-L1, and controls.

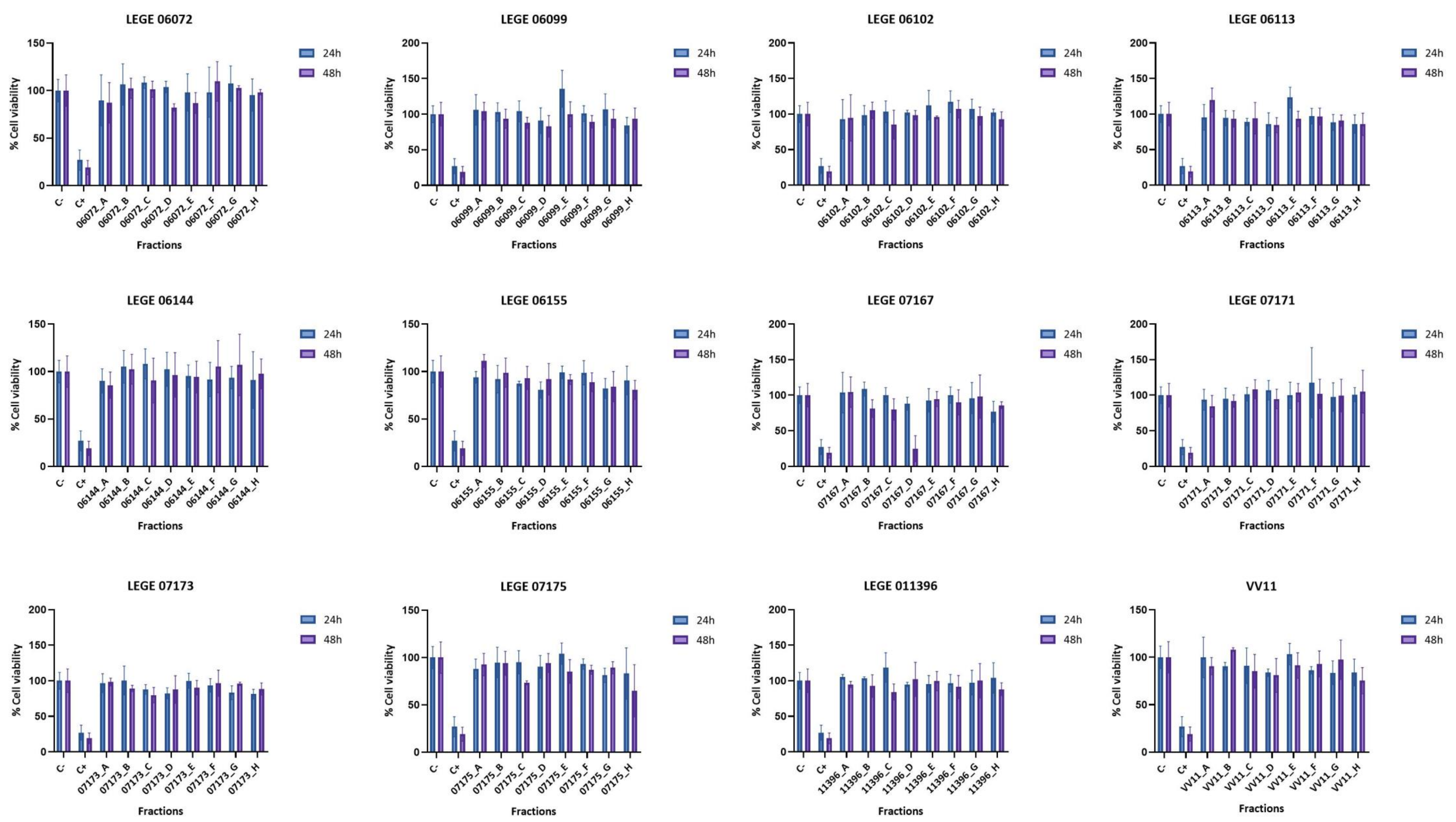



Figure 3 – Cell viability percentages after 24 and 48 hours of incubation period with the cyanobacteria fractions in the endothelial cell line hCMEC/D3 and controls.

Regarding the 3T3-L1 cell line (Figure 2), an incubation period of 48 hours with 3 cyanobacterial fractions, specifically LEGE 07167_B, LEGE 07167_C, and LEGE 07167_D, showed a cellular viability percentage below 70%. These fractions exhibited values of 48.92%, 60.02%, and 43.92%, respectively. On the other hand, when the fraction LEGE 07173_A was incubated for 24 hours, a cell viability percentage above 130% was observed, specifically 133.45%. Similarly, the fraction LEGE 06099_G showed a cellular viability of 131.71% after an incubation period of 48 hours. In general, the cellular viability percentages were higher compared to the SH-SY5Y results, which can be attributed to the fact that the neuroblastoma cell line is more sensitive and reactive. For the hCMEC/D3 endothelial cells (Figure 3), 2 cyanobacterial fractions displayed cytotoxic activity for this cell line. At the 48-hour point, the fractions LEGE 07167_D and LEGE 07175_H evoked a cellular viability percentage of 24,62% and 65,02%, respectively. The cytotoxic effect of the fraction LEGE 07175_H translates to low cytotoxicity. However, the standard deviation value was 27,59, indicating the need for the repetition of the assay with this particular fraction, in order to confirm its cytotoxic properties.

The fractions LEGE 07167_B and LEGE 07167_C showed cytotoxic effects, after an incubation period of 48 hours, in both neuroblastoma and fibroblast cell lines, while the fraction LEGE 07167_D demonstrated cytotoxic effects on the 3 cell lines. This percentage of cellular viability might indicate the presence, in these cyanobacterial fractions, of compounds with cytotoxic effects. Although these results are not aimed for in the context of this study, cytotoxic effects towards specific cell lines may offer therapeutic benefits such as bioactive anticancer properties.

As referred above, the SH-SY5Y and hCMEC/D3 cell lines are a common experimental model to study AD and the molecular events that lead to the progression of the neurodegenerative process. Understanding the cytotoxic effect of the cyanobacterial fractions in these cell lines links up to the potential therapeutic benefits that such compounds might have in the AD brain. In this work and concerning the SH-SY5Y cell line, the fraction E of strains LEGE 06099 and LEGE 06113, and fractions B and D of LEGE 07175 seem interesting for further studies. Three organic fractions obtained from HPLC fractionation of a dicloromethane:methanol (2:1) extracts from the same strains were also found to be not toxic to SH-SY5Y (74), which emphasizes the potential non-toxic nature of the strains to these cell line. In the same study the strains LEGE 06102, LEGE 06144 and LEGE 07173 were also found to be non-toxic, as in the present study.

Strains LEGE 06099 and LEGE 06113 were previously found to have high phenolic content (2.45 mg GAE g -1 and 1.41 mg GAE g -1 respectively) (75). In the same study strain LEGE 06099 was

the strain richest in total carotenoids, zeaxanthin and lutein, followed by LEGE 06102, and the highest DPPH scavenging activity was observed with strain LEGE 06099 (IC 50 = 863.82 µ g mL -1 ), followed by LEGE 06102 (IC 50= 1077.59 µ g mL -1) (75).These strains phytochemical composition and antioxidant potential could influence the cell viability and the antioxidant potential which could be interesting in future studies once it is recognized the role of oxidative stress in the development of AD.

Three fractions of strain LEGE 7167 namely B, C and D were found to be toxic to SH-SY5Y and 3T3-L1 cells. The same results were obtained using dichloromethane:methanol fractionation, which highlights the presence of toxic compounds in the strain (74).

Comparing the most promising strains considering the non-toxic effect on cell lines with the extraction yields presented in Table 10, it is interesting that strains such as LEGE 06099, LEGE 06113, LEGE 07175 and LEGE 06102 were the ones with the highest extraction yields (13,381%, 18,075%, 21,047%, and 27,226%, respectively). This result is of interest if the strains are indeed considered promising with more tests carried out, as it could mean a smaller volume of culture on an industrial scale.

### 3.14. Acetylcholinesterase assay

The acetylcholinesterase assay results are presented in Figure 4. A compilation of strains and fractions with AChE inibithion higher than 10% is presented in Table 11.

The cyanobacterial fraction's ability to inhibit the in vitro activity of the AChE eel enzyme, as a means to enhance the levels of ACh, was evaluated using the colorimetric Ellman's method with minor modifications. The AChE enzyme activity was indirectly measured by quantifying the absorption of the 5-thio-2-nitrobenzoic acid (TNB) ion, which is a product of the reaction between the thiol reagent DTNB and thiocholine. The results regarding the enzymatic inhibition are included in figure 4.

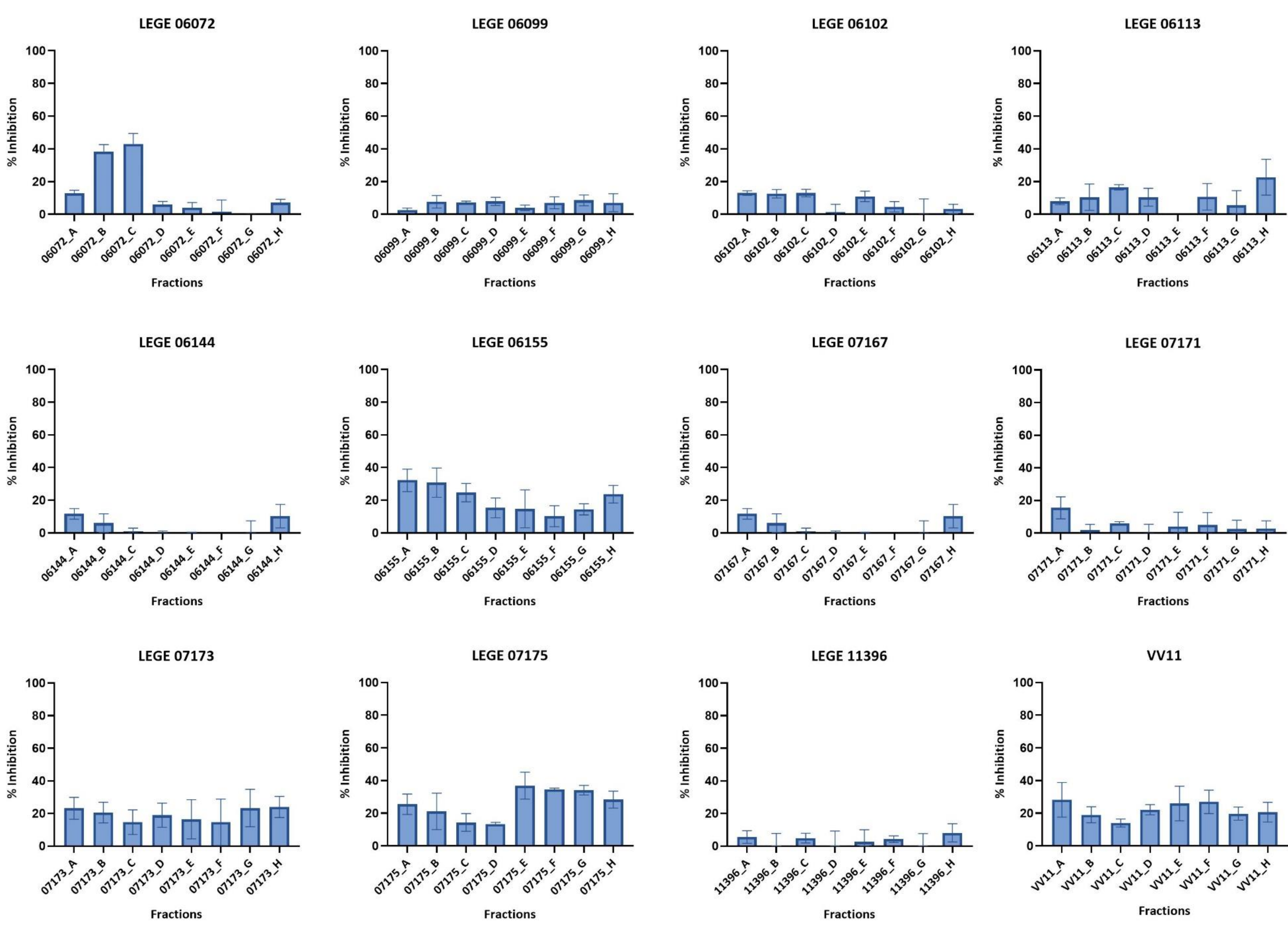



**Figure 4** – Percentage of AChE enzymatic inhibition of cyanobacterial fractions, at 500 μL/mL.

In the acetylcholinesterase assay, a considerate number of fractions demonstrated AChE enzymatic inhibitory activity, as shown in table 11. In table 12 is represented a resume of the strains and fractions that exhibited AChE inhibition activity above 30% provided along with the results of cell viability.

**Table 11.** Cyanobacterial fractions and respective percentage of AChE inhibition, at 500 μL/mL.

| Fraction | AChE inhibition (%) | Fraction | AChE inhibition (%) |
|---|---|---|---|
| LEGE 06072_A | 12,86 | LEGE 07173_A | 23,17 |
| LEGE 06072_B | 38,37 | LEGE 07173_B | 20,53 |
| LEGE 06072_C | 43,03 | LEGE 07173_C | 14,67 |
| LEGE 06102_A | 13,01 | LEGE 07173_D | 18,96 |
| LEGE 06102_B | 12,52 | LEGE 07173_E | 16,46 |
| LEGE 06102_C | 12,92 | LEGE 07173_F | 14,62 |
| LEGE 06102_E | 10,9 | LEGE 07173_G | 23,3 |
| LEGE 06113_B | 10,46 | LEGE 07173_H | 24,01 |
| LEGE 06113_C | 16,5 | LEGE 07175_A | 25,54 |
| LEGE 06113_D | 10,44 | LEGE 07175_B | 21,13 |
| LEGE 06113_F | 10,66 | LEGE 07175_C | 14,4 |
| LEGE 06113_H | 22,68 | LEGE 07175_D | 13,32 |
| LEGE 06144_A | 11,62 | LEGE 07175_E | 36,91 |
| LEGE 06144_H | 10,25 | LEGE 07175_F | 34,65 |
| LEGE 06155_A | 32,17 | LEGE 07175_G | 34,12 |
| LEGE 06155_B | 30,75 | LEGE 07175_H | 28,38 |
| LEGE 06155_C | 24,65 | LEGE VV11_A | 28,21 |
| LEGE 06155_D | 15,36 | LEGE VV11_B | 19,07 |
| LEGE 06155_E | 14,71 | LEGE VV11_C | 14,02 |
| LEGE 06155_F | 10,21 | LEGE VV11_D | 22,17 |
| LEGE 06155_G | 14,46 | LEGE VV11_E | 25,96 |
| LEGE 06155_H | 23,66 | LEGE VV11_F | 26,93 |
| LEGE 07167_A | 11,62 | LEGE VV11_G | 19,79 |
| LEGE 07167_H | 10,25 | LEGE VV11_H | 20,65 |
| LEGE 07171_A | 15,48 | | |

From the results the strains and fractions LEGE 06072_B, LEGE 06072_C, LEGE 06155_A, LEGE 06155_B, LEGE 07175_E, LEGE 07175_F, and LEGE 07175_G showed the highest potential in the AChE enzymatic assay. Although standard deviation values were considerably high, and repetition of the assay is needed these are promising results. Interestingly none of these fractions was found to induce cytotoxicity. The cognitive decline in Alzheimer's disease (AD) is partially caused by a shortage of cholinergic activity, which is a result of compromised cholinergic pathways in the cerebral cortex and basal forebrain. One way to improve cognitive function is by increasing the levels of acetylcholine in the brain through the inhibition of AChE activity, which breaks down acetylcholine (76). In this study, a significant number of cyanobacteria fractions demonstrated the ability to inhibit AChE enzymatic activity. In these strains and fractions the determination of a dose-response curve could represent the next step in a promising approach to the determination of the inhibitory potential of the fractions toward AChE by addressing the cholinergic deficit and thus ameliorating the progression of AD.

Comparing the most interesting cyanobacteria strains with the extraction yields presented in table 10 it is found that also in this case the strains present higher values, this is particularly evident to strains LEGE06155 and LEGE 07175 with extraction yields of 17,804% and 21,047%, respectively.

**Table 12.** Results of MTT cell viability assay and corresponding AChE inhibition percentage of cyanobacteria fractions.

| | SH-SY5Y | | 3T3-L1 | | hCMEC/D3 | | % AChE |
|---|---|---|---|---|---|---|---|
| | 24h | 48h | 24h | 48h | 24h | 48h | 500µg/mL |
| **LEGE 06072_B** | 102,93 | 97,20 | 122,86 | 100,80 | 103,195 | 108,12 | 38,37 |
| **LEGE 06072_C** | 104,55 | 105,56 | 136,92 | 114,10 | 111,605 | 105,115 | 43,03 |
| **LEGE 06155_A** | 121,56 | 121,41 | 118,58 | 103,01 | 101,02 | 117,32 | 32,17 |
| **LEGE 06155_B** | 107,53 | 127,48 | 108,11 | 94,40 | 107,03 | 92,55 | 30,75 |
| **LEGE 07175_E** | 107,79 | 102,32 | 97,96 | 105,50 | 123,79 | 99,51 | 36,91 |
| **LEGE 07175_F** | 109,02 | 127,34 | 86,74 | 88,83 | 98,78 | 82,22 | 34,65 |
| **LEGE 07175_G** | 96,62 | 103,55 | 88,32 | 111,42 | 89,94 | 83,69 | 34,12 |

Considering strain LEGE 06155, previous studies described a highest concentration of echinenone when compared to the other strains that were assessed. Echinenone is an essential carotenoid that plays a role in photoprotection, mainly due to its carbonyl group that has a part in protecting against photoinhibition of photosystem II. LEGE 07175, on the other hand, demonstrated the ability to enhance the growth of fibroblasts, which in turn leads to the synthesis of matrix fibers, including collagen. Additionally, this strain exhibited hyaluronidase inhibition activity, pointing to its potential role in combating skin aging and for skin care and cosmetic applications.

## 4. Conclusions

Alzheimer's disease, is a ND that affects millions of individuals and their families and caregivers, leads to cognitive impairment, memory loss, behavioural changes, and a loss of independence. Its devastating consequences represent a significant burden and a global impact. Effective treatments are still needed to improve the quality of life for individuals with Alzheimer's and to halt the neurodegenerative progress that this disease imposes. Governments and international organizations are recognizing the importance of addressing AD on a global scale through efforts that are being made by research promotion in this complex and challenging field, dementia care improvement, and the development of strategies to support affected individuals. In this light, the development of effective treatments for Alzheimer's has remained a challenge, while potential drug strategies fail to provide an effective response for this multifactorial disorder.

Cyanobacteria are known to produce a wide range of bioactive compounds with potential therapeutic applications. This biofactories and their produced bioactive compounds have demonstrated pharmacological properties that hold the potential for sustainable therapeutic approaches.

This study approached the therapeutic potential of cyanobacteria based on it's ability to inhibit AChE, an enzyme responsible for breaking down the neurotransmitter acetylcholine in the synaptic cleft, which plays an essential role in cognitive function. According to the demonstrated reduced level of acetylcholine in the brains of those with AD, the inhibition of AChE is a therapeutic strategy that mimics some of the action of the drugs already approved for this disease.

The cyanobacteria strains which demonstrated a noteworthy inhibitory AChE activity are *Oxynema acuminatum* LEGE 06072, *Synechocystis salina* LEGE 06155 and *Cyanobium* sp. LEGE 07175. *Synechocystis salina* LEGE 06155, a cyanobacterium that produces phycocyanin (77), demonstrated abilities include immune-stimulating properties, therapeutic antioxidant potential (78), and the ability to synthesize several bioactive compounds of interest (79). *Cyanobium sp.* LEGE 07175, a rich biosource of compounds with cosmetic applications (80), has demonstrated antioxidant properties and anti-inflammatory abilities and the capacity to generate a wide range of bioactive compounds by means of regulating its metabolic processes (81).

Taking this into account, and based on the reduced cytotoxicity that was verified in the MTT assay in the SH-SY5Y, 3T3, and hCMEC cell lines, and in the interesting inhibitory AChE activity verified, further studies should strive to assess the therapeutic potential of marine cyanobacteria

strains as a source of compounds that can give a therapeutic answer to the progressive nature of AD and a natural and sustainable approach in the search for a multifaceted answer to this multifactorial disease.

## Future perspectives

AD challenges require the resolution of different pieces. Cyanobacteria strains, which are of biotechnological interest due to their numerous bioactive compounds that show promise in therapeutics and pharmacology, can be considered as a potential aspect in this complex puzzle.

In the present study, the cyanobacteria strains *Oxynema acuminatum* LEGE 06072, *Synechocystis salina* LEGE 06155, and *Cyanobium* sp. LEGE 07175 exhibited the most encouraging outcomes. The demonstrated AChE inhibitory activity, along with the absence of cytotoxicity in the neuroblastoma, fibroblasts, and endothelial cell lines of the fractions, represents the optimal combination for advancing toward the achievement of novel therapeutic approaches to Alzheimer's disease. Accordingly, considering the beneficial role of AChE inhibitors in various conditions, including AD, it is imperative to persist in investigating the therapeutic capabilities of cyanobacteria and their metabolites in the context of the development of AChE inhibitors. The cyanobacteria strains Oxynema acuminatum LEGE 06072, *Synechocystis salina* LEGE 06155, and *Cyanobium* sp. LEGE 07175 are among the cyanobacteria strains that exhibited the highest extraction yields in this study. This could represent a further step in a more accessible assessment of these strains, more attainable cyanobacteria biomass production on a large scale, and the isolation of bioactive compounds with potential interesting biological activities.

Following these results, the next step in the assessment of the therapeutic potential towards AD of these microorganisms includes the evaluation of biological activies with beneficial properties including anti-inflammatory and antioxidant abilities, amyloid and tau protein oriented approaches to prevent plaque formation, neuroprotective assessment, the search for compounds which ameliorate vascular health, BACE1, BChE and $\gamma$ -Secretase inhibition assays, anti-aging potential, immunomodulating cyanobacterial products, cyanobacterial-derived neurotrophic factors, in vitro assays for drug screening and in vivo assays.

Overall, this study has shown minimal cytotoxicity towards the three cell lines and interesting AChE inhibition assays results, thus indicating that cyanobacteria still have numerous applications that can greatly benefit various fields. Whether it be in biotechnology, drug discovery, the food and nutraceutical industry, or the realm of natural products, cyanobacteria and its natural products continue to hold scientific promise and offer innovative approaches that remain vital for humanity.

## 5. Referências Bibliográficas


1. Wilson DM, Cookson MR, Van Den Bosch L, Zetterberg H, Holtzman DM, Dewachter I. Hallmarks of neurodegenerative diseases. Cell. 2023 Feb;186(4):693–714.
2. Zidan M, Arcoverde C, Araújo NB de, Vasques P, Rios A, Laks J, et al. Alterações motoras e funcionais em diferentes estágios da doença de Alzheimer. Archives of Clinical Psychiatry (São Paulo). 2012;39(5):161–5.
3. Iwasaki Y, Kinoshita M, Ikeda K, Takamiya K, Shiojima T. Cognitive impairment in amyotrophic lateral sclerosis and its relation to motor disabilities. Acta Neurol Scand. 2009 Jan 29;81(2):141–3.
4. Otaiku AI. Dream Content Predicts Motor and Cognitive Decline in Parkinson's Disease. Mov Disord Clin Pract. 2021 Oct 9;8(7):1041–51.
5. Kataoka M, Sahashi K, Tsujikawa K, Takeda J ichi, Hirunagi T, Iida M, et al. Dysregulation of Aldh1a2 underlies motor neuron degeneration in spinal muscular atrophy. Neurosci Res. 2023 Sep;194:58–65.
6. Teferedegn EY, Tesfaye D, Abebe E, Un C. Amylotrophic Lateral Sclerosis-Like Motor Impairment in Prion Diseases. Neurosci Med. 2019;10(01):15–29.
7. Soto C, Pritzkow S. Protein misfolding, aggregation, and conformational strains in neurodegenerative diseases. Nat Neurosci. 2018 Oct 24;21(10):1332–40.
8. Taoufik E, Kouroupi G, Zygogianni O, Matsas R. Synaptic dysfunction in neurodegenerative and neurodevelopmental diseases: an overview of induced pluripotent stem-cell-based disease models. Open Biol. 2018 Sep 5;8(9).
9. Seeley WW, Crawford RK, Zhou J, Miller BL, Greicius MD. Neurodegenerative Diseases Target Large-Scale Human Brain Networks. Neuron. 2009 Apr;62(1):42–52.
10. Cairns NJ, Lee VM, Trojanowski JQ. The cytoskeleton in neurodegenerative diseases. J Pathol. 2004 Nov 19;204(4):438–49.
11. Cyske Z, Gaffke L, Pierzynowska K, Węgrzyn G. Tubulin Cytoskeleton in Neurodegenerative Diseases–not Only Primary Tubulinopathies. Cell Mol Neurobiol. 2023 Jul 9;43(5):1867–84.
12. Cenini G, Lloret A, Cascella R. Oxidative Stress in Neurodegenerative Diseases: From a Mitochondrial Point of View. Oxid Med Cell Longev. 2019 May 9;2019:1–18.

13. Brice A. Unstable mutations and neurodegenerative disorders. J Neurol. 1998 Aug 3;245(8):505–10.
14. Neueder A. RNA-Mediated Disease Mechanisms in Neurodegenerative Disorders. J Mol Biol. 2019 Apr;431(9):1780–91.
15. Stephenson J, Nutma E, van der Valk P, Amor S. Inflammation in CNS neurodegenerative diseases. Immunology. 2018 Jun 17;154(2):204–19.
16. Andreone BJ, Larhammar M, Lewcock JW. Cell Death and Neurodegeneration. Cold Spring Harb Perspect Biol. 2020 Feb;12(2):a036434.
17. Rheault-Henry M, Hawes A, Léger E, Kpazaï G. La maladie d'Alzheimer et ses conséquences sur les dimensions de la santé de la personne. Diversity of Research in Health Journal. 2018 Sep 4;2:139–49.
18. Van Schependom J, D'haeseleer M. Advances in Neurodegenerative Diseases. J Clin Med. 2023 Feb 21;12(5):1709.
19. Mortada I, Farah R, Nabha S, Ojcius DM, Fares Y, Almawi WY, et al. Immunotherapies for Neurodegenerative Diseases. Front Neurol. 2021 Jun 7;12.
20. Müller T, Mueller BK, Riederer P. Perspective: Treatment for Disease Modification in Chronic Neurodegeneration. Cells. 2021 Apr 12;10(4):873.
21. Bahlakeh G, Rahbarghazi R, Mohammadnejad D, Abedelahi A, Karimipour M. Current knowledge and challenges associated with targeted delivery of neurotrophic factors into the central nervous system: focus on available approaches. Cell Biosci. 2021 Oct 12;11(1):181.
22. El Ouaamari Y, Van den Bos J, Willekens B, Cools N, Wens I. Neurotrophic Factors as Regenerative Therapy for Neurodegenerative Diseases: Current Status, Challenges and Future Perspectives. Int J Mol Sci. 2023 Feb 15;24(4):3866.
23. Ferrari C, Sorbi S. The complexity of Alzheimer's disease: an evolving puzzle. Physiol Rev. 2021 Jul 1;101(3):1047–81.
24. Villemagne VL, Doré V, Burnham SC, Masters CL, Rowe CC. Imaging tau and amyloid-β proteinopathies in Alzheimer disease and other conditions. Nat Rev Neurol. 2018 Apr 16;14(4):225–36.
25. Roda A, Serra-Mir G, Montoliu-Gaya L, Tiessler L, Villegas S. Amyloid-beta peptide and tau protein crosstalk in Alzheimer's disease. Neural Regen Res. 2022;17(8):1666.

26. Bali J, Gheinani AH, Zurbriggen S, Rajendran L. Role of genes linked to sporadic Alzheimer's disease risk in the production of β-amyloid peptides. Proceedings of the National Academy of Sciences. 2012 Sep 18;109(38):15307–11.
27. Zhao J, Huai J. Role of primary aging hallmarks in Alzheimer´s disease. Theranostics. 2023;13(1):197–230.
28. Arslan D, Mroczko J, Kulczyńska-Przybik A. Diagnosis of Alzheimers disease the role of biochemicalmarkers. Diagnostyka Laboratoryjna. 2022 Dec 16;58(3):120–9.
29. Bomasang-Layno E, Bronsther R. Diagnosis and Treatment of Alzheimer's Disease: Dela J Public Health. 2021 Sep 27;7(4):74–85.
30. Galvin JE, Aisen P, Langbaum JB, Rodriguez E, Sabbagh M, Stefanacci R, et al. Early Stages of Alzheimer's Disease: Evolving the Care Team for Optimal Patient Management. Front Neurol. 2021 Jan 22;11.
31. Breijyeh Z, Karaman R. Comprehensive Review on Alzheimer's Disease: Causes and Treatment. Molecules. 2020 Dec 8;25(24):5789.
32. Alkadhi K, Eriksen J. Editorial [Hot Topic: The Complex and Multifactorial Nature of Alzheimers Disease (Guest Editors: Karim Alkadhi and Jason Eriksen)]. Curr Neuropharmacol. 2011 Dec 1;9(4):586–586.
33. Chen G fang, Xu T hai, Yan Y, Zhou Y ren, Jiang Y, Melcher K, et al. Amyloid beta: structure, biology and structure-based therapeutic development. Acta Pharmacol Sin. 2017 Sep 17;38(9):1205–35.
34. Sun BL, Li WW, Zhu C, Jin WS, Zeng F, Liu YH, et al. Clinical Research on Alzheimer's Disease: Progress and Perspectives. Neurosci Bull. 2018 Dec 28;34(6):1111–8.
35. Vassar R, Bennett BD, Babu-Khan S, Kahn S, Mendiaz EA, Denis P, et al. β-Secretase Cleavage of Alzheimer's Amyloid Precursor Protein by the Transmembrane Aspartic Protease BACE. Science (1979). 1999 Oct 22;286(5440):735–41.
36. Hardy J, Selkoe DJ. The Amyloid Hypothesis of Alzheimer's Disease: Progress and Problems on the Road to Therapeutics. Science (1979). 2002 Jul 19;297(5580):353–6.
37. Mandelkow EM, Mandelkow E. Biochemistry and Cell Biology of Tau Protein in Neurofibrillary Degeneration. Cold Spring Harb Perspect Med. 2012 Jul 1;2(7):a006247–a006247.
38. Chen H, Xu J, Xu H, Luo T, Li Y, Jiang K, et al. New Insights into Alzheimer's Disease: Novel Pathogenesis, Drug Target and Delivery. Pharmaceutics. 2023 Apr 3;15(4):1133.

39. Medeiros R, Baglietto-Vargas D, LaFerla FM. The Role of Tau in Alzheimer's Disease and Related Disorders. CNS Neurosci Ther. 2011 Oct;17(5):514–24.
40. H. Ferreira-Vieira T, M. Guimaraes I, R. Silva F, M. Ribeiro F. Alzheimer's disease: Targeting the Cholinergic System. Curr Neuropharmacol. 2016 Jan 22;14(1):101–15.
41. McHardy SF, Wang HYL, McCowen S V., Valdez MC. Recent advances in acetylcholinesterase Inhibitors and Reactivators: an update on the patent literature (2012-2015). Expert Opin Ther Pat. 2017 Apr 3;27(4):455–76.
42. Bartus RT, Dean RL, Beer B, Lippa AS. The Cholinergic Hypothesis of Geriatric Memory Dysfunction. Science (1979). 1982 Jul 30;217(4558):408–14.
43. Mesulam MM, Mufson EJ, Levey AI, Wainer BH. Cholinergic innervation of cortex by the basal forebrain: Cytochemistry and cortical connections of the septal area, diagonal band nuclei, nucleus basalis (Substantia innominata), and hypothalamus in the rhesus monkey. J Comp Neurol. 1983 Feb 20;214(2):170–97.
44. Terry A V., Buccafusco JJ. The Cholinergic Hypothesis of Age and Alzheimer's Disease-Related Cognitive Deficits: Recent Challenges and Their Implications for Novel Drug Development. Journal of Pharmacology and Experimental Therapeutics. 2003 Sep;306(3):821–7.
45. Wang R, Reddy PH. Role of Glutamate and NMDA Receptors in Alzheimer's Disease. Journal of Alzheimer's Disease. 2017 Apr 19;57(4):1041–8.
46. Bak LK, Schousboe A, Waagepetersen HS. The glutamate/GABA-glutamine cycle: aspects of transport, neurotransmitter homeostasis and ammonia transfer. J Neurochem. 2006 Aug;98(3):641–53.
47. Yeung JHY, Walby JL, Palpagama TH, Turner C, Waldvogel HJ, Faull RLM, et al. Glutamatergic receptor expression changes in the Alzheimer's disease hippocampus and entorhinal cortex. Brain Pathology. 2021 Nov 16;31(6).
48. Castaneda A, Ferraz R, Vieira M, Cardoso I, Vasconcelos V, Martins R. Bridging Cyanobacteria to Neurodegenerative Diseases: A New Potential Source of Bioactive Compounds against Alzheimer's Disease. Mar Drugs. 2021 Jun 16;19(6):343.
49. Medzhitov R. Inflammation 2010: New Adventures of an Old Flame. Cell. 2010 Mar;140(6):771–6.

50. Ferrero-Miliani L, Nielsen OH, Andersen PS, Girardin SE. Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1β generation. Clin Exp Immunol. 2007 Jan 5;147(2):227–35.
51. Fiebich BL, Akter S, Akundi RS. The two-hit hypothesis for neuroinflammation: role of exogenous ATP in modulating inflammation in the brain. Front Cell Neurosci. 2014 Sep 1;8.
52. Garbuz DG, Zatsepina OG, Evgen'ev MB. Beta Amyloid, Tau Protein, and Neuroinflammation: An Attempt to Integrate Different Hypotheses of Alzheimer's Disease Pathogenesis. Mol Biol. 2021 Sep 19;55(5):670–82.
53. Nazem A, Sankowski R, Bacher M, Al-Abed Y. Rodent models of neuroinflammation for Alzheimer's disease. J Neuroinflammation. 2015 Dec 17;12(1):74.
54. Aranda-Abreu G, Hernández-Aguilar M, Herrera-Rivero M, Garca-Hernándes L. Drugs for Alzheimer's. J Addict Res Ther. 2011;s5.
55. Du X, Wang X, Geng M. Alzheimer's disease hypothesis and related therapies. Transl Neurodegener. 2018 Dec 30;7(1):2.
56. Gademann K, Portmann C. Secondary Metabolites from Cyanobacteria: Complex Structures and Powerful Bioactivities. Curr Org Chem. 2008 Mar 1;12(4):326–41.
57. Sciuto K, Moro I. Cyanobacteria: the bright and dark sides of a charming group. Biodivers Conserv. 2015 Apr 20;24(4):711–38.
58. Tan LT, Phyo MY. Marine Cyanobacteria: A Source of Lead Compounds and their Clinically-Relevant Molecular Targets. Molecules. 2020 May 8;25(9):2197.
59. Żymańczyk-Duda E, Samson SO, Brzezińska-Rodak M, Klimek-Ochab M. Versatile Applications of Cyanobacteria in Biotechnology. Microorganisms. 2022 Nov 23;10(12):2318.
60. Kultschar B, Llewellyn C. Secondary Metabolites in Cyanobacteria. In: Secondary Metabolites - Sources and Applications. InTech; 2018.
61. Liu J, Chen W, Xu Y, Ren S, Zhang W, Li Y. Design, synthesis and biological evaluation of tasiamide B derivatives as BACE1 inhibitors. Bioorg Med Chem. 2015 May;23(9):1963–74.
62. Patočka J, Gupta RC, Kuča K. ANATOXIN-A(S): NATURAL ORGANOPHOSPHORUS ANTICHOLINESTERASE AGENT. Military Medical Science Letters. 2011 Sep 9;80(3):129–39.

63. Becher PG, Beuchat J, Gademann K, Jüttner F. Nostocarboline: Isolation and Synthesis of a New Cholinesterase Inhibitor from *Nostoc* 78-12A. J Nat Prod. 2005 Dec 1;68(12):1793–5.

64. Carvalho LR, Costa-Neves A, Conserva GAA, Brunetti RL, Hentschke GS, Malone CFS, et al. Biologically active compounds from cyanobacteria extracts:in vivo and in vitro aspects. Revista Brasileira de Farmacognosia. 2013 May;23(3):471–80.

65. Monserrat JM, Yunes JS, Bianchini A. Effects of *Anabaena spiroides* (cyanobacteria) aqueous extracts on the acetylcholinesterase activity of aquatic species. Environ Toxicol Chem. 2001 Jun;20(6):1228–35.

66. Zelík P, Lukešová A, Voloshko LN, Štys D, Kopecký J. Screening for acetylcholinesterase inhibitory activity in cyanobacteria of the genus *Nostoc*. J Enzyme Inhib Med Chem. 2009 Apr 1;24(2):531–6.

67. Bortolini DG, Maciel GM, Fernandes I de AA, Pedro AC, Rubio FTV, Branco IG, et al. Functional properties of bioactive compounds from Spirulina spp.: Current status and future trends. Food Chemistry: Molecular Sciences. 2022 Dec;5:100134.

68. Riegerová P, Brejcha J, Bezděková D, Chum T, Mašínová E, Čermáková N, et al. Expression and Localization of AβPP in SH-SY5Y Cells Depends on Differentiation State. Journal of Alzheimer's Disease. 2021 Jul 21;82(2):485–91.

69. Dubey SK, Ram MS, Krishna KV, Saha RN, Singhvi G, Agrawal M, et al. Recent Expansions on Cellular Models to Uncover the Scientific Barriers Towards Drug Development for Alzheimer's Disease. Cell Mol Neurobiol. 2019 Mar 23;39(2):181–209.

70. Surin AM, Sharipov RR, Krasil'nikova IA, Boyarkin DP, Lisina OYu, Gorbacheva LR, et al. Disruption of functional activity of mitochondria during MTT assay of viability of cultured neurons. Biochemistry (Moscow). 2017 Jun 15;82(6):737–49.

71. Ghasemi M, Turnbull T, Sebastian S, Kempson I. The MTT Assay: Utility, Limitations, Pitfalls, and Interpretation in Bulk and Single-Cell Analysis. Int J Mol Sci. 2021 Nov 26;22(23):12827.

72. Liu DM, Xu B, Dong C. Recent advances in colorimetric strategies for acetylcholinesterase assay and their applications. TrAC Trends in Analytical Chemistry. 2021 Sep;142:116320.

73. Nhat TP, Trinh-Dang M. Optimization of Spirulina's Phycocyanin Extraction Yield Using Response Surface Method. Biotechnology Journal International. 2023 Apr 7;27(1):52–9.

74. Costa M, Garcia M, Costa-Rodrigues J, Costa M, Ribeiro M, Fernandes M, et al. Exploring Bioactive Properties of Marine Cyanobacteria Isolated from the Portuguese Coast: High Potential as a Source of Anticancer Compounds. Mar Drugs. 2013 Dec 31;12(1):98–114.
75. Morone J, Lopes G, Preto M, Vasconcelos V, Martins R. Exploitation of Filamentous and Picoplanktonic Cyanobacteria for Cosmetic Applications: Potential to Improve Skin Structure and Preserve Dermal Matrix Components. Mar Drugs. 2020 Sep 22;18(9):486.
76. McGleenon, Dynan, Passmore. Acetylcholinesterase inhibitors in Alzheimer's disease. Br J Clin Pharmacol. 1999 Oct;48(4):471–80.
77. Assunção J, Amaro HM, Malcata FX, Guedes AC. Factorial Optimization of Ultrasound-Assisted Extraction of Phycocyanin from Synechocystis salina: Towards a Biorefinery Approach. Life. 2022 Sep 6;12(9):1389.
78. Nováková M, Fábryová T, Vokurková D, Dolečková I, Kopecký J, Hrouzek P, et al. Separation of the Glycosylated Carotenoid Myxoxanthophyll from Synechocystis Salina by HPCCC and Evaluation of Its Antioxidant, Tyrosinase Inhibitory and Immune-Stimulating Properties. Separations. 2020 Dec 15;7(4):73.
79. Assunção J, Amaro HM, Tavares T, Malcata FX, Guedes AC. Effects of Temperature, pH, and NaCl Concentration on Biomass and Bioactive Compound Production by Synechocystis salina. Life. 2023 Jan 9;13(1):187.
80. Pagels F, Almeida C, Vasconcelos V, Guedes AC. Cosmetic Potential of Pigments Extracts from the Marine Cyanobacterium Cyanobium sp. Mar Drugs. 2022 Jul 27;20(8):481.
81. Pagels F, Bonomi-Barufi J, Vega J, Abdala-Díaz R, Vasconcelos V, Guedes AC, et al. Light quality triggers biochemical modulation of Cyanobium sp.—photobiology as tool for biotechnological optimization. J Appl Phycol. 2020 Oct 13;32(5):2851–61.

# 7. Appendix

## 7.1. MTT assay

Fractions A-H

(3 wells x 100µg per well x 4 plaques)

Vf = 1500 µL

0,01 x 1500 µL = 15 µL of cyanobacterial fraction

1500 µL -15 µL = 1485 µL of medium

Control

(12 wells x 100µg per well x 4 plaques)

Vf = 5000 µL

Negative Control: 1% DMSO

0,01 x 5000 µL = 50 µL of DMSO

5000 – 50 = 4950 µL of medium

Positive Control: 20% DMSO

0,2 x 5000 µL = 1000 µL of DMSO

5000 – 1000 = 4000 µL of medium