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Stalling the course of Alzheimer's Disease: can cyanobacteria be a new approach toward therapy?

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Stalling the course of Alzheimer's Disease: can cyanobacteria be a new approach toward therapy?

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Resumo

A doença de Alzheimer (DA) é uma doença neurodegenerativa debilitante, e é a principal causa de demência. É marcada pela perda progressiva de neurónios, acompanhada pela presença de placas amiloides, tranças neurofibrilares e outras alterações patológicas, incluindo deficiência colinérgica. Atualmente, não há cura para a DA e, embora existam tratamentos disponíveis para aliviar os sintomas, estes não impedem o avanço da doença. Os inibidores da acetilcolinesterase (AChE), como o donepezil, a galantamina e a rivastigmina têm sido a principal linha de tratamento, mas são necessários fármacos novos, mais eficazes e toleráveis. A biotecnologia azul lidera a procura de novos compostos bioativos e as cianobactérias, um grupo heterogéneo de microrganismos fotossintéticos, são uma fonte prolífica de compostos que já demonstraram potencial contra a DA. A *Blue Biotechnology and Ecotoxicology Culture Collection* (LEGE-CC) do CIIMAR, composta maioritariamente por estirpes de cianobactérias, é um recurso valioso que tem sido explorado para várias aplicações biomédicas. Neste trabalho, 22 estirpes de cianobactérias da LEGE-CC foram avaliadas quanto à sua citotoxicidade em células de neuroblastoma (SH-SY5Y), células endoteliais representativas da barreira hematoencefálica (hCMEC/D3) e fibroblastos (3T3-L1), e quanto ao seu potencial de inibir a AChE. A abordagem seguida incluiu o rastreio de frações de cianobactérias já existentes na biblioteca de produtos naturais (LEGE-NPL), a cultura de novas estirpes para obtenção de biomassa, preparação de extratos metanólicos e fracionamento destes extratos por HPLC. Por cada estirpe foram obtidas 8 frações (A-H), tendo no total sido testadas 176 frações. Os resultados de viabilidade celular, baseados no ensaio do MTT, mostraram que a maioria das frações não é citotóxica à concentração de 50 μg/mL, já que apenas 15 mostraram toxicidade com inibição da viabilidade celular acima de 30%. Nos ensaios de inibição da AChE *in vitro,* baseados no método de Ellman, as frações *Spirulina* sp. LEGE 11439_A e *Scytonema* sp. LEGE 07189_A (500 μg/mL) mostraram uma inibição baixa a moderada, de 29,15% e 21,73%, respetivamente, o que indica algum potencial numa triagem preliminar. Mais estudos devem ser realizados para explorar outras possíveis atividades contra a DA e para elucidar os perfis químicos das frações.

Palavras-chave: Doença de Alzheimer; cianobactérias; citotoxicidade; acetilcolinesterase; biotecnologia azul.

Abstract

Alzheimer's Disease (AD) is a severe, age-related neurodegenerative disease and it is the primary cause of dementia. It is marked by the progressive loss of neurons, accompanied by the presence of amyloid plaques, neurofibrillary tangles, and other pathological changes, including cholinergic deficiency. Currently, there is no cure for AD, and although treatments are available to alleviate symptoms, they do not hinder the advancement of the disease. Acetylcholinesterase inhibitors (AChEIs), such as Donepezil, Galantamine, and Rivastigmine are the main course of treatment, but new, more efficacious, and tolerable medications are needed. Blue biotechnology is leading the search for new bioactive compounds. Cyanobacteria, a heterogeneous group of photosynthetic microorganisms, are a prolific source that has shown promise in the field of AD. The Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC) housed at CIIMAR, composed mainly of cyanobacteria strains, is a valuable resource that has been exploited for various biomedical applications. In this work, 22 cyanobacteria strains from the LEGE-CC were evaluated for their cytotoxicity in neuroblastoma cells (SH-SY5Y), endothelial cells representative of the blood-brain barrier (hCMEC/D3), and fibroblasts (3T3-L1), and for their potential to inhibit AChE. The approach included the screening of existing cyanobacterial fractions in the natural products library (LEGE-NPL), cultivating new strains to obtain biomass, preparing methanolic extracts, and HPLC fractionation. For each strain, 8 fractions (A-H) were obtained, with a total of 176 fractions tested. The cell viability results, based on the MTT assay, showed that most of the fractions are not cytotoxic at a concentration of 50 μg/mL, as only 15 showed toxicity, with inhibition of cell viability above 30%. In the *in vitro* AChE inhibition assay, based on Ellman's method, the fractions *Spirulina* sp. LEGE 11439_A and *Scytonema* sp. LEGE 07189_A (500 μg/mL) showed low to moderate inhibition of 29.15% and 21.73%, respectively, which indicates some potential in a preliminary screening. Further research can be undertaken to investigate additional anti-AD activities and to elucidate the chemical profiles of the fractions.

Keywords: Alzheimer's Disease; cyanobacteria; cytotoxicity; acetylcholinesterase; blue biotechnology.

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List of Abbreviations

3T3-L1 – Embryonic mouse fibroblast cell line

Aβ – Amyloid-β peptide

ACh – Acetylcholine

AChE – Acetylcholinesterase

AChEIs – Acetylcholinesterase inhibitors

AD – Alzheimer's Disease

Akt – Serine/threonine protein kinase

ALR – Augmenter of the liver regeneration

ApoE – Apolipoprotein E

APP – Amyloid precursor protein

ATCI – Acetylcholine iodide

BACE-1 – β-secretase

Bax – Bcl-2 Associated X-protein

BBB – Blood-brain barrier

BChE – Butyrylcholinesterase

Bcl-2 – B-cell lymphoma-2

BDNF – Brain-derived neurotrophic factor

BMMA – β-N-methylamino-L-alanine

BOGA – Bioterium of aquatic organisms

BSA – Albumin

CAT – Catalase

Cct4 – Chaperonin containing TCP1 subunit 4 gene

ChAT – Choline acetyltransferase

ChEIs – Cholinesterase inhibitors

CIIMAR – Interdisciplinary Center of Marine and Environmental Research

CNS – Central nervous system

COX – Cyclooxygenase

CSF – Cerebrospinal fluid analysis

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

DTNB – 5,5-bisdithionitrobenzoic acid

EDPC – Enzyme-digested C-phycocyanin

Endog – Endonuclease G gene

FDA – Food and Drug Administration

GFAP – Glial fibrillary acid protein

GSH – Glutathione

GSH-Px – Glutathione peroxidase

GSK3β – Glycogen synthase kinase 3

Hbq1a – Hemoglobin theta 1A gene

hCMEC/D3 – Human cerebral microvascular endothelial cell line

HDAC3 – Histone deacetylase 3

HPLC – High-performance liquid chromatography

IBA-1 – Ionized calcium-binding adapter molecule 1

IC_{50} – Half-maximal inhibitory concentration

IGF-1 – Insulin-like growth factor 1

IgG – Immunoglobulin G

IL – Interleukin

INS – Insulin

IRS-1 – Insulin receptor substrate 1

KDEL – Endoplasmic reticulum retention signal

LEGE-CC – Blue Biotechnology and Ecotoxicology Culture Collection

LEGE-NPL – Cyanobacteria Natural Products Library

MAP – Microtubule-associated protein

Map9 – Microtubule associated protein 9 gene

MCI – Mild cognitive impairment

MDA – Malondialdehyde

MeCN – Acetonitrile

Mia40 – Mitochondrial intermembrane space assembly protein 40

mRNA – Messenger RNA

MTA – Material transfer agreement

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

NAD(P)H – Nicotinamide adenine dinucleotide phosphate

ND – Neurodegenerative disease

NF-kβ – Nuclear factor

NFTs – Neurofibrillary tangles

NMDA – N-methyl-D-aspartate

PBS – Phosphate-buffered saline

PET – Positron emission tomography

PI3K – Phosphatidylinositol-3 kinase

Pik3cg – Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma gene

Prnp – Prion protein gene

PSEN-1 – Presenilin-1

PSEN-2 – Presenilin-2

PTEN – Phosphatase and tensin homolog gene

PTFE – Polytetrafluoroethylene

ROS – Reactive oxygen species

SFE-EtOH – Supercritical fluid extraction with ethanol

SH-SY5Y – Human neuroblastoma cell line

SOD – Superoxide dismutase

SPLN – *S. platensis*-loaded noisome

sTREM-2 –Soluble triggering receptor expressed on myeloid cells-2

TAC –Total antioxidant capacity

TBARS –Thiobarbituric acid reactive substances

TNB^2 – 5-thio-2-nitrobenzoate

TNF-α – Tumor necrosis factor alfa

Tris–HCl – Tris-hydrochloride

Vegfd – Vascular endothelial growth factor D gene

Zfand5 – Zinc finger AN1-type containing 5 gene

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1. Introduction

Dementia is a clinical syndrome marked by significant cognitive and functional decline that surpasses that of the normal aging process. It represents a serious public health concern, with over 55 million cases worldwide and ranking as the seventh leading cause of death. As the population and life expectancy increase, it is expected that these numbers will rapidly escalate and reach 139 million cases by 2050 (1). Portugal exceeds the general European trend, with 193 thousand cases in 2018, and is expected to reach 346 thousand by 2050 (2). It is considered to be the 4th country with the highest number of cases per thousand inhabitants (3). Dementia is also a major cause of disability and dependency, with an estimated economic burden of 1.3 trillion US dollars (1).

Alzheimer's Disease (AD) is the primary cause of dementia, accounting for 60–70% of all cases, and thus statistics of AD are often intertwined with those of dementia (1, 4). AD is a complex, age-related neurodegenerative disease (ND) with a multifactorial etiology. The primary hallmarks are the buildup of toxic amyloid-β (Aβ) species and the formation of hyperphosphorylated tau protein tangles in the brain. These, along with other degenerative pathways, cause progressive and irreversible damage to neurons, primarily affecting regions responsible for memory, language, and cognitive functions (4, 5).

The diagnosis and treatment of AD are challenging. Although considerable efforts have been made, only a few drugs have been approved to provide temporary symptom relief, rather than a cure. Disease-modifying therapies that can alter the progression of the disease are still in the early stages (6). As a result, patients may live for up to 20 years while experiencing cognitive, behavioral, social, and functional changes that are ultimately fatal (5).

The high failure rate of AD drug development, with 99% of trials failing, emphasizes the need to identify novel and effective pharmacological candidates and targets (7). Natural products derived from plants, algae, macrofungi, invertebrates, and microorganisms have traditionally been key contributors to drug development due to their great diversity and structural complexity (8). In fact, natural compounds, synthetic derivatives, and pharmacophore-inspired drugs account for more than 60% of all approved drugs (9).

Cyanobacteria are primitive photosynthetic microorganisms that serve as an excellent example of a naturally abundant source of bioactive compounds. They offer a sustainable and versatile option, producing a wide range of metabolites with diverse pharmacological properties, such as

neuroprotective, antioxidant, anti-inflammatory, and immunomodulatory ones (10–12), which can be an asset in the treatment of NDs.

The Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC, https://lege.ciimar.up.pt) at the Interdisciplinary Center of Marine and Environmental Research (CIIMAR) comprises more than 700 strains well distributed throughout the cyanobacterial tree of life, which were primarily isolated from aquatic (freshwater and marine) and terrestrial (subaerial and soil) environments in Portugal, as well as from other various geographical locations around the world (13, 14). Research on LEGE-CC cyanobacteria has led to the identification of multiple compounds and extracts with biotechnological applications, namely anticancer, cosmetics, and antifouling (14–17). However, their potential against neurodegeneration is still largely untapped, making them a valuable resource for biomedical research in this field.

Considering that AD is a growing cause of mortality and morbidity, finding new effective treatment options is of great socioeconomic significance. As the exploitation of cyanobacteria as a source of lead compounds represents a promising approach, this project specifically aimed to evaluate the therapeutic potential of cyanobacteria from the LEGE-CC against AD.

1.1. Alzheimer's Disease (AD)

AD is classified as an ND, which refers to a group of neurological disorders that result in progressive and irreversible loss of neurons, leading to impairment of brain function (18).

The discovery of AD is attributed to the psychiatrist Alois Alzheimer, who in 1907, during an autopsy of a patient with dementia, observed unusual anatomical features, including the presence of abnormal fibrous inclusions, which served as the basis for the description of the disease (19). A century later, the concept of AD has evolved significantly. Originally viewed as a neuropathological entity of a clinical syndrome characterized by amnestic dementia and invariably linked to a specific pathologic dyad, the disease is now recognized as a biological disorder that can develop gradually from normal cognition to dementia, encompassing a diverse array of clinical phenotypes that may affect memory, cognition, behavior, sensory, and/or motor function (19, 20).

AD is a heterogeneous disorder, making it impossible to attribute its pathology to a single process. The complexity of this disorder renders it challenging to prevent, diagnose, and treat (4). The following sections provide an overview of the pathophysiology, etiology, diagnosis, and treatment of AD.

1.1.1. Pathophysiology and therapeutical targets

The pathophysiology of AD is a complex and multifaceted process that unfolds over an extended period, and it is characterized by pathophysiological changes that may emerge as far as two decades prior to the onset of clinical symptoms (5). The key neuropathological alterations include positive lesions resulting from the accumulation of Aβ-plaques, neurofibrillary tangles (NFTs), and other deposits, as well as negative lesions resulting from atrophy, such as neural and synaptic losses (21) (Figure 1). These occur alongside other pathological processes such as neuroinflammation, oxidative stress, mitochondrial dysfunction, cholinergic neuronal injury, excitotoxicity, vascular and ion channel dysfunction, all of which lead to neuronal death and atrophy, primarily in the entorhinal cortex and hippocampus (4, 21).

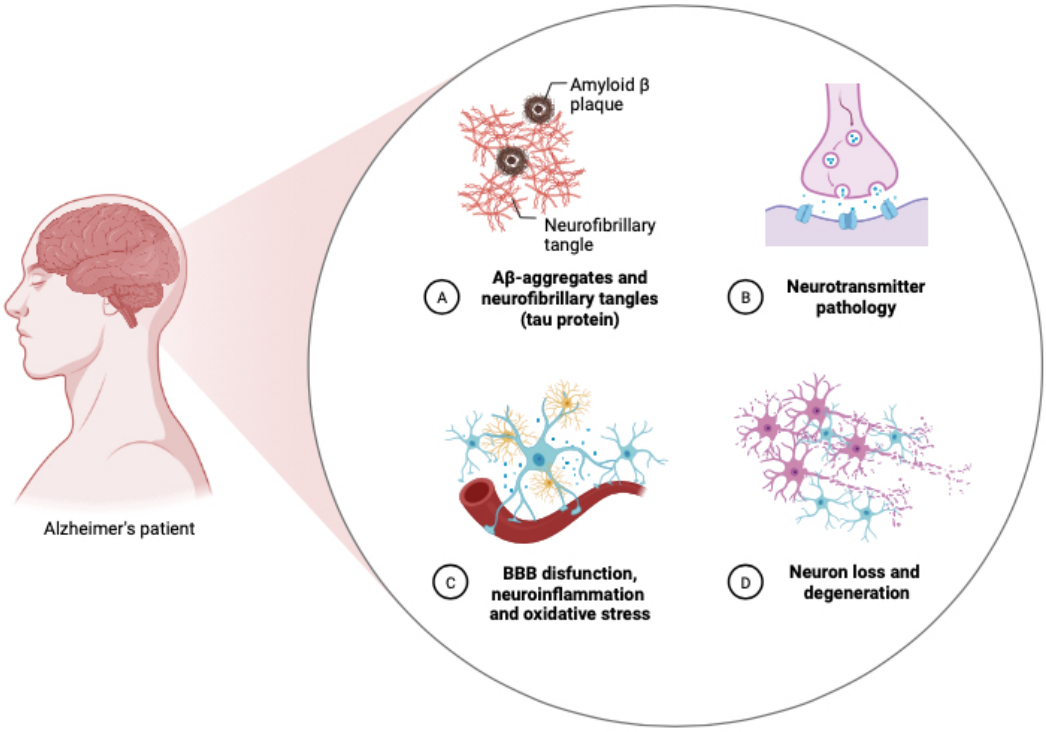


Figure 1. Main neuropathological features of Alzheimer's Disease. Created in Biorender.com.

1.1.1.1. Amyloid-β (Aβ) pathology

Aβ is a peptide that, under physiological conditions, is believed to play a role in neuronal cytoprotective pathways and synaptic functions (22). It is derived from the amyloid precursor protein (APP), which can be processed through either the non-amyloidogenic pathway, which generates non-neurotoxic peptides, or the amyloidogenic pathway, where APP is cleaved by β-secretase (BACE-1) and subsequently by γ-secretase, resulting in peptides with 40 or 42 amino acids ($A\beta_{40}$ and $A\beta_{42}$). $A\beta_{42}$ is more neurotoxic and prone to aggregation than $A\beta_{40}$, and in AD the

ratio $A\beta_{42}$/ $A\beta_{40}$ is increased (22). The neurotoxicity can be mediated through toxic small, soluble oligomers that disseminate throughout the brain, or through the accumulation of larger, insoluble fibrils that form plaques. The Aβ-plaques, which can be diffuse or classical, are insoluble clusters of Aβ fibrils, neurites, and glial elements in the extracellular spaces between nerve cells, particularly in the hippocampus, cerebral cortex, brainstem, and eventually the cerebellum (23).

The Aβ cascade hypothesis is a well-established theory, and it states that the buildup of Aβ in the brain is a central event in AD (24). According to this theory, an increase in Aβ aggregation and a decrease in Aβ clearance lead to the accumulation of Aβ, which triggers a neurodegenerative cascade. This is the driving force behind other pathological changes, such as tau phosphorylation, oxidative stress, neuroinflammation, and synaptic dysfunction, ultimately resulting in neuronal death (22, 24).

However, it was noted that Aβ-plaques also accumulate in the brains of healthy aging individuals and that direct inhibition of Aβ formation is not successful, leading to the proposal of alternative hypotheses (23, 25).

Aβ-plaques are still promising targets, and several approaches can be pursued, such as inhibitors of β and γ-secretase, anti-aggregation drugs, drugs that regulate protease activity, and immunotherapy (23, 26).

1.1.1.2. Tau pathology

Tau is a microtubule-associated protein (MAP) with a critical function in the stabilization of microtubules, the preservation of cytoskeletal integrity, and the transport processes within neurons. There are six isoforms of tau, and AD is associated with disruptions in the equimolar ratio of 3R and 4R tau isoforms (27). In AD, tau also undergoes post-translational modifications, specifically hyperphosphorylation in the serine/threonine residues, due to the increased activity of kinases (GSK3β) and the dysregulation of phosphatases. This leads to conformational changes that cause it to detach from microtubules and aggregate into paired helical filaments, causing the formation of NFTs, a twisted and tangled structure within the neuron's cell body, dendrites, and axon. These disrupt the normal cytoskeletal architecture of neurons, ultimately leading to neuronal death (28).

The spread of tau pathology is thought to occur in a prion-like manner, with affected areas starting in the hippocampus and entorhinal cortex. Abnormal tau protein seeds can be released

from damaged neurons and taken up by neighboring cells, leading to the progression of the disease (29).

The tau hypothesis centers around the part played by tau in the pathology of AD (30). Hyperphosphorylated tau directly hinders cellular functions, such as axonal transport, and disrupts synaptic function. Additionally, it drives other processes like neuroinflammation and oxidative stress and interacts bidirectionally with Aβ to worsen neurodegeneration. In fact, NFTs directly correlate with cognitive impairment (26, 28).

Several types of drugs that target tau protein can be explored as potential treatments for AD including tau kinase inhibitors, phosphatase enhancers, tau assembly inhibitors, microtubule stabilizers, and immunotherapies (27, 29).

1.1.1.3. Cholinergic pathology

The cholinergic hypothesis is a pivotal concept that poses that the impairment in cholinergic neurotransmission contributes to cognitive deficits in AD. Acetylcholine (ACh) is a neurotransmitter required for cognitive functions. In AD patients, there is a pronounced degeneration of cholinergic neurons, mainly in the *nucleus basalis of Meynert*, as these neurons are particularly vulnerable to the pathological changes that occur in AD, namely Aβ-plaques and NFTS. Hence, there is an imbalance in ACh metabolism, as the number of ACh receptors and the expression of the synthesis enzyme, the acetyltransferase (CHAT), are reduced, while the activity of the hydrolysis enzyme, the acetylcholinesterase (AChE) remains incredibly efficient, contributing to a very fast depletion of ACh in the synaptic cleft (31, 32). This has a negative impact on cognitive functions, including memory, attention, and problem-solving, and it is linked to aggregation of Aβ, increased phosphorylation, neuroinflammation, and an imbalance in other neurotransmitters (33).

This theory is supported by the current commercial use of AChE inhibitors (AChEIs), which reduce the rate of ACh degradation by AChE, thus increasing the availability of ACh at synapses, leading to temporary enhancement of cholinergic neurotransmission and concomitant cognitive benefits. Other alternative treatments under investigation include choline supplementation and modulators of ACh receptors (34).

1.1.1.4. Excitotoxicity

The glutamatergic hypothesis in AD delves deeper into the role of the neurotransmitter glutamate. Glutamate is the primary excitatory neurotransmitter in the central nervous system and plays a crucial role in synaptic plasticity. In AD patients, there is damage to glutamatergic neurons and a disruption in glutamate clearance and recycling mechanisms (35). This can be due to excessive glutamate release from presynaptic neurons related to disruptions in calcium homeostasis; a compromise in the function/expression of glutamate transporters, such as those present in astrocytes; or changes in the expression and function of glutamate receptors, particularly the N-methyl-D-aspartate (NMDA) receptor, which become more susceptible to excitotoxicity (35). The elevated synaptic glutamate levels promote an overactivation of receptors, creating a rapid and prolonged influx of calcium and sodium ions into neurons, resulting in persistent depolarization and excitotoxicity. This triggers the activation of apoptotic cell death pathways that culminate in synaptic injury and neuronal death (36).

Excessive glutamate is related to mitochondrial dysfunction and ROS overproduction and has been shown to enhance the accumulation of Aβ and NFTs, further exacerbating neurodegeneration (35, 37).

This theory is confirmed by the use of NMDA receptor antagonists, which prevent the activation of this pathway. Glutamate transporter enhancers or synaptic modulators are other possible therapeutic strategies (26, 37).

1.1.1.5. Neuroinflammation

The neuroinflammation hypothesis explores the intricate interplay between the immune system and the AD brain. Whilst acute neuroinflammation is protective, chronic neuroinflammation is detrimental, as it can independently and directly influence the functioning of brain networks, being implicated in the genesis of AD (38).

The inflammatory response in the brain is mediated by microglia and astrocytes, the innate immune cells of the central nervous system (CNS). Harmful *stimuli*, such as Aβ plaques and NFTs, persistently activate microglia and shift it toward a pro-inflammatory phenotype, triggering the release of inflammatory mediators such as interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α) and reactive oxygen species (ROS), and a decrease in phagocytic activity. In response, astrocytes become activated and release more inflammatory mediators, contributing to synaptic

impairment and blood-brain barrier (BBB) dysfunction. This allows peripheral immune cells, such as T cells, to infiltrate the CNS, amplifying the local immune response (39, 40).

A chronic neuroinflammatory state contributes to the disruption of neuronal signaling, oxidative stress, tau phosphorylation, and Aβ pathology, thereby perpetuating a vicious cycle of neurodegeneration (41, 42).

The use of anti-inflammatory drugs, which inhibit the enzyme cyclooxygenase (COX), or monoclonal antibodies targeting specific immune factors may be useful to alleviate AD symptoms (34, 42)

1.1.1.6. Oxidative stress

The oxidative stress hypothesis is a widely established concept in AD pathology. Oxidative stress is caused by an imbalance between the production of ROS and the antioxidant defense system, promoting cellular injury by lipid, protein, and DNA damage (43). The CNS is particularly vulnerable to oxidative stress owing to its high metabolic rate and oxidizable substrate content (43, 44).

In AD, there is excessive ROS production, even prior to the onset of clinical symptoms (45). Major contributors are impaired redox systems, mitochondrial dysfunction, and loss of metal homeostasis, as high levels of free copper, iron, and zinc ions in AD brains act as effective catalysts of ROS generation (46). Mitochondrial dysfunction, resulting from impaired mitochondrial dynamics and quality control, leads to a disruption of the respiratory chain and energy metabolism, with an increase in ROS production. It also activates cellular apoptosis pathways, increasing neuronal death (47).

Furthermore, in a complex and reciprocal interplay, oxidative stress causes and is caused by pathological events such as Aβ aggregation, tau pathology, and neuroinflammation (48).

Strategies to mitigate oxidative stress include metal chelators, drugs to improve mitochondrial dysfunction, like fission inhibitors, and drugs to enhance the antioxidant system, which comprises enzymatic antioxidants like superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), or nonenzymatic antioxidants like vitamin C (34, 46, 47).

1.1.1.7. Other hypothesis

Several other hypotheses linked to AD have also been frequently discussed. Conditions such as vascular damage, that impairs blood flow and consequent oxygen and nutrient supply to the brain, metabolic disturbances, including insulin resistance and glucose dysregulation, exposure to

heavy metals, pesticides, and air pollution, untreated viral or bacterial infections, and dysbiosis and gut inflammation may all trigger inflammation, oxidative stress, and immune responses that amplify neurodegeneration and contribute to AD pathology (34).

1.1.2. Risk factors

The development of AD is not attributable to a single cause, but rather a combination of factors, with age being the most significant risk factor. Other contributing factors can be categorized as either modifiable or non-modifiable, and include genetic, acquired, and environmental factors (49).

The incidence of AD increases exponentially with age (50), with nearly 95% of cases occurring in individuals aged 65 years or older, known as late-onset AD, while the remaining 5% occur in individuals under the age of 65, referred to as early onset AD (51). The likelihood of developing AD also appears to be higher in women than in men (52).

Genetics is another major risk factor. AD can be categorized as either sporadic or familial autosomal dominant, with the latter being the rarest (53). The apolipoprotein ε4 (ApoE ε4) gene variant is the most significant genetic risk factor, being expressed in more than half of AD patients. Although the exact mechanism of action is unclear, it is linked to impaired Aβ clearance and other pathological processes (54). Several other critical genes have been identified, such as the gene that encodes APP, and the presenilin-1 (PSEN-1) and presenilin-2 (PSEN-2) genes, both of which encode two subunits of the γ-secretase complex responsible for the processing of APP. These mutations lead to increased production and/or aggregation of Aβ (53). Down syndrome is also a risk factor because the extra chromosome 21 contains an extra copy of the gene that encodes APP (53).

Other factors that increase the risk of developing AD include cerebrovascular disease; metabolic factors such as hypertension, diabetes *mellitus*, obesity, and hypercholesterolemia; and other conditions such as traumatic brain injury or depression. Tobacco and alcohol consumption, sleep habits and stress, as well as exposure to air pollution and heavy metals, can also contribute to the disease (55).

On the other hand, some factors, such as protective genes like the ApoE ε2 allele, may reduce the risk of developing AD. A higher cognitive level, regular physical activity, a healthy, antioxidant-rich diet, and participation in leisure and social activities are all beneficial (55).

1.1.3. Diagnosis and clinical staging

The diagnosis of AD is based on clinical and biological criteria. This requires evidence of both a specific clinical phenotype and pathological biomarkers. A patient should undergo several tests, including a neurological examination to determine the presence and severity of cognitive impairment, as well as examinations such as positron emission tomography (PET) or cerebrospinal fluid analysis (CSF) of known biomarkers of amyloid and tau pathology (CSF $A\beta_{42}$ and p-tau). The clinical staging of AD is performed through the AD continuum, which has three major phases, as summarized in Table 1 (56). AD is ultimately fatal due to complications, and patients live an average of 4 to 8 years after diagnosis (5).

Table 1. AD staging and main characteristics.

Phase		Characteristics
Pre-Clinical AD		Biomarker evidence such as CSF/PET $A\beta_{42}$ and p-tau (at risk of developing AD); Asymptomatic.
Mild Cognitive Impairment (MCI) due to AD		Pathological changes in the hippocampus; Patients with memory, language, and thinking impairments that do not interfere with daily life.
Dementia due to AD	**Mild AD**	Pathological changes reach the cerebral cortex; Patients with cognitive and executive functioning impairment that may interfere with daily life.
	Moderate AD	Pathological changes spread further; Patients with worse cognitive, functional, social, and behavioral symptoms may require assistance.
	Severe AD	Pathological changes affect the entire cortex; Patients lose control of basic bodily functions and their independence, needing around-the-clock care.

1.1.4. Treatment

Despite ongoing efforts, only a limited number of drugs have been approved by the U.S. Food and Drug Administration (FDA) for the treatment of AD. While these medications may help to alleviate some symptoms, such as cognitive and behavioral dysfunction, they are unable to stop the progression of the disease (6).

The most commonly used class of drugs is AChEIs. AChE is the enzyme responsible for ACh hydrolysis, turning it into inactive choline and acetate metabolites. It is highly efficient from a kinetic perspective, leading to a rapid turnover time and a decrease in ACh levels within the synaptic cleft (31). ACh is also degraded by butyrylcholinesterase (BChE). Although AChE is more

prevalent in the brain tissue of AD patients, the activity of BChE is elevated in these individuals, thus making it an important target as well. Furthermore, AChE plays a role in multiple other processes, including the formation of Aβ aggregates (33).

Thus, cholinesterase inhibitors (ChEIs) prevent the degradation of ACh, increasing its synaptic levels and therefore enhancing the deficient brain cholinergic neurotransmission. They can be classified as nonspecific when they inhibit AChE or BuChE, and specific when they inhibit only one of them (57). They can also form a short, medium (reversible), or long-term (irreversible) bond with the enzyme, with only those that form reversible bonds being considered potential therapeutic agents (58).

AChEIs include donepezil, galantamine, and rivastigmine (57). Donepezil is a reversible, non-competitive inhibitor of AChE and is also effective in reducing glutamate-induced excitotoxicity. It has received approval for the symptomatic management of mild to moderate cases of AD and has been demonstrated to enhance cognitive function and improve basic activities of daily living. Galantamine is an isoquinoline alkaloid that has been approved for the treatment of mild to moderate AD. It acts as a selective, competitive, and reversible inhibitor of AChE, which helps to improve cognitive and behavioral symptoms in patients with the disease. Rivastigmine is an effective pseudo-inhibitor of both AChE and BChE. It is also indicated for the treatment of mild and moderate cases of AD, due to its ability to enhance cognitive function and improve daily living activities (6, 57).

In general, AChEIs have demonstrated the ability to enhance cholinergic neurotransmission while reducing Aβ protein deposits, resulting in stabilization of cognitive decline, improvement in overall condition, and decrease in mortality in individuals with mild to moderate AD. However, they have been associated with several adverse effects, including nausea, vomiting, diarrhea, abdominal pain, anorexia, headache, insomnia, muscle cramps, bradycardia, and syncope, which may lead to discontinuation of treatment (59).

Another class of pharmaceuticals currently employed are NMDA receptor antagonists, including memantine. This agent functions by blocking receptor activity and reducing the deleterious impact of elevated glutamate levels. Memantine is approved for the management of moderate to severe AD, and its use has been associated with improvements in cognitive function, behavior, and mood (6). Combinatorial strategies are also a viable option. The combination of memantine with donepezil (Namzaric®) is approved for the symptomatic treatment of moderate to severe AD

and is more effective as it prevents the toxic effects of excess glutamate and the breakdown of ACh in the brain (6, 60).

Recently, immunotherapies, which are disease-modifying treatments, have been approved by the FDA. Aducanumab (Aduhelm™) is a human IgG1 monoclonal antibody that specifically targets soluble Aβ protein fibrils and oligomers, thereby improving Aβ clearance. It was approved in 2021 for MCI and mild AD cases. However, it has since been withdrawn from Europe (61, 62). Lecanemab-irmb (Leqembi™) was granted approval in 2023. It is a humanized IgG1 monoclonal antibody that targets aggregated soluble and insoluble forms of the Aβ protein, thereby reducing Aβ plaques. It is used to treat MCI and mild AD (63).

Many more treatments have been tested in clinical trials but have failed, while others are undergoing, indicating that there is an opportunity for research in this field (7). Altogether, it is imperative to develop newer and more effective drugs to improve treatment outcomes.

1.2. Cyanobacteria

Cyanobacteria, also known as green-blue algae, are a diverse phylum of gram-negative, prokaryotic organisms. They differ from other bacteria in that they are the only prokaryotes that can perform oxygenic photosynthesis and possess *chlorophyll-a* (64). Cyanobacteria were among the first organisms to live on Earth, with more than 3.5 billion years of fossil records. These organisms are key oxygen producers and nitrogen fixers that play important roles in ecosystems and in shaping the biosphere (65). Cyanobacteria exhibit diverse morphologies, from single cells to colonies and filaments, and can be present in high densities, such as in crusts or blooms. They thrive in a wide range of environments, including freshwater, marine, and terrestrial ecosystems, even those deemed hostile to life (64).

Cyanobacteria's ability to adapt and survive is a result of their metabolic diversity, flexibility, and reactivity, which involves unique biochemical pathways that yield a variety of metabolites including proteins, essential fatty acids, vitamins, minerals, flavonoids, carotenoids, chlorophylls, and phycobiliproteins (65, 66). Cyanobacteria also offer economical and sustainable advantages, as they have fast-growing potential with high yields without the need for many resources, making them an appealing option for biomedical research (67).

Cyanobacteria's health benefits have long been documented, as *Nostoc* species have been used to treat gout, fistulas, and cancer since 1500 B.C., and Aztecs employed *Spirulina* strains as a food source (67, 68). Several cyanobacteria-derived metabolites have been identified, exhibiting

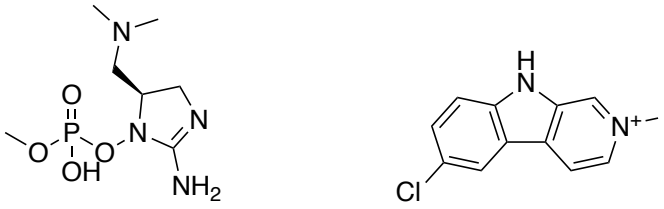
anticancer, antiviral, antibacterial, and antidiabetic properties, among others. Some of these, such as the anticancer drug Adcetris™, are in commercial use, whereas others are undergoing preclinical and clinical trials (69, 70).

Regarding neuroprotection, cyanobacteria produce several neuroactive compounds that have been linked to ecological roles, such as enhancing competitiveness in grazing defense by reducing palatability and repelling predators (71). However, the effects of cyanobacteria-derived products can vary widely, from the medicinal potential of phycocyanin to lethal cyanotoxins like microcystins, nodularin, and β-N-methylamino-L-alanine (BMAA), whose exposure has been associated with the onset of NDs (71, 72).

1.3. Cyanobacteria against Alzheimer's Disease

The potential of cyanobacteria against AD is vast, as reviewed by Castaneda et al. (2021) (71). Recent studies have reinforced this hypothesis (Table 2).

One of the most explored treatment approaches for AD is restoring cholinergic signaling, through the use of ChEIs (33). Cyanobacteria-derived AChE and BChE inhibitors have been reported. Anatoxin-a(S) **(1)** (Figure 2) from *Anabaena flos-aquae* is an irreversible AChE inhibitor, but it is also a potent neurotoxin that can cause severe cholinergic poisoning when administered to rats (0.1-1.0 mg/kg) (73). Nostocarboline **(2)** (Figure 2) from *Nostoc* is an inhibitor of AChE and BChE, with half-maximal inhibitory concentration (IC_{50}) values of 5.3 μM (74) and 13.2 μM (75), respectively. However, it is also a neurotoxin, showing moderate toxicity when tested in crustaceans (74). Although described as a potent neurotoxin produced by cyanobacteria, anatoxin-a(S) is also one of the least understood and monitored (76). In fact, as recently reviewed, studies involving cyanobacteria neurotoxins such as anatoxin-a (S) in standardized neuronal cell lines and mammals are still scarce and results are inadequate to confirm its real toxicity (77).



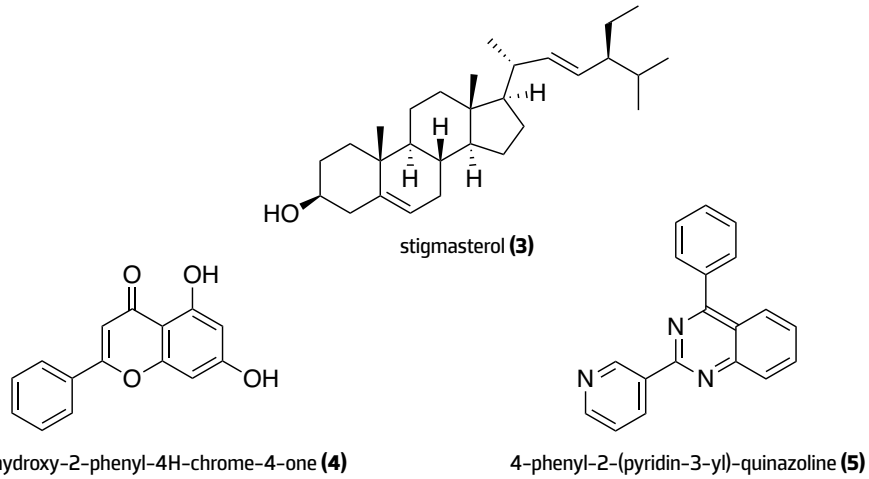
anatoxin-a(S) **(1)** nostocarboline **(2)**

Figure 2. Structure of anatoxin-a(S) **(1)** and nostocarboline **(2)**, cyanobacteria-derived AChE and BChE inhibitors.

A phytosterol-rich extract of *Phormidium autumnale* obtained through supercritical fluid extraction with ethanol (SFE-EtOH) revealed moderate to high inhibitory activity against AChE

(IC_{50} = 65.80 µg/mL) and lipoxygenase (IC_{50} = 58.20 µg/mL) while showing a high antioxidant capacity (IC_{50} = 7.40 µg/mL). The presence of the phytosterol stigmasterol **(3)** (Figure 3) in the extract significantly correlates with AChE inhibition, as it showed interactions with several AChE binding sites in molecular docking assays (78).

Refaay et al. (2022) (79) found that fraction 7 of the *Anabaena variabilis* methylene chloride/methanol (1:1) extract effectively reduced AChE activity (73.6%). This can be due to the presence of two aromatic compounds, the flavonoid 5,7-dihydroxy-2-phenyl-4H-chrome-4-one **(4)** and the alkaloid 4-phenyl-2-(pyridin-3-yl)-quinazoline **(5)**, shown in Figure 3, which interact with the allosteric binding site of AChE in molecular docking studies.



stigmasterol **(3)**

5,7-dihydroxy-2-phenyl-4H-chrome-4-one **(4)**

4-phenyl-2-(pyridin-3-yl)-quinazoline **(5)**

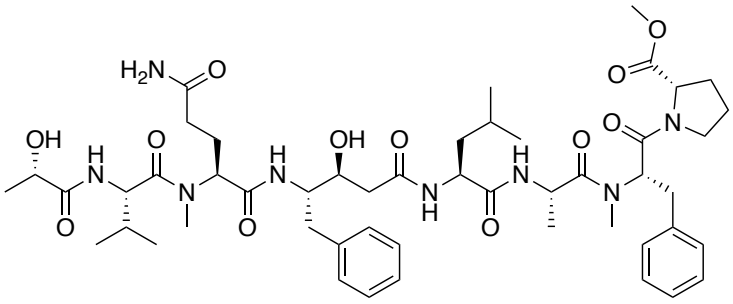
Figure 3. Structure of stigmasterol **(3)**, 5,7-dihydroxy-2-phenyl-4H-chrome-4-one **(4)** and 4-phenyl-2-(pyridin-3-yl)-quinazoline **(5)**, which interact with AChE *in silico*.

In another *in vitro* experiment, a crude methylene chloride/methanol (1:1) extract of *Oscillatoria sancta* lowered AChE activity by 60.7% (80). The ethanolic extract of *Nostoc* sp. also showed significant inhibitory action against AChE (69.9%) at 3 mg/mL and against BChE (72.7%) at 5 mg/mL, as well as a high radical scavenging ability (81).

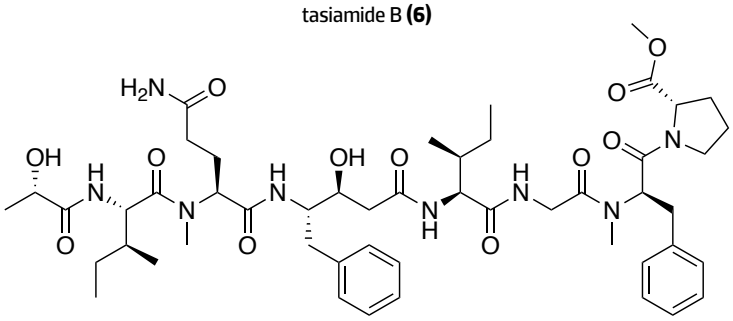
Other possible therapeutic targets include lowering the Aβ load, which can be accomplished by hindering Aβ formation (22). Luo and Jing (2020) (82) showed that phycocyanin (0.5–50 µg/mL) from *Spirulina* sp. spontaneously inhibits the Aβ formation process of bovine serum albumin (BSA) by interacting in a gomphosis structure. Another study found that phycocyanin at a 5:1 (Aβ: phycocyanin) molar ratio had anti-amyloidogenic activity, as seen by its ability to inhibit $A\beta_{40/42}$ fibrillation (83).

The inhibition of the amyloidogenic pathway enzymes is an important strategy for reducing Aβ-peptide synthesis. This stops the conversion of APP into Aβ-peptide via sequential proteolytic

cleavages by BACE-1 and γ-secretase enzymes (22). BACE-1 inhibitors derived from cyanobacteria have been identified, such as tasiamide B **(6)** (Figure 4) isolated from *Symploca* sp. (84, 85) and its analog tasiamide F **(7)** (Figure 4) from *Lyngbya* sp. (86). Tasiamide B (IC_{50} = 80 nM) is eight times more effective than tasiamide F (IC_{50} = 690 nM) due to modifications in the residues that engage in hydrophobic interactions with the receptor's pocket and provide the inhibitory effect (86). These can be the starting point for the design of more potent and selective BACE-1 inhibitors (84, 85).



tasiamide B **(6)**



tasiamide F **(7)**

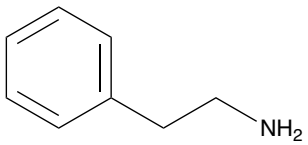
Figure 4. Structure of the cyanobacterial BACE-1 inhibitor tasiamide B **(6)** and its analog tasiamide F **(7)**.

Phycobiliproteins from cyanobacteria also have potential as BACE-1 inhibitors. Molecular docking studies show that phycocyanin from *Leptolyngbya* sp. N62DM interacts with BACE-1 in an energetically favorable manner (87). In the same study, an experiment was conducted using *Caenorhabditis elegans* CL4176, a transgenic model of AD that expresses $A\beta_{1-42}$ in its muscle cells. It was found that phycocyanin administered through the medium (100 µg/mL) was able to rescue paralysis worms (87). Similarly, Chaubey et al. (2019) (88) found that phycoerythrin from *Lyngbya* sp. A09DM exhibited significant interaction and binding affinity with BACE-1 in molecular docking studies and protein-protein interactions *in vitro*. These results were also further supported by *in vivo* experiments on *C. elegans* CL4176, where treatment with phycoerythrin (100 µg/mL) led to a reduction in Aβ deposition and senile plaque formation.

A study looked at the effects of oral pre-treatment with a 70% ethanol extract of *Spirulina maxima* (SM70EE) on rats with cognitive impairment caused by intracerebroventricular injection

of $A\beta_{1-42}$. The extract (150 and 450 mg/kg/day) decreased the levels of APP and BACE-1, thereby reducing APP processing and lowering Aβ accumulation in the hippocampus. It also improved cognition, reduced AChE activity, and suppressed hippocampal oxidative stress by improving the antioxidant system. The treatment stimulated the brain-derived neurotrophic factor (BDNF)/phosphatidylinositol-3 kinase (PI3K)/serine/threonine protein kinase (Akt) signaling pathway, which reduced GSK3β phosphorylation, contributing to BACE-1 suppression (89).

Galizzi et al. (2023) (90) studied the effects of KlamExtra®, a supplement derived from *Aphanizomenon flos-aquae*, in a high-fat diet rodent model of neurodegeneration. KlamExtra® is a combination of the patented extracts Klamin® and AphaMax®. Klamin® contains a concentrated dose (15-18 mg) of phenylethylamine **(8)** (Figure 5), a compound that modulates both the nervous and immune systems, as well as phycocyanins, mycosporine-like amino-acids and phytochrome, which are neuroprotectants and selective monoamine oxidase B inhibitors (91). Additionally, AphaMax® is rich in phycocyanins (25-30%) and polyphenols, which are powerful antioxidants, and anti-inflammatory molecules (92). Specially polyphenols were also found to be involved in the regulation of autophagy in various NDs (93). Treatment with KlamExtra® (0.9 mg/mouse), induced a pattern of decreased BACE-1 and PSEN-1 expression, resulting in reduced APP processing and accumulation of Aβ. It also safeguarded neural function and synaptic transmission by elevating synaptophysin levels and maintaining normal neuronal morphology. Furthermore, the extract improved the levels of metabolic markers related to glucose metabolism and showed anti-inflammatory properties by increasing IL-10 and modulating the astrocyte and microglia activation, with a decrease in the astrocyte marker glial fibrillary acid protein (GFAP) and an increase in soluble triggering receptor expressed on myeloid cells-2 (sTREM-2) (90). Especially the increase in the immunosuppressive cytokine IL-10 has been described as promising in NDs therapeutics as recently reviewed (94).



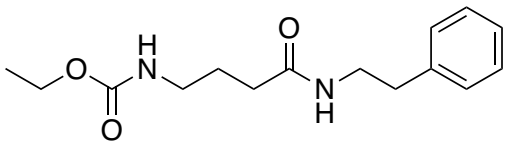
phenethylamine **(8)**

Figure 5. Structure of phenethylamine **(8)**, one of the main components of the Klamin® extract.

Neurofibrillary tangles, which are composed of hyperphosphorylated tau protein, are also a hallmark of AD. Kinases, mainly GSK3β, are responsible for tau phosphorylation, and thus, reducing enzymatic activity can reduce tau load (95). In a study with Wistar rats treated with

nicotine, a daily intraperitoneal injection with *Spirulina platensis*-lipopolysaccharides (100 μg/kg) provided neuroprotection by suppressing the up-regulation of phosphorylated-tau ratio expression by 2-folds while showcasing antioxidant, anti-inflammatory, and anti-apoptotic activities (96). Dietary supplementation of 1% and 2% *S. platensis* dry powder in high-fat diet mice lowered the tau burden by reducing both phosphorylated-tau and phosphorylated-GSK levels, while it also decreased $A\beta_{1-42}$ concentrations, APP and BACE-1 levels in the hippocampus (97).

AD has also been linked to mitochondrial dysfunction and endoplasmic reticulum stress. Santacruzamate A **(9)** (Figure 6), a compound produced by a marine cyanobacterium cf. *Symploca* sp. has shown therapeutic potential *in vitro* and *in vivo*. It inhibited the $A\beta_{25-35}$-induced apoptosis in PC12 cells (2 μM STA), by reversing the endoplasmic reticulum and unfolded protein response stress. It regulated the endoplasmic reticulum retention signal (KDEL) receptor, which increased chaperone luminal retention. Compound **9** also restored the mitochondrial intermembrane space assembly pathway and regulated the expression of the mitochondrial intermembrane space assembly protein 40 (Mia40) and the augmenter of the liver regeneration (ALR) system, resulting in a reduction in the mitochondrial fission and apoptosis pathways (98). This was confirmed by *in vivo* studies in APPswe/PS1dE9 mice, a common AD mouse model bearing mutant transgenes of APP and PSEN-1, which leads to an early-onset increase in parenchymal Aβ-levels and other clinically relevant AD-like symptoms (99). Treatment with santacruzamate A **(9)** (5 and 10 mg/kg/day) promoted memory performance in behavioral tests and enhanced KDELR and Mia40-ALR functions in the brain tissue (98).



santacruzamate A **(9)**

Figure 6. Structure of santacruzamate A **(9)**, a carbamate derivative with neuroprotective activity.

Another pathological aspect of AD is heavy metal bioaccumulation, and reversing its toxicity can improve disease outcomes. In Wistar rats, tablets of *S. platensis* (1500 mg/kg) revealed neuroprotective potential against brain degeneration induced by aluminum chloride ($AlCl_3$). While lowering the number of illuminated Aβ protein aggregates, the treatment also reduced histopathological alterations in the cerebral cortex and hippocampus, with close to normal neuron morphology and fewer neurodegenerative features. It also improved metabolic indices and

demonstrated anti-inflammatory activity through the reduction of TNF-α. The tablets showed strong antioxidant potential by decreasing thiobarbituric acid reactive substances (TBARS) levels while restoring glutathione (GSH), thiol levels, and total antioxidant capacity (TAC) (100).

In a study by Abdelghany et al. (2023) (101), an *S. platensis*-loaded niosome (SPLN) formulation was explored as a drug delivery system in an $AlCl_3$-induced AD rat model. The use of nanoparticles enables more effective, controlled, and targeted brain treatment. *S. platensis*-loaded niosome (300 mg/kg) improved recognition and working memory and demonstrated neuroprotective activity by maintaining normal morphology in hippocampal brain tissue. Additionally, it restored AChE activity, ACh, and monoamine levels in the brain and also improved the oxidative state, as it lowered the malondialdehyde (MDA) levels and TAC (101).

Growing data suggest that AD is associated with dysbiosis of the human gut microbiota via neuroinflammatory processes across the microbiota-gut-brain axis, suggesting that modifying the gut microbiota could be a strategy for treating the condition (102). According to Zhou et al. (2021) (97), dietary supplementation with 1% and 2% *S. platensis* dry powder in high-fat diet mice alleviated cognitive impairment and restored gut microbial dysbiosis by increasing the Shannon, ACE, and Chao indices while decreasing the Simpson Index, indicating enhanced microbial community richness and diversity. It improved the intestinal environment by balancing microbiota, increasing the abundance of beneficial microorganisms, such as *Verrucomicrobia*, while reducing the presence of harmful microorganisms, like *Firmicutes*. Supplementation also lowered inflammatory lipopolysaccharide levels in feces and serum and raised fecal levels of short-chain fatty acids, which improves neuronal homeostasis. Furthermore, it showed anti-inflammatory benefits by lowering inflammatory markers such as GFAP, TNF-α, IL-1β, IL-6, and ionized calcium-binding adapter molecule 1 (IBA-1) in the hippocampus (97).

Aside from the modes of action outlined above, cyanobacteria, particularly *Spirulina* and its component phycocyanin, largely work through gene modulation.

In a study conducted in rodents intracerebroventricularly injected with $A\beta_{25-35}$, the oral pre-treatment with a proteolysis product of phycocyanin (EDPC) from *S. platensis* (750 mg/kg) improved cognitive impairment in a Y maze spontaneous alternation test and modulated the gene expression profile in a DNA microarray analysis. It counteracted the aberrant expression of 35 genes, including Prnp, Cct4, Vegfd, Map9, Pik3cg, Zfand5, Endog, and Hbq1a, which are directly linked to AD or other neurological diseases (103).

In C57BL/6 mice injected with oligomeric $A\beta_{1-42}$, treatment with phycocyanin (200 mg/kg) from *S. platensis* improved spatial memory and reversed the epigenetic dysregulation. It restored the expression of the regulatory miRNA-335, which was downregulated by 76%, and the expression of the BDNF gene, which was reduced to 24% in Aβ-mice. On the other hand, it downregulated the histone deacetylase 3 (HDAC3) gene, whose expression was amplified 3-fold in Aβ-mice. The treatment also showed anti-apoptotic and anti-inflammatory effects, by restoring protein B-cell lymphoma-2 (Bcl-2)/ Bcl-2 Associated X-protein (Bax) equilibrium, decreasing caspase-3 and caspase-9 release, as well as lowering inflammatory cytokine levels (IL-6 and IL-1β) (104).

Agrawal et al. (2020) (105) demonstrated that phycocyanin administration (100 mg/kg) in an intracerebroventricular streptozotocin-induced AD-mice model improved spatial memory and reduced memory impairment in behavioral tests. It improved metabolic parameters, by restoring the gene expression of insulin signaling molecules such as the insulin (INS) gene, insulin receptor substrate 1 (IRS-1), PI3K, and Akt. Thereby, it increased the activation of the insulin-PI3K-Akt pathway, while it lowered the expression of one of its inhibitors, the phosphatase and tensin homolog (PTEN) gene. In addition, the treatment upregulated the anti-apoptotic marker Bcl-2, whereas the pro-apoptotic marker Bax was downregulated. It also altered ACh metabolism by lowering AChE activity while increasing ChAT in the hippocampus and mitigated neuroinflammation by reducing TNF-α and nuclear factor (NF)-kβ levels (105).

In another study, treatment with *S. platensis*-loaded niosome (300 mg/kg) modulated gene expression, restoring the mRNA levels of the enzymes AChE and monoamine oxidase and reversing the $AlCl_3$-induced decrease in Bcl-2 and increase in Bax mRNA levels (101).

Human clinical trials have also validated the potential of *Spirulina* as a nutraceutical. Patients with mild cognitive impairment who consumed 1 g/day of *S. maxima* 70% ethanol extract (SM70EE) capsules experienced statistically significant gains in visual learning and working memory, according to a randomized, double-blind, placebo-controlled clinical trial (106). Another randomized, double-blind, controlled clinical trial investigated the cognitive and metabolic status of patients with AD who took *S. platensis* capsules twice daily (500 mg/day). Supplementation considerably improved the Mini-Mental State Examination score, indicating an enhancement in cognitive function. It also had a favorable impact on metabolic status by lowering C-reactive protein, fasting glucose, insulin levels, and insulin resistance while increasing insulin sensitivity (107).

Table 2. Cyanobacteria-derived products/extracts studied in AD disease models.

Strain	Compound/Extract	Effect	*In Vitro* Assays	*In Vivo* Assays	Reference
Anabaena flos-aquae NRC-525-17	Anatoxin-a(s) **(1)**	AChE and BChE inhibition	AChE and BChE inhibition assay		(73)
Nostoc 78-12A	Nostocarboline **(2)**	BChE inhibition	AChE and BChE inhibition assay		(75)
Phormidium autumnale	SFE-EtOH extract	AChE and LOX inhibition. Antioxidant.	AChE inhibition assay. LOX inhibition assay. ORAC assay.		(78)
Anabaena variabilis	Methylene chloride/ methanol extract (Fraction 7)	AChE inhibition	AChE inhibition assay		(79)
Oscillatoria sancta	Methylene chloride/ methanol (1:1) extract	AChE inhibition	AChE inhibition assay		(80)
Nostoc sp.	Ethanolic Extract	AChE and BChE inhibition. Antioxidant.	AChE and BChE inhibition assay. DPPH assay.		(81)
Spirulina sp.	Phycocyanin	Inhibition of Aβ formation	Fluorimetric assay. Kinetic analysis. Circular dichroism analysis.		(82)
Spirulina sp.	Phycocyanin	Inhibition of $A\beta_{40/42}$ amyloid fibrillation	Fibrillar and amorphous aggregation assays. Transmission electron microscopy imaging.		(83)
Symploca sp.	Tasiamide B **(6)**	BACE-1 inhibition	BACE-1 inhibition assay		(84)
Lyngbya sp.	Tasiamide F **(7)**	BACE-1 inhibition	BACE-1 inhibition assay		(86)
Leptolyngbya sp. *N62DM*	Phycocyanin	BACE-1 inhibition	Protein-complex interface identification	*Caenorhabditis elegans* CL4176 transgenic AD-model: Paralysis assay	(87)
Lyngbya sp. *A09DM*	Phycoerythrin	BACE-1 inhibition	Surface plasmon resonance. Isothermal titration calorimetry. Enzyme activity by kinetic parameters.	*Caenorhabditis elegans* CL4176 transgenic AD-model: Thioflavin-T staining assay	(88)
Spirulina platensis	Lipopolysaccharide	Downregulation of p-tau expression. Antioxidant. Anti-inflammatory.		Wistar albino rats exposed to nicotine: Biochemical assessments (Oxidative and inflammatory markers). RT-PCR. Western Blot (p-tau).	(96)
Spirulina maxima	70% ethanol extract	AChE inhibition. Reduced Aβ, APP, and BACE-1 levels. BDNF/PI3K/Akt pathway activation. Antioxidant. Improved cognition.		ICR mice injected with $A\beta_{1-42}$: Passive Avoidance Test. Morris Water Maze Test. Biochemical Analysis ($A\beta_{1-42}$, GSH, BDNF, AChE). Western Blot.	(89)

Table 2. *Cont.*

Strain	Compound/Extract	Effect	*In Vitro* Assays	*In Vivo* Assays	Reference
Aphanizomenon flos-aquae	KlamExtra®	Reduced Aβ, APP and BACE-1 levels. Anti-inflammatory and anti-gliosis. Improved metabolic parameters. Protection of neuronal morphology and synapses.		High-Fat Diet C57BL/6J mice: Metabolic parameters analysis. Western Blot (IR, Akt, PSEN-1, BACE-1, PSD-95, synaptophysin, TNF-α, GFAP, IL-10, TREM-2). Histopathology and Immunohistochemistry (GFAP, TREM-2, Aβ). Thioflavin T staining. TUNEL assay.	(90)
Spirulina platensis	Diet supplementation	Decreased Aβ1-42, APP, BACE-1, p-tau, and p-GSK levels. Anti-inflammatory. Improved microbiota dysbiosis. Improved metabolic parameters. Improved locomotor and cognitive function.		High-Fat Diet C57BL/6J mice: Barnes Maze test. Morris Water Maze test. ELISA ($A\beta_{1-42}$, TNF-α, IL-1β, IL-6, LPS). RT-PCR. Western Blot (APP, BACE-1, p-tau, p-GSK, IBA-1). Microbial diversity analysis. GC (SCFAs).	(97)
cf. *Symploca* sp.	Santacruzamate A **(9)**	Anti-apoptotic. Anti-UPR and ER stress. Improvement of the mitochondrial fission pathway. Modulation of KDELR and Mia40-ALR. Memory improvement.	PC12 cells: Cell viability and apoptosis assays. Electrophysiological recordings. Immunoblot analyses. Measurement of mitochondrial permeability transition pore. Opening and mitochondrial membrane potentials.	APPswe/PS1dE9 mice: Open-Field test. Morris Water Maze test. RT-PCR (Mia40, KDEL).	(98)
Spirulina platensis	Diet supplementation (tablets)	Protection of neuronal morphology. Reduction of Aβ accumulation. Improvement of metabolic parameters. Antioxidant. Anti-inflammatory.		Wistar rats treated with $AlCl_3$: TBARS assay. GSH content assay. Total thiol content assay. TAC assay. GPx, GST, SOD activity assay. Lipid profile determination. ELISA (TNF-α). Histology. Immunofluorescence (Aβ).	(100)
Spirulina platensis	*S. platensis*-loaded niosome	Protection of neuronal morphology. Restored levels of AChE and ACh. Gene modulation. Recognition and working memory improvement.		Wistar rats treated with $AlCl_3$: Novel object recognition test. Y-maze test. TAC assay. MDA assay. AChE assay. Histology. HPLC (ACh, NE, 5HT, DA, DOPAC). qPCR (Bax, Bcl-2, AChE, MAO).	(101)
Spirulina platensis	Enzyme Digested Phycocyanin (EDPC)	Cognitive function improvement. Gene modulation.		Male Slc:ddY SPF mice injected with $A\beta_{25-35}$: Y Maze test. DNA microarray.	(103)

Table 2. *Cont.*

Strain	Compound/Extract	Effect	*In Vitro* Assays	*In Vivo* Assays	Reference
Spirulina platensis	Phycocyanin	Gene and miRNA modulation. Anti-inflammatory. Anti-apoptotic. Memory improvement.		Male C57BL/6 mice injected with oligomeric $A\beta_{1-42}$: Eight-arm radial maze. RT-PCR (caspase-3, caspase-9, miR-335). Western Blot (HDAC3, Bcl-2, Bax, IL-6, IL-1β). Immunohistochemistry (Bcl-2, Bax). Immunofluorescence (BDNF, HDAC3).	(104)
Spirulina platensis	Phycocyanin	AChE inhibition. ChAT activity increase. Gene modulation. Increased PI3K/Akt pathway. Anti-inflammatory. Memory improvement.		Female Wistar Rats injected with STZ: Morris Water Maze. Memory consolidation test. Novel object recognition test. Open field test. AChE and ChAT activity assays. ELISA (TNF-α, NF-kB p56, Bcl-2, Bax, BDNF, IGF-1). qRT-PCR (IRS-1, INS, PI3K, Akt, PTEN).	(105)
Spirulina maxima	70% ethanolic extract (SM70EE) pills	Memory and vocabulary improvement.		Randomized, double-blind, placebo-controlled clinical trial: Visual learning, visual working memory, and verbal learning tests.	(106)
Spirulina platensis	Dietary supplementation	Improved cognitive function. Improved metabolic status.		Randomized, double-blind, placebo. -controlled clinical trial: Mini-mental state exam. ELISA (hs-CRP, Insulin). Biochemical analysis (NO, TAC, GSH, MDA, FPG, lipid profile).	(107)

Abbreviations: AChE - Acetylcholinesterase. BChE – Butyrylcholinesterase. SFE-EtOH – Supercritical Fluid Extraction with Ethanol. LOX – Lipoxygenase. ORAC - Oxygen Radical Absorbance Capacity. DPPH - 2,2-diphenyl-1-picrylhydrazyl. BACE-1 - Beta Secretase 1. AD – Alzheimer's Disease. Aβ - Beta-amyloid peptide. APP - Amyloid-beta Precursor Protein. BDNF - Brain-derived Neurotrophic Factor. PI3K - Phosphoinositide 3-kinase. Akt - Protein kinase B. TBARS - Thiobarbituric Acid Reactive Substances. GSH – Total Glutathione. IR - Insulin receptor. PSEN-1 - Presenilin-1. PSD-95 – Postsynaptic density protein 95. TNF-α - Tumor Necrosis Factor α. GFAP - Glial fibrillary acidic protein. IL-10 – Interleukin. TREM-2 - Triggering receptors expressed on myeloid cells-2. p-tau – Phosphorylated Tau. p-GSK - Phosphorylated Glycogen Synthase. LPS – Lipopolysaccharide. RT-PCR - Reverse Transcription Polymerase Chain Reaction. Iba-1 - Ionized calcium-binding adaptor molecule 1. GC - Gas Chromatography. SCFAs - Short-chain fatty acids. UPR - Unfolded Protein Response. ER – Endoplasmic Reticulum. KDELR - Endoplasmic Reticulum Protein Retention Receptor. Mia40 - Mitochondrial Intermembrane Space Assembly Protein 40. ALR - Augmenter of the Liver Regeneration. AlCl3 – Aluminum Chloride. TAC – Total Antioxidant Capacity. GPx - Glutathione Peroxidase. GST - Glutathione S-transferase. SOD - Superoxide Dismutase. ELISA - Enzyme-Linked Immunosorbent Assay. MDA – Malondialdehyde. HPLC - High-Performance Liquid Chromatography. ACh – Acetylcholine. NE – Norepinephrine. 5HT – Serotonin. DA – Dopamine. DOPAC - 3,4-Dihydroxyphenylacetic acid. Bcl-2 - B-cell Lymphoma-2. Bax - Bcl-2 Associated X-protein. MAO - Monoamine oxidase. HDAC3 - Histone deacetylase 3. STZ – Streptozotocin. ChAT - Choline acetyltransferase. NF-kβ - Nuclear Factor Kappa β. IGF-1- Insulin-like growth factor 1. IRS-1 - Insulin receptor substrate 1. INS – Insulin Gene. PTEN - Phosphatase and Tensin Homolog. hs-CRP – High sensitivity C-reactive protein. NO – Nitric Oxide. FPG - Fasting Plasma Glucose.

2. Aims

The exploitation of cyanobacteria presents a promising avenue for the extraction of bioactive compounds with potential therapeutic applications in AD. LEGE-CC holds a wide range of cyanobacteria strains, which are still underexplored for anti-AD purposes. This work intends to take a first approach to the potential of cyanobacteria in the treatment of AD, starting by evaluating the cytotoxicity of fractions toward cell lines widely used in AD research, including the neuroblastoma cell line SH-SY5Y and the human cerebral microvascular endothelial cell line hCMEC/D3, and by evaluating their potential inhibitory effect on AChE. For this purpose, a library of fractions from 22 cyanobacteria strains from the LEGE-CC was selected.

Specifically, the project included:

- Cyanobacteria culture for biomass production;
- Preparation of ultrasound-assisted methanolic extracts and HPLC fractionation;
- Assessment of the cytotoxicity of fractions through the MTT cell viability assay using the neuroblastoma cell line SH-SY5Y, the human cerebral microvascular endothelial cell line hCMEC/D3, and the fibroblast cell line 3T3-L1;
- Evaluation of the potential of fractions to inhibit AChE, a key enzyme involved in AD, through the Ellman's method.

3. Materials and methods

3.1. Selection of cyanobacteria strains

In this study, 22 cyanobacteria strains from the LEGE-CC were selected for the evaluation of the cytotoxicity against the neuroblastoma cell line SH-SY5Y, the human cerebral microvascular endothelial cell line hCMEC/D3, and the fibroblast cell line 3T3-L1, and for the screening of the potential to inhibit the enzyme AChE. The selection of cyanobacteria took into consideration:

- the diversity of taxonomic groups and traits to ensure a broad and representative sample;
- the previously demonstrated bioactivity of the strains in anticancer, antifouling, and cosmetic studies;
- the novelty of the fractions, which were never tested against AD.

Fractions or methanolic extracts from 16 strains were used directly from the Cyanobacteria Natural Products Library (LEGE-NPL) repository (Table 3). Extracts from 6 of these strains were requested from the solid LEGE-NPL and subjected to fractionation, including *Synechocystis salina* LEGE 00040, *Phormidium* sp. LEGE 05292, *Cyanobium* sp. LEGE 06097, *Leptolyngbya mycoidea* LEGE 06108, *Synechococcus* sp. LEGE 07172 and *Cyanobium* sp. LEGE 07183. The other 10 strains were directly selected from the liquid LEGE-NPL in the form of fractions and were ready for bioactivity assays. To contribute to enlarging the LEGE-NPL collection 6 new strains were requested from the LEGE-CC and went through the process of culturing, harvesting, ultrasonic-assisted extraction with methanol, and HPLC fractionation. These included *Oscillatoriales cyanobacterium* LEGE 181159, unidentified VV5, *Synechococcales cyanobacterium* LEGE 181150, *Synechococcales cyanobacterium* LEGE 181151, *Leptothoe* sp. LEGE 181153, and *Leptothoe* sp. LEGE 181156 (Table 4). A schematic representation of the workflow is presented in Figure 7.

Furthermore, strains other than the ones included in the screening were also cultured, for logistical purposes and to maintain the stock of LEGE-NPL. Information about these strains is listed in Table I, Appendix I, and a log of all 4L cultures is presented in Table II, Appendix II.

For abbreviation purposes, from this point forward, all references to the strains included in this project will be designated solely by their LEGE code.

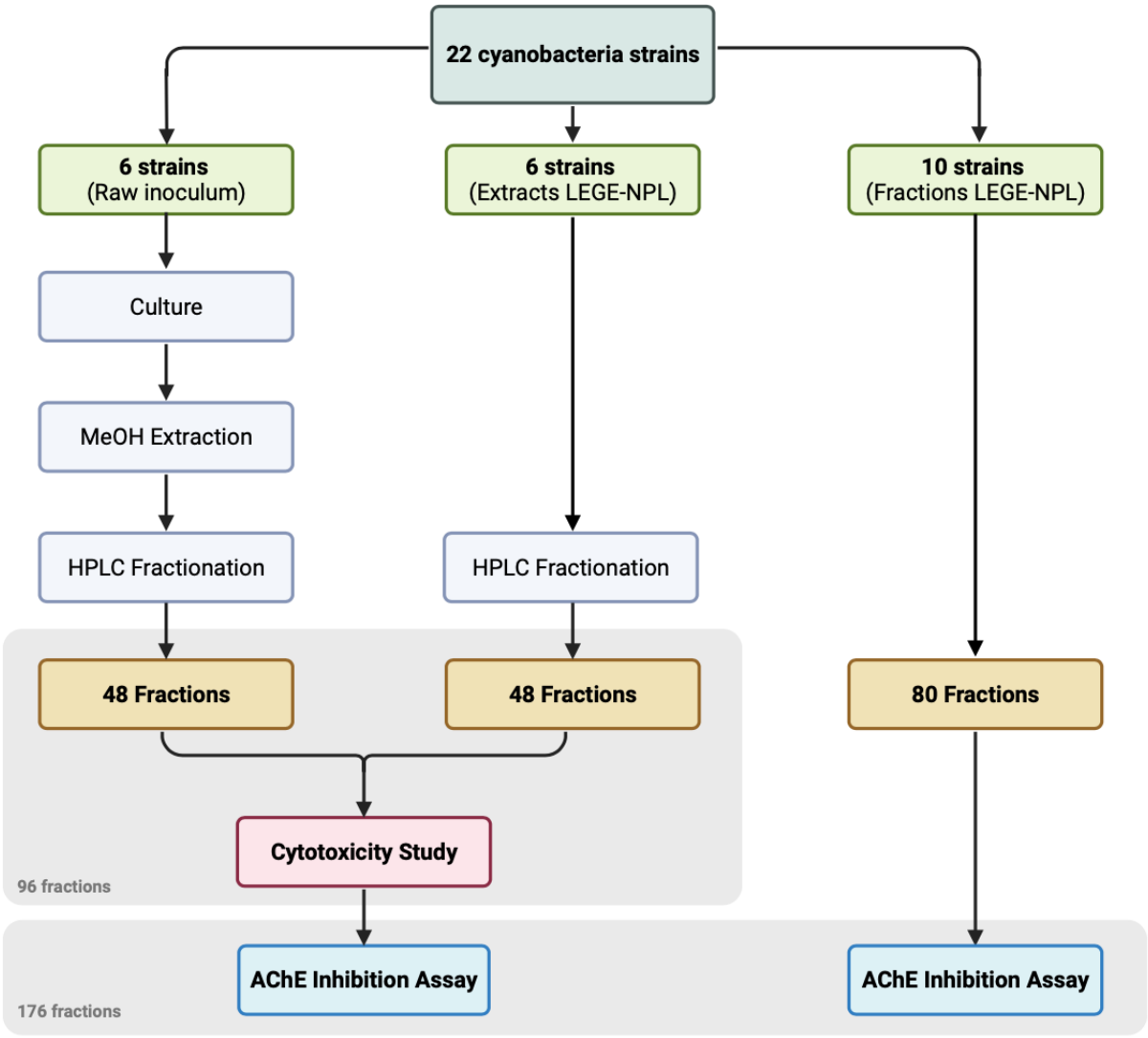
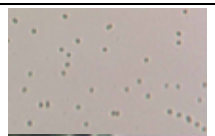
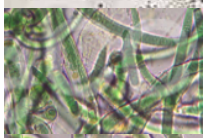
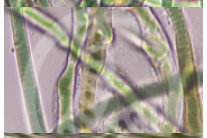
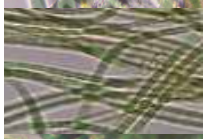
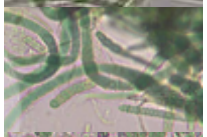
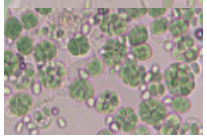


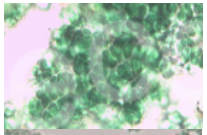
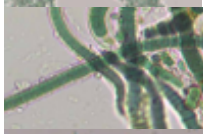
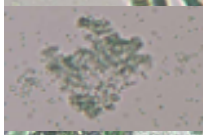
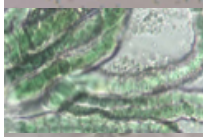
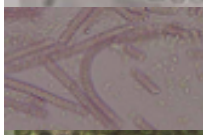
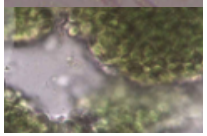
Figure 7. Schematic representation of the workflow followed in this work. Created in Biorender.com.

Table 3. Information about the selected strains from the Natural Product Library (LEGE-NPL).*

Microphotograph 1000×	Strain ID	Identification	Sampling	Lifestyle	Bioactivity	Reference
	LEGE 00040	*Synechocystis salina*	Caminha, Portugal (*Patella* sp. shell surface)	Aquatic, marine		
	LEGE 05292	*Phormidium* sp.	Porto, Portugal (indoor aquarium wall)	Aquatic, freshwater	Antifouling; Anticancer	(17, 108, 109)
	LEGE 06097	*Cyanobium* sp.	Vila do Bispo, Portugal (green macroalgae)	Aquatic, marine	Anticancer	(15)
	LEGE 06100	*Calothrix* sp.	Albufeira, Portugal (rock surface)	Aquatic, marine		
	LEGE 06105	*Plectonema* cf. *radiosum*	Lagos, Portugal (green macroalga)	Aquatic, marine		
	LEGE 06108	*Leptolyngbya mycoidea*	Lagos, Portugal (rock surface)	Aquatic, marine	Anticancer	(15)
	LEGE 06122	*Calothrix* sp.	Esposende, Portugal (submerged stone)	Aquatic, marine		
	LEGE 06123	*Gloeocapsopsis crepidinum*	Lagos, Portugal (green algae)	Aquatic, marine		

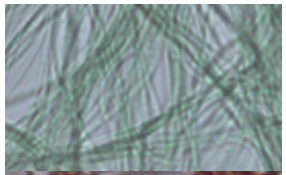
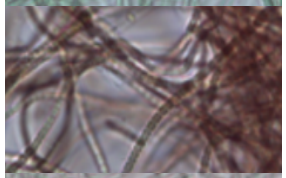
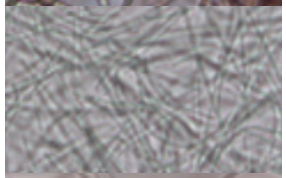
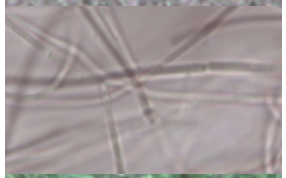
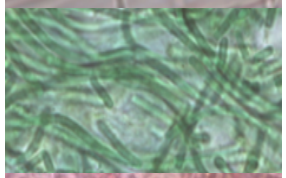
* Images, identification, and origin were courtesy of the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE CC, http://lege.ciimar.up.pt), CIIMAR.

Table 3. *Cont.*

Microphotograph 1000×	Strain ID	Identification	Sampling	Lifestyle	Bioactivity	Reference
	LEGE 06150	unidentified *Nostocales*	Esposende, Portugal (rock surface)	Aquatic, marine		
	LEGE 07172	*Synechococcus* sp.	Albufeira, Portugal (submerged rock)	Aquatic, marine	Anticancer	(15)
	LEGE 07177	*Rivularia* sp.	Vila do Bispo, Portugal (green macroalga)	Aquatic, marine		
	LEGE 07183	*Cyanobium* sp.	Albufeira, Portugal (rock surface)	Aquatic, marine		
	LEGE 07189	*Scytonema* sp.	Caminha, Portugal (rock surface)	Aquatic, marine		
	LEGE 11386	unidentified *Nostocales*	Portugal (marine sponge)	Aquatic, marine		
	LEGE 11439	*Spirulina* sp.	Matosinhos, Portugal (subtidal sample)	Aquatic, marine		
	LEGE 13413	*Nostoc* sp.	Porto, Portugal (tablet *spirulina* supplements)			

* Images, identification, and origin were courtesy of the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE CC, http://lege.ciimar.up.pt), CIIMAR.

Table 4. Information about the cyanobacteria strains selected from the LEGE-CC collection for culture.*

Microphotograph 1000×	Strain ID	Identification	Sampling	Lifestyle	Bioactivity	Reference
	LEGE 181150	*Synechococcales* cyanobacterium	São Vicente Island, Cape Verde (rock surface)	Aquatic, marine	Hyaluronidase Inhibition; Antioxidant; Anticancer	(13, 16, 110)
	LEGE 181151	*Synechococcales* cyanobacterium	São Vicente Island, Cape Verde	Aquatic, marine		(110)
	LEGE 181153	*Leptothoe* sp.	São Vicente Island, Cape Verde	Aquatic, marine		(110, 111)
	LEGE 181156	*Leptothoe* sp.	Santo Antão Island, Cape Verde (rock surface)	Aquatic, marine	Tyrosinase Inhibition; Antioxidant	(16, 110)
	LEGE 181159	*Oscillatoriales* cyanobacterium	São Vicente Island, Cape Verde (rock surface)	Aquatic, marine	Antioxidant	(16, 110)
	VV5	Not Determined	São Vicente Island, Cape Verde	Aquatic, marine		(110)

*Images, identification, and origin were courtesy of the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE CC, http://lege.ciimar.up.pt), CIIMAR, and (110).

3.2. Cyanobacteria culture

Cyanobacterial cultures were carried out at CIIMAR using the Bioterium of Aquatic Organisms (BOGA) microalgal culture room facilities. In order to grow the selected cyanobacteria, an internal Material Transfer Agreement (MTA) form was filled out to guarantee the correct and ethical usage of the strains. All material and handling of the medium and samples was done under sterile conditions.

All cultures were performed in liquid Z8 medium (112) supplemented with 25% synthetic sea salts (Tropic Marin®, Berlin, Germany) and 1% vitamin B12. The culture was performed through a 10-fold scale-up process. The raw inoculum of each strain of cyanobacteria was transferred from the LEGE-CC to a 40 mL starter culture. Over time of growth, it was upscaled to 175 mL, 400 mL, and finally to 4 L cultures. Cultures were performed at 25 °C and luminance with light/dark cycles of 16/8h at a light intensity of 10-30 µmol photons m^2/s. Cultures in 4 L medium were maintained under constant aeration. The standard culture conditions are presented in Table III, Appendix III, and the composition of the medium is listed in Tables IV-XI, Appendix III.

During the exponential growth phase, from 25 to 50 days, the cyanobacterial biomass was harvested by centrifugation at 4700 rpm for 10 min (Sorvall BIOS 16 Centrifuge, Thermo Scientific, Germany) for unicellular strains, or by filtration through an appropriately sized mesh for filamentous strains. The concentrated biomass was washed with distilled water to remove the excess salt. The biomass was then frozen and freeze-dried (LyoQuest, Telstar, Terrassa, Spain) under reduced pressure (0.1 mbar with the condenser at -47°C) for 4-6 days (depending on the strain and water content of the material). Freeze-dried biomass was stored at - 20 °C until further use.

3.3. Ultrasound-assisted organic extraction

The LEGE-NPL solid inventory consists of methanolic extracts of cyanobacterial strains. For the organic extraction, freeze-dried biomass of 6 strains was extracted with methanol, as described by Ferreira et al. (13). Briefly, biomass was macerated using a mortar and pestle and transferred to an Erlenmeyer flask. Extraction was carried out by adding 50 mL of methanol to the grounded biomass and submitting the Erlenmeyer to an ultrasonic bath (Vibra Cell, USA) for five minutes (keeping the temperature below 30 °C). The supernatant was then decanted and filtered through a vacuum filtering system (Figure 8) into a round flask.

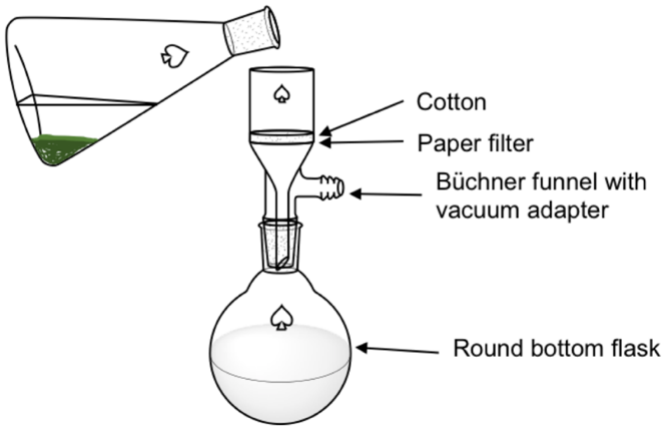


Figure 8. Representation of the extraction system. *

*Courtesy of the Blue Biotechnology and Ecotoxicology Group.

Afterward, the previous steps were repeated two more times, changing the amount of methanol added (25 mL). The obtained extract was concentrated at 70 mbar and 30 ºC using a rotary evaporator (BUCHI Interface I-300, Switzerland), to remove methanol. The concentrated content was then filtered through a Pasteur pipette with pressed cotton to capture any non-soluble particles, before being transferred to a previously weighed vial. Under the same conditions, the solvent was evaporated again to thoroughly dry. Finally, the glass vial was weighed and deposited in the solid library of LEGE-NPL. The extraction yields were calculated using the following expression:

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

3.4. High performance liquid chromatography (HPLC) fractionation

Following the methodology described in Ferreira et al. (13), 12 methanolic extracts were fractionated by reverse-phase HPLC using a Waters Alliance e2695 Separations Module instrument coupled to a photodiode array detector (Waters 2998 PDA) and an automatic Waters Fraction Collector III (Waters, Mildford, MA, USA). The extracts were resuspended in HPLC-grade methanol (40 mg/mL) in 2 mL HPLC glass vials, vortexed, and filtered through a 0.22 µm PTFE filter. The eluents used were filtered through a 0.22 µm nylon membrane filter (Fioroni Filters, Ingré, France) using a vacuum pump (Dinko D-95, Barcelona, Spain) and degassed for 15 min in an ultrasonic bath (Vibra Cell, USA). The glass vials were loaded on the HPLC system, and 500 µL of each extract was then injected through a 1 mL loop and separated on an ACE 10 C8 column (50

x 10 mm, ACE, Reading, UK), using an H_2O: MeCN gradient. The blank was HPLC-grade methanol. Hence, each extract was separated into 8 different fractions (A-H), which were collected by the automatic sample collector into deep 48-well plates (Riplate, Ritter, Schwabmünchen, Germany) every 1.3 min, with a volume of around 4 mL. The experience condition is described in Table 5.

Table 5. HPLC chromatographic and collection program for generating the fractions. Adapted from (13).

Fraction	Time (min)	Flow (mL/min)	MeCN (%)	H_2O (%)	Collection Time (min)
A	0.0	3.0	10	90	1.00-2.30
B	2.0	3.0	80	20	2.30-3.60
C	3.0	3.0	80	20	3.60-4.90
D	4.0	3.0	100	0	4.90-6.20
E	8.9	3.0	100	0	6.20-7.50
F	9.2	3.5	100	0	7.50-8.80
G	12.0	3.5	100	0	8.80-10.26
H	12.3	3.0	100	0	10.36-11.50
	14.0	3.0	100	0	
	15.0	3.0	10	90	
	18.0	3.0	10	90	

The fractions were dried using a CentriVap Concentrator (LabConco, Kansas City, MO, USA) to remove the solvent. They were then solubilized in 500 μL of DMSO, at a final concentration of approximately 5 mg/mL, transferred to 96-deep well microplates (Nest Scientific, Woodbridge Township, NJ, USA), and stored at – 80 °C, thus forming the mother plates of the LEGE-NPL liquid library. A general scheme of the LEGE-NPL workflow is shown in Figure 9.

Out of each mother plate, two working plates were prepared, by transferring 200 μL of each fraction to a 96-well plate, to pursue the bioactivity assays, without disturbing the whole stock. One of the working plates was freeze-dried (LyoQuest, Telstar, Terrassa, Spain) under reduced pressure (0.1 mbar with the condenser at -47°C) for 4-6 days, to eliminate all the DMSO. The fractions contained within this plate were subsequently resuspended in buffer (50 mM Tris–HCl) and 10% methanol and were later used for AChE assays.

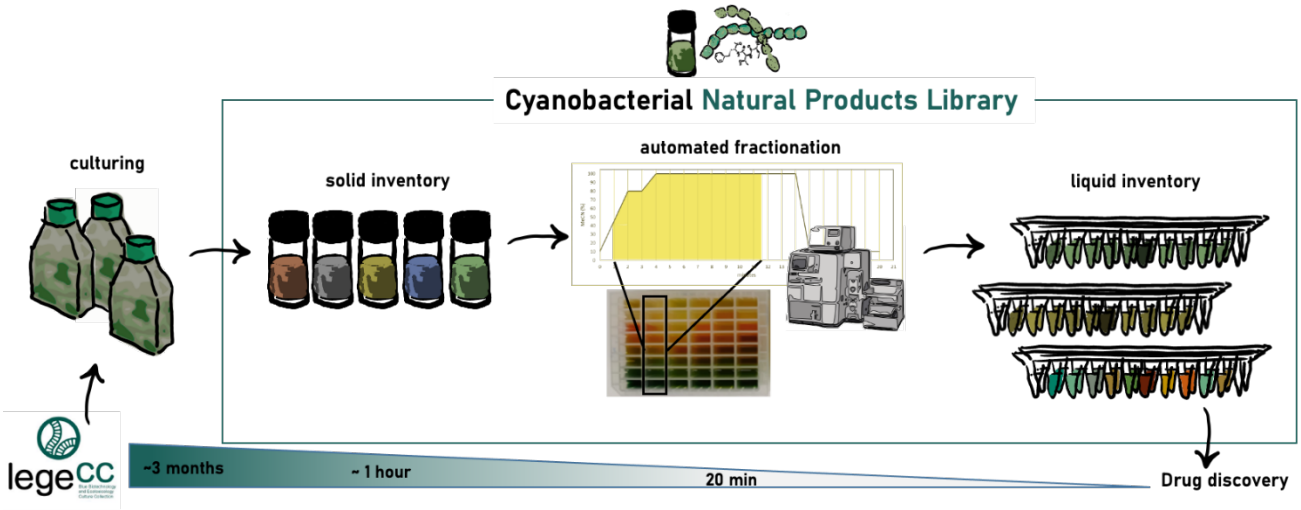


Figure 9. Schematic Representation of the Natural Products Library Workflow.*
*Courtesy of the Blue Biotechnology and Ecotoxicology Group.

A representation of one of the mother plates obtained is presented in Table 6. The other mother plates are represented in Tables XII–XIII, Appendix IV.

Table 6. Schematic representation of mother plate 1.

	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO	181159_A	VV5_A	181150 _A	181151_A	181153_A	181156_A					DMSO
B	DMSO	181159_B	VV5_B	181150 _B	181151_B	181153_B	181156_A					DMSO
C	DMSO	181159_C	VV5_C	181150 _C	181151_C	181153_C	181156_A					DMSO
D	DMSO	181159_D	VV5_D	181150 _D	181151_D	181153_D	181156_A					DMSO
E	DMSO	181159_E	VV5_E	181150 _E	181151_E	181153_E	181156_A					DMSO
F	DMSO	181159_F	VV5_F	181150 _F	181151_F	181153_F	181156_A					DMSO
G	DMSO	181159_G	VV5_G	181150 _G	181151_G	181153_G	181156_A					DMSO
H	DMSO	181159_H	VV5_H	181150 _G	181151_H	181153_H	181156_A					DMSO

3.5. Cytotoxicity evaluation

3.5.1. Cell lines and cell culture

Two cell lines related to the nervous system were selected for this study: the human neuroblastoma cell line SH-SY5Y, which is a common model for neurotoxicity studies (113, 114), and the human cerebral microvascular endothelial cell line hCMEC/D3, representative of the BBB

(114). The mouse fibroblast cell line 3T3-L1 was used as a representation of the main cellular component of the conjunctive tissue (115).

The three cell lines (Table 7) were cultured using routine procedures and exposed to cyanobacterial fractions for cytotoxicity evaluation.

Table 7. Information about the cell lines included in the cytotoxicity studies.

Cell	Cell line	Origin
Neuroblastoma	SH-SY5Y	American Type Culture Collection (ATCC)
Fibroblasts	3T3-L1	American Type Culture Collection (ATCC)
Endothelial cells	hCMEC/D3	Donated by Dr. PO Couraud (INSERM, France).

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% CO_2. The culture medium was replaced every two days. When the cells hit 80-90% confluence a cell passage was performed. The first step was to remove the previous medium and wash the cells with 2 mL of warm phosphate-buffered saline (PBS) (Gibco, Germany). Then, 1 mL of TrypLE express enzyme (1x) (Gibco, Denmark) was added to detach cells from the flask's wall 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 cm^2 with 4mL or 75 cm^2 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.

3.5.2. MTT assay

Due to time constraints and logistic problems with cell cultures, only 96 fractions obtained by HPLC fractionation were subjected to cytotoxicity evaluation. Cell viability was determined by the widely used 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For

the assay cells were seeded in 96-well culture plates at a density of $1.0x10^5$ cells/mL for hCMEC/D3 cells, $2.0x10^4$ cells/mL for SH-SY5Y cells, and $3.3x10^4$ cells/mL for 3T3-L1 cells, with each well containing 100 μL of medium. After 24 h of incubation to allow adhesion, the cells were treated with 100 μL of fresh media supplemented with 1 μL of fractions to a final concentration of 50 μg/mL in the well. The positive control consisted of 20% DMSO and 1% DMSO was used as the negative control. After a 24 and 48 h incubation, 20 μL of MTT (AppliChem, GmbH: Ottoweg, Darmstadt, Germany) at a concentration of 1 mg/mL was added to each well, to a final concentration of 0.2 mg/mL. After 3 h of incubation, the medium was carefully aspirated and the purple-colored formazan salts, which are insoluble in the medium, were dissolved in 100 μL DMSO.

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 (mean ± SD), following the equation below.

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

Three independent assays were performed in triplicates.

3.6. Acetylcholinesterase (AChE) inhibition assay

Fractions were tested for their AChE inhibition potential following Ellman's method (116), with some modifications. The reagents used in this assay included the enzyme AChE from *Electrophorus electricus* (electric eel Type-VI-S, Sigma Chemical Co, St. Louis, MO, USA), the subtract acetylcholine iodide (ATCI, A5751-5G sigma), the Ellman's Reagent or 5,5'-bisdithionitrobenzoic acid (DTNB, Sigma Chemical Co, St. Louis, MO, USA) and the Buffers A (50 mM Tris–HCl, pH 8), B (50 mM Tris–HCl (pH 8) with 0.1% of BSA) and C (50mM Tris-HCl (pH 8) with 0.1% NaCl and 0.02M $MgCl_2.6H_2O$).

Briefly, the freeze-dried fractions were dissolved in buffer A with 10% methanol, to a concentration of 5 mg/mL in the working plate. Then, 25 μL of the previously dissolved fractions were added to the wells with 125 μL of DTNB reagent (3 mM), 50 μL of buffer B, 25 μL of ATCI (15mM), and 25 μL of AChE (0.44 U/mL), to a final concentration of 500 μg/mL in the well. A description of the composition of the testing samples, the blank of the samples, the negative control, and the blank of the negative control is presented in Table 8.

Table 8. Composition of the testing samples, the blank of the samples, the negative control, and the blank of the negative control used in 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 + 10%	25 μL of Buffer A + 10%
125 μL of DTNB	125 μL of DTNB	Methanol	Methanol
50 μL of Buffer B	50 μL of Buffer B	125 μL of DTNB	125 μL of DTNB
25 μL of ATCI	25 μL of ATCI	50 μL of Buffer B	50 μL of Buffer B
25 μL AChE	25 μL of Buffer B	25 μL of ATCI	25 μL of ATCI
		25 μL AChE	25 μL Buffer B

The hydrolysis of ATCI by AChE was monitored through the reaction of DTNB with the thiocholine released, which forms the yellow-colored anion 5-thio-2-nitrobenzoate (TNB^{2-}). Readings were performed at 405 nm using a Synergy HT Multi-detection Microplate Reader running GEN5™ software, operating in kinetic mode, at room temperature, for 2 min.

The percentage of inhibition was determined by comparing the rates of reaction with those of the negative control (buffer A + 10% methanol) following the expression below:

$$\%\ \text{inhibition} = \frac{\left(\text{slope}_{\text{control}} - \text{slope}_{\text{blank of controls}}\right) - \left(\text{slope}_{\text{samples}} - \text{slope}_{\text{blank of samples}}\right)}{\left(\text{slope}_{\text{control}} - \text{slope}_{\text{blank of control}}\right)} \times 100$$

One independent assay was performed in triplicate. Data was presented as mean ± SD.

3.7. Statistical analysis

Statistical analysis was performed using GraphPad PRISM software (GraphPad® software, CA, USA) (Version 9 for MacOS). Data was presented as mean ± SD. Data was analyzed for normality and homogeneity of variances by Kolmogorov-Smirnov and Leven's tests. Cytotoxicity data was submitted to a two-way ANOVA followed by Tuckey's HSD multiple comparisons test and AChE inhibition data was submitted to a Kruskal-Wallis test.

4. Results and discussion

In this study, 176 fractions from 22 cyanobacteria strains were evaluated for their potential as AChEI, as part of an effort to evaluate the potential of cyanobacteria in the therapy of AD. When choosing the strains, it was intended to include strains with different morphologies, strains sampled from different environments, and strains for which there is already reference to bioactivities such as anticancer, antioxidant, and antifouling (Tables 3,4). 6 strains went through the process of integrating the LEGE-NPL, which involved culturing, harvesting, ultrasonic-assisted extraction with methanol, and HPLC fractionation. Extracts from 6 other strains were requested from the solid LEGE-NPL and subjected to fractionation. The other 10 strains were selected from the liquid LEGE-NPL and were directly used for the AChE inhibition assay. 96 fractions were evaluated for cytotoxicity (Figure 7) in two cell lines widely used in AD-related studies, the neuroblastoma cell line SH-SY5Y and the human cerebral microvascular endothelial cell line hCMEC/D3, which is used as a BBB model cell line. The fibroblast cell line 3T3-L1 was also studied and was used as a representative of the connective tissue which in the nervous system is present in the meninges layers. While SH-SY5Y and hCMEC/D3 cells are of human origin, 3T3-L1 cells are of mouse origin. However, as we were unable to obtain fibroblasts of human origin, we opted to use the available cell line. All cyanobacteria fractions (176) were investigated for their ability to inhibit AChE (Figure 7). The results of these studies are presented below.

4.1. Cyanobacteria culture, extraction and fractionation

Traditionally, the exploitation of natural organisms for the extraction of bioactive compounds has been a standard practice in biomedical research (8). Cyanobacteria, a heterogeneous group of microorganisms, are recognized for their potential to harbor such compounds, making them a worthy subject of inquiry (117). Cyanobacteria culture collections, such as LEGE-CC maintained at CIIMAR, are a valuable biotechnological asset, providing access to their microbial diversity, associated data, and natural compounds (14). It is important to recognize the value of this particular resource, which has resulted in the identification of numerous bioactive compounds, such as chlorosphaerolactylates (antibacterial), nocuolin A (anticancer), and portoamides (antifouling and cytotoxic) (13, 14). However, there is a scarcity of research on the application of LEGE-CC strains in combating neurodegenerative disorders, particularly AD, making this study an opportunity to delve into this field.

For the bioactivity screening of multiple strains, this study made use of the LEGE NPL. This tool is a library that consists of both a solid collection of methanolic extracts and a liquid assortment of 8 chromatographic fractions (A-H) with different polarities, both of which are available for exploration. This methodology enables a swifter, more practical, and more efficient screening process, thereby facilitating the isolation of bioactive compounds (13).

To initiate the process of inclusion in the LEGE-NPL, the raw materials were sourced from 4L cultures of the marine cyanobacteria LEGE 181159, VV5, LEGE 181150, LEGE 181151, LEGE 181153 and LEGE 181156. The duration of growth prior to harvesting and freeze-drying varied between 1 to 2 months. Table 9 presents the amount of dry biomass obtained from the 4L culture of each cyanobacteria strain. The results revealed that cultures yielded an average of 1.9 g, and biomass yields are dependent on strain, as VV5 produced the least amount of biomass, with a value of 0.4020 g, while LEGE 181150 generated the highest amount, which was 4.7532 g.

Table 9. Weight of dry biomass (g), methanolic extract (g) and yield of extraction (%).

Code	Dry Biomass (g)	Methanolic Extract (g)	Extraction Yield (%)
LEGE 181159	0,5341	0,06111	11,44
VV5	0,4020	0,03498	8,70
LEGE 181150	4,7532	0,64554	13,58
LEGE 181151	1,0929	0,16084	14,85
LEGE 181153	1,0077	0,11125	11,04
LEGE 181156	3,8601	0,60144	15,58

The biomass obtained was subsequently subjected to an ultrasound-assisted methanol extraction, and the resulting extracts were added to the solid LEGE-NPL. The extraction yield and its composition are influenced by the extraction conditions, such as the affinity of the different compounds to the solvent (118). In this sense, methanol was chosen as the solvent due to its proficiency in extracting components with differing polarities, resulting in a very complex extract. Ultrasounds were employed to aid in the rupture of cell walls, which facilitates the release of bioactive compounds from the biological matrix, thereby enhancing the extraction process (13, 118). The yields of extraction are listed in Table 9. The average extraction yield was found to be 12.5%, with LEGE 181156 displaying the highest yield (15.58%). It is also noteworthy to consider the strain LEGE 181159 in this context, as it produces comparable yields while utilizing significantly less biomass. The extraction yield obtained for both strains was significantly higher

than the ones obtained with acetonic extraction (16). Furthermore, both of these strains have already been recognized for their bioactivity, particularly their antioxidant properties (16), which may have potential valuable applications in biotechnology.

The liquid inventory of the LEGE-NPL was created by fractionating the cyanobacteria methanolic extracts into 8 distinct fractions, A to H, via a semi-automated process utilizing an HPLC system. Hence, the 6 methanol extracts obtained earlier, and 6 other methanol extracts from the LEGE-NPL were separated by a gradient of H_2O: MeCN on a C8 column. This allows for the separation of complex sample mixtures or with unknown composition, through the compounds' polarity, allowing for a better separation between lipophilic and medium polarity compounds (13). These yielded a total of 96 fractions, which were incorporated into the liquid LEGE-NPL. Because each fraction is eluted under different conditions, they are expected to contain a distinct set of compounds based on their polarity, leading to unique results for each one.

4.2. Cytotoxicity

Cytotoxicity assays are a crucial part of the research process, as they enable the evaluation of potentially harmful and toxic effects of a compound, fraction, or extract on cells. This can be achieved by measuring the cell viability levels, which serve as an indirect indicator of cell proliferation. *In vitro* cell viability assays are advantageous due to their ease, speed, affordability, and non-animal-based nature (119). The MTT assay, in particular, is a widely used colorimetric assay that quantifies cell viability through the evaluation of mitochondrial function. MTT is a water-soluble yellow tetrazole salt that, in metabolically active cells is reduced by NAD(P)H-dependent oxidoreductase mitochondrial enzymes to purple formazan crystals, which can be quantified by visible light spectrophotometry (119).

As AD is a neurological disorder, the cytotoxicity of fractions was evaluated using cell lines related to the nervous system. The human neuroblastoma cell line SH-SY5Y is a widely used model for AD research, namely concerning the activity of AChE or the toxicity of Aβ species in the presence of extracts or compounds (120), due to its high proliferative capacity, ease of handling, affordability, and ability to differentiate into various adult neuron phenotypes (113, 114). The human cerebral microvascular endothelial cell line hCMEC/D3, derived from human temporal lobe microvessels, has been widely used for its brain endothelial phenotype. It is a stable and easily cultivated model with properties closely resembling that of the BBB, exhibiting almost all endothelial marker characteristics. It is very relevant given the highly vascularized nature of the

brain and the suspected role of brain endothelium dysfunction in the progression of AD (114, 121). As such, the fractions must not exhibit cytotoxicity toward any of these cell lines.

Only the 96 fractions obtained by HPLC fractionation were tested for cytotoxicity. Due to time constraints, and logistic problems with cell culture, it was not possible to conduct cytotoxicity tests on the 80 fractions requisited directly from the liquid LEGE-NPL. Given the objective of conducting an expedited assessment and the volume of test fractions, only a single concentration of 50 μg/mL was studied. Cells were exposed to the cyanobacteria fractions for 24 and 48 hours and the fractions were deemed toxic when cell viability was reduced to 70% or less. The results provided below are presented as the percentage of cell viability normalized to the negative control (1% DMSO), which represents 100% viability, and were obtained in three independent experiments. The positive control comprised 20% DMSO, which is recognized for its cytotoxic properties, resulting in the death of a large proportion of cells.

Cell viability results of SH-SY5Y cells after exposure to the fractions are present in Figure 10. As observed, most fractions were found to be not toxic, with cell viability higher than 70%. However, 4 fractions were found to be highly toxic at both 24 and 48 hours, namely LEGE 181151_G, LEGE 181151_H, LEGE 05292_B, and LEGE 05292_C, with a reduction in cell viability around 40-50%. Although these results are not particularly interesting within the scope of this project, they may hold significance in future anticancer research, given that SH-SY5Y cells are derived from a bone marrow biopsy of a neuroblastoma patient, a type of cancer that is common in infants (112). On the other hand, some fractions induced cell proliferation since cell viability was higher than the control. An increase in cell viability was particularly evident for fractions LEGE 181159_A, LEGE 00040_G, and LEGE 181151_A, showing cell viability at 24h of 118.50%, 115.08%, and 114.36%, respectively.

The results regarding cell viability of hCMEC/D3 after exposure to the fractions are present in Figure 11. In hCMEC/D3, most fractions did not induce cytotoxicity, with cell viability ranging higher than 70%. 4 fractions were found to induce cytotoxicity namely LEGE 05292_B, LEGE 05292_C, and LEGE 07172_E with percentages of cell viability of 27.70%, 49.83%, and 65.69%, respectively, at 24h, and LEGE 06108_G with 69.21% at 48h. However, some fractions did induce an increase in cell viability at 24h, with emphasis on LEGE 181159_E (129.61%), VV5_G (122.70%), and VV5_E (122.29%).

The embryonic mouse fibroblast cell line 3T3-L1 was another cell line studied. Fibroblasts are the primary cells of the connective tissue and play a pivotal role in the provision of structural support

to various tissues. In the CNS, fibroblasts are localized in the meninges, but they can also be found in the perivascular Virchow-Robin space and choroid plexus (115, 122). They were previously thought to be solely structural cells but are now known to be involved in CNS development, neuroinflammation, aging, neurodegenerative diseases, and injury. Specifically, it has been observed that there is a significant loss of fibroblasts in AD brains (115, 122). Also, for being one of the most widely distributed cell types, fractions must not show toxicity to reduce possible systemic side effects.

The results of 3T3-L1 viability after exposure to the fractions are present in Figure 12. Even though most fractions were non-cytotoxic (viability higher than 70%), this was the cell line where a higher number of toxic fractions was found, with 14 fractions displaying toxicity. 6 fractions were toxic at 24h and 48h, namely LEGE 05292_B, LEGE 05292_C, LEGE 181151_G, LEGE 181151_H, LEGE 181156_G and LEGE 181156_H, while the others were toxic at 48h, including LEGE 00040_E, LEGE 00040_G, LEGE 05292_E, LEGE 05292_G, LEGE 07172_E, LEGE 07172_G, LEGE 07183_E and LEGE 181153_G. Nevertheless, some fractions enhanced cell viability with the highest being LEGE 181156_D, LEGE 181153_B, and LEGE 181151_C with viability values of 116.43%, 113.92%, and 112.93% at 24h, respectively.

A summary of all the results is presented in Table XIV, Appendix V. Reviewing the combined results from all three cell lines, most fractions did not show cytotoxicity except for 15 distinct fractions. Among strains that exhibited cytotoxic properties, LEGE 05292 fractions B and C were found to be the most cytotoxic by inducing a reduction in cell viability around 50% or higher in all three cell lines. These fractions contain cytotoxic compounds, which are likely to be portoamides, as they have been isolated from *Phormidium* sp. LEGE 05292 and reported to be cytotoxic in both carcinogenic and non-carcinogenic cell lines (13, 109). Other fractions, such as LEGE 181151_G, LEGE 181151_H, LEGE 181156_G, and LEGE 181156_H, displayed some level of toxicity. These fractions are the least polar and, although their chemical makeup is currently unknown, they may house similar nonpolar compounds that are eluted at the same timeframe across strains, leading to the observed toxicity.

On the other hand, it is noteworthy to mention that the more polar fractions of the strains LEGE 181151 (mainly fraction A and C), LEGE 181156 (mainly fraction D), and LEGE 181159 (mainly fraction A) are among the fractions that induced cell proliferation. This finding may be interesting for neuroprotection as, for example, these fractions may promote angiogenesis in the case of the

hCMEC/D3 cell line or support the structural integrity of CNS in the case of 3T3-L1 cells. These effects may be worthy of further investigation.

Furthermore, aqueous and acetonic extracts from these cyanobacteria strains were found to have antioxidant potential in the superoxide anion radical ($O_2^{\cdot-}$) scavenging activity (16). Thus both of these properties could be relevant in the field of AD, as oxidative stress caused by excessive ROS species, such as $O_2^{\cdot-}$, is one of the main culprits in the pathophysiology of AD, and strategies to mitigate oxidative stress may be beneficial (123).

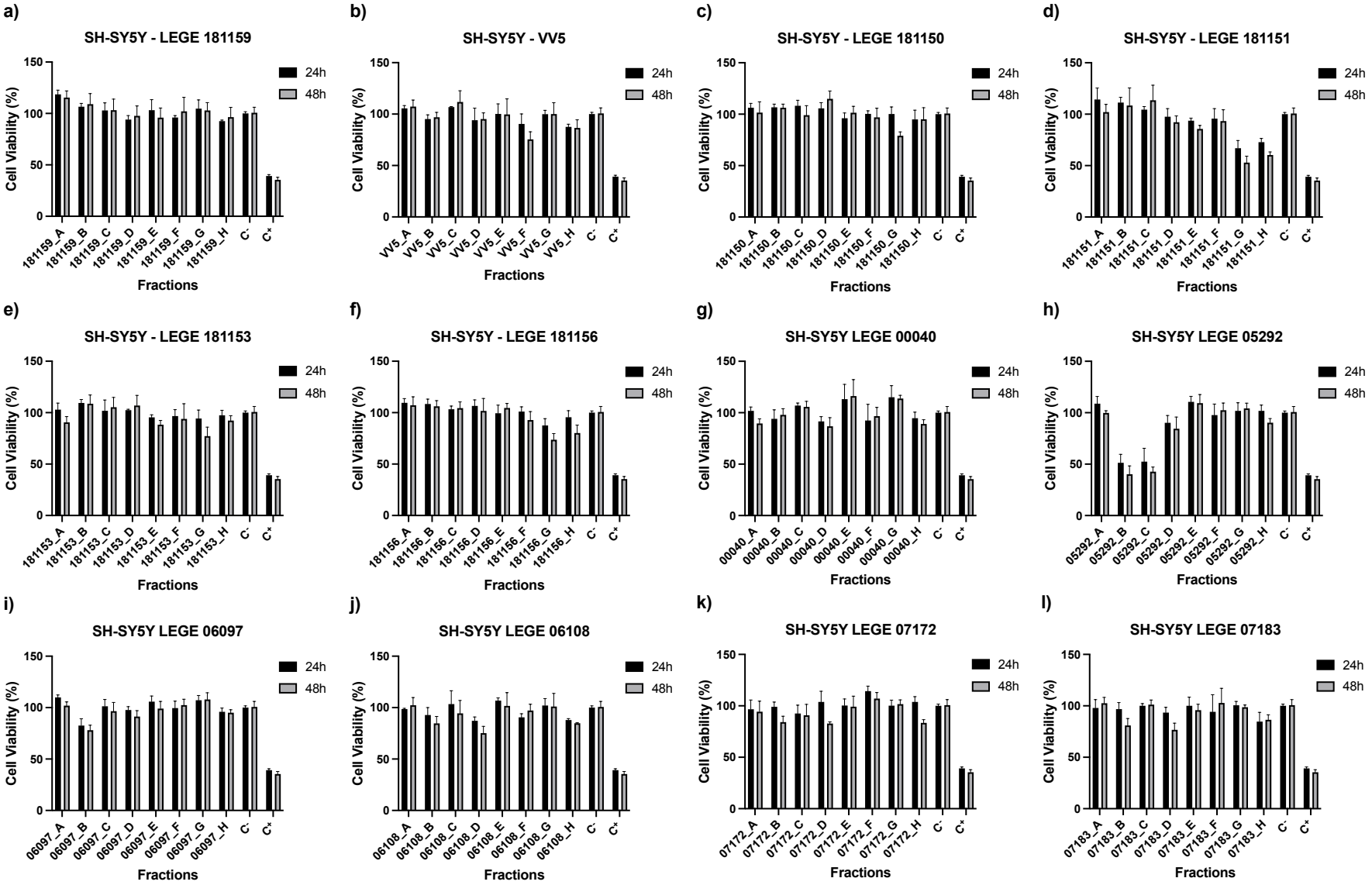


Figure 10. Cell viability (%) at 24h and 48h of the neuroblastoma cell line SH-SY5Y exposed to the 8 fractions (50 μg/mL) of a) *Oscillatoriales cyanobacterium* LEGE 181159; b) VV5; c) *Synechococcales cyanobacterium* LEGE 181150; d) *Synechococcales cyanobacterium* LEGE 181151; e) *Leptothoe* sp. LEGE 181153; f) *Leptothoe* sp. LEGE 181156; g) *Synechocystis salina* LEGE 00040; h) *Phormidium* sp. LEGE 05292; i) *Cyanobium* sp. LEGE 07097; j) *Leptolyngbya mycoidea* LEGE 06108; j) *Synechococcus* sp. LEGE 07172; l) *Cyanobium* sp. LEGE 07183. Negative control (C-) was 1% DMSO (100% viability) and Positive Control (C+) was 20% DMSO. Results are expressed as mean±SD, of three independent experiments, in triplicate.

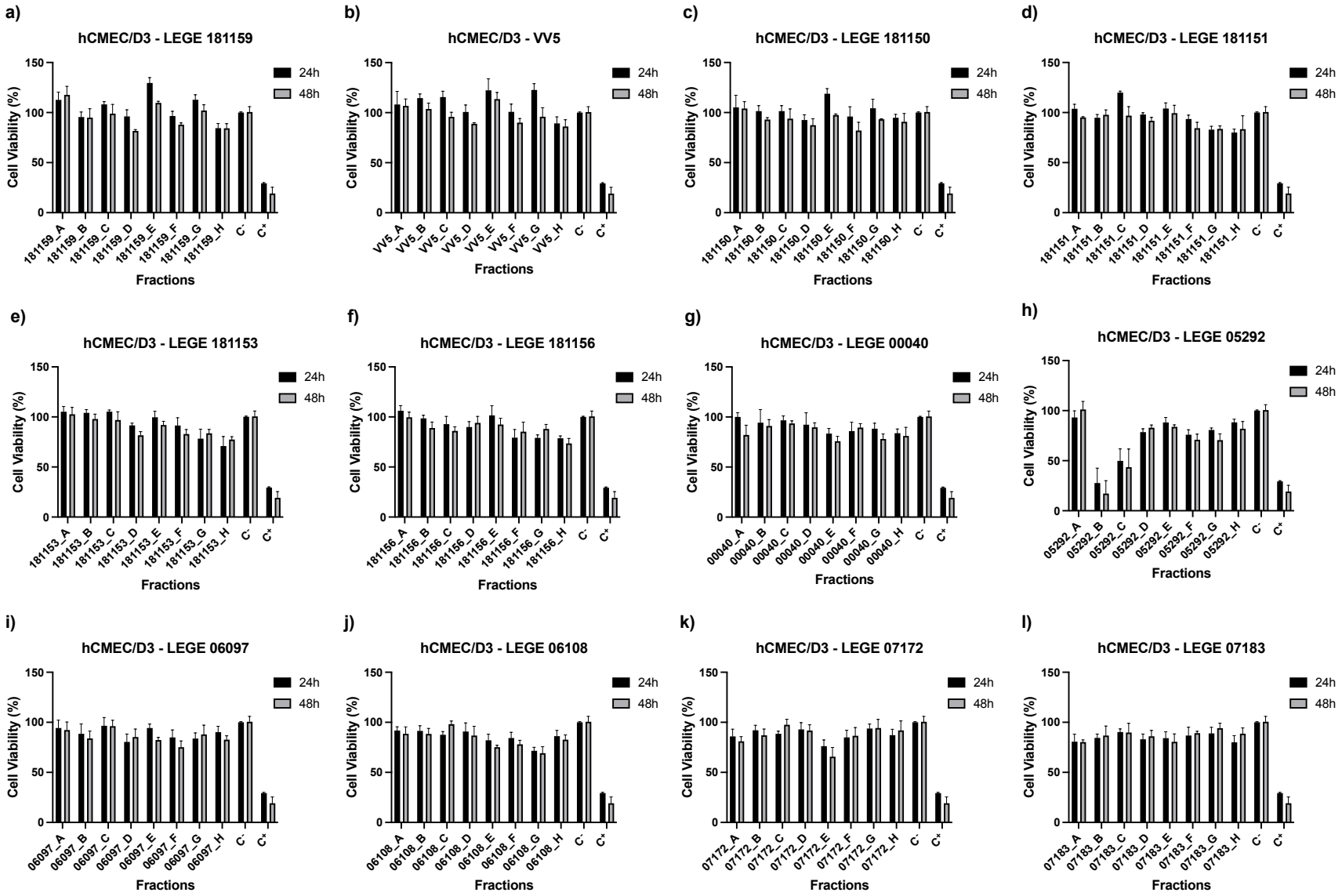


Figure 11. Cell viability (%) at 24h and 48h of the human cerebral microvascular endothelial cell line (hCMEC/D3) exposed to the 8 fractions (50 μg/mL) of a) *Oscillatoriales cyanobacterium* LEGE 181159; b) VV5; c) *Synechococcales cyanobacterium* LEGE 181150; d) *Synechococcales cyanobacterium* LEGE 181151; e) *Leptothoe* sp. LEGE 181153; f) *Leptothoe* sp. LEGE 181156; g) *Synechocystis salina* LEGE 00040; h) *Phormidium* sp. LEGE 05292; i) *Cyanobium* sp. LEGE 07097; j) *Leptolyngbya mycoidea* LEGE 06108; j) *Synechococcus* sp. LEGE 07172; l) *Cyanobium* sp. LEGE 07183. Negative control (C–) was 1% DMSO (100% viability) and Positive Control (C+) was 20% DMSO. Results are expressed as mean±SD, of three independent experiments, in triplicate.

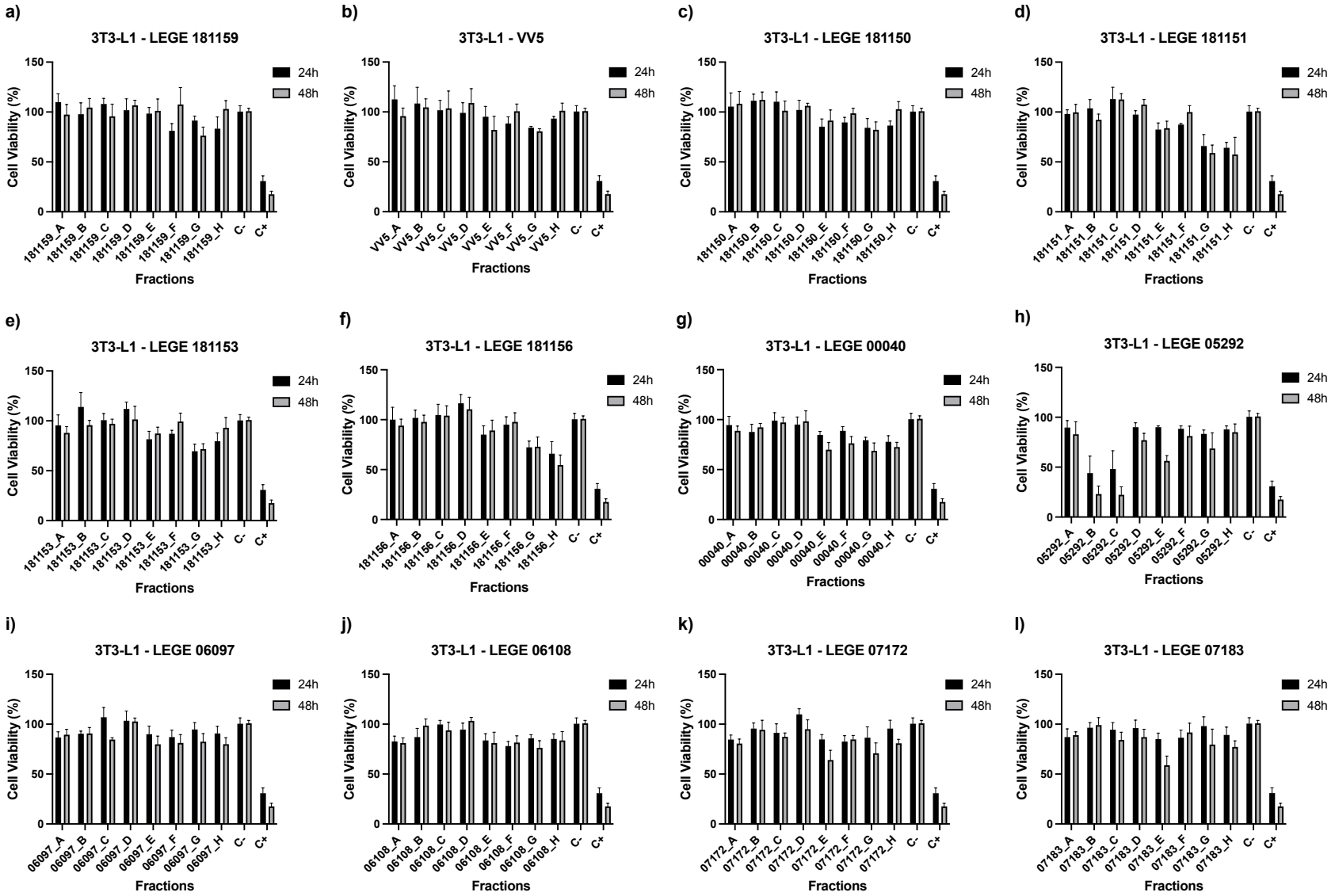


Figure 12. Cell viability (%) at 24h and 48h of fibroblast cell line (3T3-L1) exposed to the 8 fractions (50 μg/mL) of a) *Oscillatoriales cyanobacterium* LEGE 181159; b) VV5; c) *Synechococcales cyanobacterium* LEGE 181150; d) *Synechococcales cyanobacterium* LEGE 181151; e) *Leptothoe* sp. LEGE 181153; f) *Leptothoe* sp. LEGE 181156; g) *Synechocystis salina* LEGE 00040; h) *Phormidium* sp. LEGE 05292; i) *Cyanobium* sp. LEGE 07097; j) *Leptolyngbya mycoidea* LEGE 06108; j) *Synechococcus* sp. LEGE 07172; l) *Cyanobium* sp. LEGE 07183. Negative control (C-) was 1% DMSO (100% viability) and Positive Control (C+) was 20% DMSO. Results are expressed as mean±SD, of three independent experiments, in triplicate.

4.3. Acetylcholinesterase (AChE) inhibition

AChE is a critical enzyme that plays a significant role in the cholinergic nervous system. It is bound to the postsynaptic membrane of cholinergic junctions and primarily functions by hydrolyzing ACh. This enzyme is recognized as an exceptionally efficient one from a kinetic standpoint, resulting in a rapid turnover time and thus a decrease in ACh levels within the synaptic cleft (31). Therefore, the regulation of ACh levels through the inhibition of its hydrolysis rate by AChE is an important approach for treating AD symptoms associated with ACh deficiency, including cognitive impairment (31, 57). As a result, AChEIs are the first line of treatment for patients diagnosed with AD. As previously mentioned, three AChEIs are in commercial use, namely galantamine, donepezil, and rivastigmine. However, there is a pressing need to develop more effective and safe alternatives (57, 59).

It is a well-known fact that natural products are an excellent source of lead compounds, and many have shown potential as AChEIs. In fact, galantamine is a natural alkaloid obtained from the *Galanthus* species, and rivastigmine was developed from a natural alkaloid, physostigmine (124, 125). Cyanobacteria are also a great natural source of AChEIs, like nostocarboline and anatoxin-a(S) (73, 74). In particular, it has been found that AChEIs in cyanobacteria can restrict the colonization of colonies and filaments of other organisms by inhibiting the settlement of invertebrate larvae (126). Therefore, the screening of AChEIs in cyanobacteria presents a favorable prospect.

Ellman's method is one of the most standard assays for AChEIs screening due to its affordability, accessibility, and ease of use in a high throughput setting. However, it is limited by its low sensitivity and tendency to produce false-positive results (127). The method is based on the production of a yellow-colored product, the anion TNB^{2-} through the reaction of DNTB with thiocholine, the product of the hydrolysis of acetylthiocholine by AChE. This yellow product can be measured by spectrophotometry (127, 128). The assay employs AChE derived from *Electrophorus electricus*, as AChE is conserved across multiple species and thus displays similar substrate selectivity and specificity of inhibition (31, 127).

In this work, the 176 fractions derived from the 22 cyanobacteria strains included in the study were screened for the potential to inhibit AChE. Due to limitations on the amount of available volume of each fraction, a single concentration of 500 μg/mL was tested.

The percentage of AChE inhibitory activity can be classified as potent (>50%), moderate (30–50%), or low (<30%), according to Vinutha et al. (129). In this screening, a percentage below 10% was considered null, and thus Figure 13 only displays the results of fractions that showed more than 10% inhibition. In general, most fractions did not exhibit AChE inhibitory activity, as they were unable to suppress enzyme activity by more than 10% (data not shown). Out of the 176 fractions evaluated, only 28 displayed inhibitory activity against AChE higher than 10% but lower than 30% (Figure 13). Among these, the highest activity was found in LEGE 11439_A with 29.15% inhibition, and LEGE 07189_A with 21.73% of inhibition.

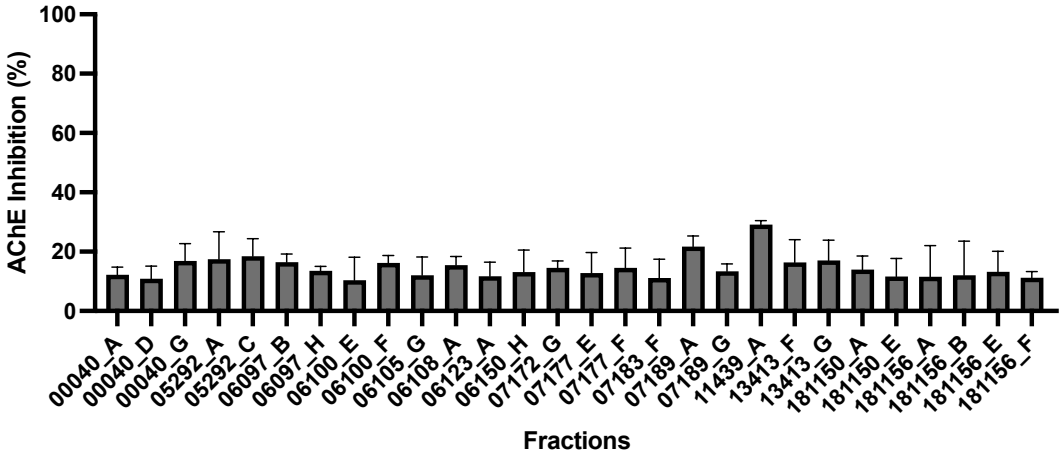


Figure 13. Inhibitory activity (%) against acetylcholinesterase (AChE), determined by the Ellmans method, of fractions (500 μg/mL) that showed inhibition above 10%. Values are expressed as mean ± SD, of one independent experiment, in triplicate.

However, it must be emphasized that the results were obtained from a single independent experiment carried out in triplicate, which is insufficient. Therefore, it is imperative to conduct additional experiments to confirm the validity of these results.

Interestingly the most active fraction in this work comes from *Spirulina* sp. LEGE 11439. *Spirulina* species are one of the most extensively studied genera of cyanobacteria regarding its potential in the field of AD, with impressive demonstrations of antioxidant, anti-inflammatory, and neuroprotective properties (10, 11). In fact, it has been substantiated by previous investigations that *Spirulina* extracts have AChE inhibitory capacity. For instance, both the 70% ethanol extract of *Spirulina maxima* (89, 130) and a fermented *Spirulina maxima* extract (131) have been shown to reduce AChE activity in mice hippocampal tissue. This highlights the potential of *Spirulina* in this domain.

When comparing the cytotoxicity data with the AChE inhibition data (Table XV, Appendix VI), it can be deduced that none of the active fractions against AChE were cytotoxic and that most non-cytotoxic fractions did not exhibit significant AChE inhibition potential at the concentrations tested, with the highest percentage of inhibition being seen in LEGE 05292_A (17.43%) and LEGE 05292_D (18.47%), as shown in Table 10.

Table 10. Combination of the results of cell viability and the correspondent percentage of AChE inhibition of fractions that did not show cytotoxicity and inhibited AChE by over 10%.

	SH-SY5Y		hCMEC/D3		3T3-L1		AChE inhibition (%)
	24h	48h	24h	48h	24h	48h	
LEGE 00040_A	101.79	89.61	100.07	82.10	94.55	88.78	12.14
LEGE 00040_D	91.53	86.75	92.29	89.74	95.04	98.37	10.89
LEGE 05292_A	108.79	99.85	93.23	101.13	89.74	82.95	17.43
LEGE 05292_D	90.16	84.42	78.50	82.97	90.08	77.00	18.47
LEGE 06097_B	82.53	78.06	88.50	83.89	90.46	90.73	16.49
LEGE 06097_H	95.99	95.01	90.22	82.67	90.67	79.97	13.50
LEGE 06108_A	98.52	102.26	91.82	88.54	82.71	81.04	15.44
LEGE 07183_F	94.23	102.86	86.76	89.14	86.31	91.66	11.11
LEGE 181159_A	118.51	115.49	112.74	117.71	109.96	97.47	13.98
LEGE 181159_E	103.19	95.98	129.61	109.68	98.40	101.24	11.63
LEGE 181156_A	109.62	107.24	106.23	99.64	100.04	94.10	11.50
LEGE 181156_B	108.42	106.21	98.58	88.93	101.81	97.87	12.02
LEGE 181156_E	99.49	104.58	101.62	92.43	85.07	89.24	13.10
LEGE 181156_F	101.12	92.81	79.26	85.36	95.02	97.79	11.19

In general, it appears that the fractions with more activity tend to be those from A to D, as opposed to E to H. This correlation may be attributed to the polarity of the compounds present in these fractions, as fractions A to D are the first to be eluted, comprising the most polar compounds, while fractions E to H comprise the least polar compounds. Consequently, the compounds in fractions E to H are not easily soluble in the utilized solvent, which is primarily polar (buffer A + 10% methanol). In fact, solubility issues were encountered at the concentration tested, which suggests that the compounds may not be completely soluble and thus less available to effectively exert their effect.

Upon examination of the overall results, it is evident that LEGE 11439_A and LEGE 07189_A represent the most promising fractions in this context. It would be beneficial to undertake additional testing on other concentrations of these fractions, to try and find their IC_{50}, and to evaluate their efficacy against BChE to fully grasp their potential as ChEIs. It is, however,

important to acknowledge the absence of cytotoxicity results for these two fractions and the necessity of conducting further assays to address this aspect.

In other studies, the research on finding new natural AChE inhibitors has focused on screening mostly crude extracts from cyanobacteria. However, fractionation plays a crucial role in simplifying the complexity of the matrix, thereby enabling a more efficient screening, as the most productive fraction is selected for compound isolation (132). AChE inhibition is often attributed to the presence of one or more active compounds (132, 133). For example, the fractionation of the active crude water-methanolic extract of *Nostoc* sp. str. Lukešová 27/97 led to the identification of one active fraction (97.8% inhibition) with one peak, corresponding to the active compound nostrotrebin 6 (133, 134). In another study, the fractionation of the active chloride/methanol crude extract of *Anabaena variabilis* also led to the identification of the most active fraction (73.6% inhibition) with two peaks, which were identified as a flavonoid and an alkaloid, two active compounds (79).

Following this perspective, it would also be valuable to study the chemical composition of the LEGE 11439_A and LEGE 07189_A fractions with mass spectrometry, to elucidate the compounds responsible for their biological activity. For instance, alkaloids, terpenoids, phenolic compounds, and flavonoids are believed to possess anti-AChE activity (124).

In general, this study has demonstrated that several fractions of cyanobacteria possess some degree of enzyme-inhibitory properties, particularly concerning the inhibition of AChE. Enzymes play a crucial role as molecular targets and enzyme inhibitors are highly sought-after therapeutic agents in various biotechnological applications (135). It this note, certain strains exhibiting some AChE inhibition, such as LEGE 181150 and LEGE 181156, have been reported in other studies to also possess additional enzymatic inhibition properties, such as the inhibition of skin metalloproteinases namely hyaluronidase, elastase, collagenase, and tyrosinase (16). This highlights their vast biotechnological potential.

5. Conclusion

The escalating impact of AD on aging populations demands immediate attention, as this debilitating condition encompasses significant health, economic, and social burdens. The fact that AD remains incurable and lacks effective treatments only exacerbates the necessity of exploring alternative interventions.

Cyanobacteria are ancient prokaryotic oxygenic phototrophs. Given that they possess remarkable chemical prolificacy and the capability to produce neuroprotective compounds, exploring cyanobacteria strains for their potential in combating AD represents a noteworthy opportunity.

This work took advantage of the diversity of the LEGE-CC collection and the practicality of the LEGE-NPL tool, which facilitates the identification of bioactive compounds. Consequently, 176 fractions from 22 cyanobacterial strains were evaluated for their potential to target AD.

LEGE 181156 represented the most favorable economic outlook, as it exhibited good biomass production potential and together with LEGE 181159 and LEGE 181151 attained the highest extraction yield among the alternatives. This is interesting for scale-up purposes and for isolating compounds, which is a process that requires a lot of substrate.

With regards to toxicity, most fractions were deemed non-cytotoxic in the SH-SY5Y, hCMEC/D3, and 3T3-L1 cell lines, with only 15 being considered toxic (less than 70% viability). Focusing on the potential of the fractions as AChE inhibitors, an important category of drugs in AD treatment, the fractions *Spirulina* sp. LEGE 11439_A and *Scytonema* sp. LEGE 07189_A demonstrated some promise, with low to moderate inhibition of 29.15% and 21.73%, respectively.

Nonetheless, this work only constitutes a preliminary endeavor. Further exploration of other properties, such as anti-inflammatory, antioxidant, and anti-excitotoxicity capacities, utilizing more extensive and complex assays that adequately capture the intricacies of AD is crucial for identifying the true potential of these strains in the AD field.

In summary, there is potential for cyanobacteria to be utilized as a treatment option, however, the specific strains utilized in this project are not the most promising. Further screenings like this study are necessary to gain a deeper understanding of the potential cyanobacteria in the treatment of NDs, which is still an emerging field.

6. Future perspectives

Notwithstanding the limited efficacy of these strains as AChE inhibitors, there is still an opportunity to investigate the potential of these cyanobacteria fractions against AD. Due to laboratory and time constraints, it was not possible to further assess their full therapeutic potential. Nonetheless, a plethora of additional therapeutic targets can be explored in this regard. For example, evaluating the inhibitory potential of the fractions against other critical enzymes in AD such as BACE-1, the enzyme responsible for the rate-determining step in Aβ formation, represents a great therapeutical target. Other potential tests include assessing their potential to reduce glutamate-induced cytotoxicity in cell-based assays, as excitotoxicity is a significant contributor to neurodegeneration in AD. Furthermore, the assessment of the anti-inflammatory and antioxidant properties of the fractions is of utmost importance, as neuroinflammation and oxidative stress play a crucial role in the pathology of AD. In fact, some strains investigated in this study have been found to possess antioxidant activity, such as LEGE 181150, LEGE 181156, and LEGE 181159 (16).

Another approach could be to conduct *in vitro* experiments in more complex AD cell models, or even *in vivo* experiments utilizing simple animal models that are commonly employed in the study of NDs, such as *C. elegans*, yeast, and zebrafish. These models can provide valuable insight into the effects of the fractions on cognitive and behavioral symptoms associated with AD.

Lastly, in the event of a hit in any of the previously mentioned assays, the identification and isolation of the chemical compound responsible for the activity is crucial.

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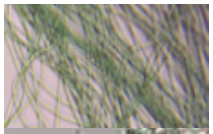
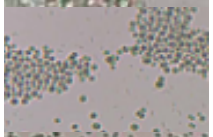
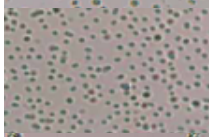
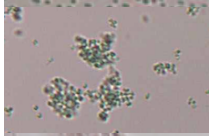
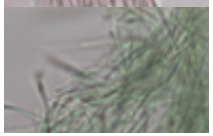
Appendices

Appendix I. Information about the strains cultured

Table I. Information about the LEGE-CC strains that were cultured in 4L cultures but were not included in the screening.*

Microphotograph 1000×	Strain ID	Identification	Sampling	Lifestyle
	LEGE 06102	*Nodosilinea nodulosa*	Esposende, Portugal (submerged stone)	Aquatic, marine
	LEGE 06155	*Synechocystis salina*	Esposende, Portugal (rock surface)	Aquatic, marine
	LEGE 07171	*Synechococcus nidulans*	Burgau, Portugal (rock surface)	Aquatic, marine
	LEGE 07173	*Cyanobium* sp.	Albufeira, Portugal (submerged stone)	Aquatic, marine
	LEGE 07175	*Cyanobium* sp.	Vila do Bispo, Portugal (seawater sample)	Aquatic, marine
	LEGE 181157	*Synechococcales cyanobacterium*	São Vicente Island, Cape Verde	Aquatic, marine
	VR7B	*Unidentified*	São Vicente Island, Cape Verde	Aquatic, marine

*Courtesy of the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE CC, http://lege.ciimar.up.pt), CIIMAR and (110).

Appendix II. Log of the 4L cultures

Table II. Log of the 4L cultures performed at BOGA, and the obtained dry biomass (g).

Strain ID	Volume	Medium	Conditions	Date of inoculation	Date of harvest	Days of growth	Dry biomass (g)
LEGE 06155	4L	Z8 + 25% TM + 1% B_{12}	Standard	12/10/22	17/11/22	36	3.1545
LEGE 07173	4L	Z8 + 25% TM + 1% B_{12}	Standard	10/10/22	17/11/22	36	1.5205
LEGE 07171	4L	Z8 + 25% TM + 1% B_{12}	Standard	10/10/22	17/11/22	36	3.0495
LEGE 07175	4L	Z8 + 25% TM + 1% B_{12}	Standard	10/10/22	17/11/22	36	2.0276
LEGE 06155	4L	Z8 + 25% TM + 1% B_{12}	Standard	15/11/22	13/12/22	28	1.1912
LEGE 181150	4L	Z8 + 25% TM + 1% B_{12}	Standard	17/11/22	14/12/22	27	4.7532
LEGE 181156	4L	Z8 + 25% TM + 1% B_{12}	Standard	17/11/22	14/12/22	27	3.8601
LEGE 06102	4L	Z8 + 25% TM + 1% B_{12}	Standard	28/11/22	12/01/23	45	1.5672
LEGE 181153	4L	Z8 + 25% TM + 1% B_{12}	Standard	28/11/22	14/01/23	45	1.0077
LEGE 181159	4L	Z8 + 25% TM + 1% B_{12}	Standard	02/12/22	13/01/23	42	1.3837
LEGE 07175	4L	Z8 + 25% TM + 1% B_{12}	Standard	02/12/22	13/01/23	42	3.7260
LEGE 181156	4L	Z8 + 25% TM + 1% B_{12}	Standard	14/12/22	12/01/23	29	1.3447
LEGE 181151	4L	Z8 + 25% TM + 1% B_{12}	Standard	14/12/22	13/01/23	30	1.0929
LEGE 181150	4L	Z8 + 25% TM + 1% B_{12}	Standard	14/12/22	12/01/23	29	1.6420
VV5	4L	Z8 + 25% TM + 1% B_{12}	Standard	12/01/23	17/02/23	36	0.4020
VR7B	4L	Z8 + 25% TM + 1% B_{12}	Standard	12/01/23	13/02/23	32	0.8337
LEGE 181159	4L	Z8 + 25% TM + 1% B_{12}	Standard	20/01/23	17/02/23	28	0.5341
LEGE 06102	4L	Z8 + 25% TM + 1% B_{12}	Standard	20/01/23	15/02/23	26	1.2290

LEGE 07175	4L	Z8 + 25% TM + 1% B_{12}	Standard	20/01/23	16/02/23	27	1.3057
LEGE 181156	4L	Z8 + 25% TM + 1% B_{12}	Standard	17/02/23	04/04/23	46	2.1329
LEGE 181157	4L	Z8 + 25% TM + 1% B_{12}	Standard	17/02/23	04/04/23	46	2.7241
LEGE 06155	4L	Z8 + 25% TM + 1% B_{12}	Standard	17/02/23	05/04/23	47	2.3256
LEGE 181150	4L	Z8 + 25% TM + 1% B_{12}	Standard	17/02/23	05/04/23	47	4.6673
VR7B	4L	Z8 + 25% TM + 1% B_{12}	Standard	17/02/23	05/04/23	47	1.2244
VV5	4L	Z8 + 25% TM + 1% B_{12}	Standard	09/03/23	05/05/23	57	1.0365
VR7B	4L	Z8 + 25% TM + 1% B_{12}	Standard	09/03/23	05/05/23	57	1.4850

Appendix III. Standard culture conditions and composition of the Z8 medium

Table III. Standard culture growth conditions.

Standard Growth Conditions	
Culture Medium	Z8 25% TM sea salt w/ vitamin B12
Temperature	25-30°C
Light	10-30 µmol/ms²/s
Photoperiod	16/8 (light /dark cycles)
Aeration	

Table IV. Composition of the Z8 medium.

Z8 Medium	
Reagents	**mL/L dH_2O***
Solution A	10
Solution B	10
Fe-EDTA Solution	10
Micronutrients Solution	1

Table V. Chemical composition of Solution A.

Solution A	
Reagents	**g/L dH_2O***
Sodium Nitrate ($NaNO_3$)	46.7
Calcium Nitrate Tetrahydrate ($Ca(NO_3)_2.4H_2O$)	5.9
Magnesium Sulphate Heptahydrate ($MgSO_4.7H_2O$)	2.5

Table VI. Chemical composition of Solution B.

Solution B	
Reagents	**g/L dH_2O***
Potassium Phosphate Dibasic (K_2HPO_4)	3.1
Sodium Carbonate (Na_2CO_3)	2.1

Table VII. Composition of Fe-EDTA Solution.

Fe-EDTA Solution	
Reagents	**mL/L dH_2O***
Iron (III) Chloride ($FeCl_3$)	10
Sodium EDTA (EDTA-Na)	9.5

Table VIII. Chemical composition of the $FeCl_3$ and EDTA-Na reagents.

$FeCl_3$		EDTA-Na	
Reagents	**g/100 mL HCl (0.1N)**	**Reagents**	**g/100 mL NaOH (0.1N)**
Ferric Chloride Hexahydrate ($FeCl_3.6H_2O$)	2.80	Ethylenediamine tetraacetic acid (EDTA)	3.90

Table IX. Composition of the Micronutrients Solution.

Micronutrients Solution	
Reagents	**mL/L dH_2O***
1 a 12	10
13 a 14	100

Table X. Chemical composition of the Micronutrients Solution.

Micronutrients	
Reagents	**g/L dH_2O***
1- Sodium tungstate dihydrate ($Na_2WO_4.2H_2O$)	0.33
2- Ammonium paramolybdate dihydrate ($(NH_4)6Mo_7O_{24}.2H_2O$)	0.88
3- Potassium bromide (KBr)	1.2
4- Potassium iodide (KI)	0.83
5- Zinc Sulfate Heptahydrate ($ZnSO_4.7H_2O$)	2.87
6- Cadmium nitrate tetrahydrate ($Cd(NO_3).4H_2O$)	1.55
7- Cobalt (II) Nitrate Hexahydrate ($Co(NO_3)_2.6H_2O$)	1.46
8- Copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$)	1.25
9- Nickel (II) ammonium bi-sulfate hexahydrate ($NiSO_4(NH_4)_2SO_4.6H_2O$)	1.98
10- Chromium (III) Nitrate Nonahydrate ($Cr(NO_3)_3.9H_2O$)	0.41
11- Vanadium pentoxide (V_2O_5)	0.089
12- Aluminium potassium bi-sulfate dodecahydrate ($Al_2(SO_4)3K_2SO_4.24H_2O$)	4.74
13- Boric acid (H_3BO_3)	3.1
14- Manganese (II) Sulfate Tetrahydrate ($MnSO_4.4H_2O$)	2.23

Table XI. Composition of Salt Z8 medium for marine strains.

Salt Z8 Medium	
Reagent	**g/L dH_2O***
TM sea salt	25
Reagents	**mL/L dH_2O***
Solution A	10
Solution B	10
Fe-EDTA Solution	10
Micronutrients Solution	1
Vitamin B12	1

**dH_2O - Deionized water.*

Appendix IV. Schematic representation of mother plaques

Table XII. Schematic representation of mother plaque 2 retrieved from the solid LEGE-NPL and HPLC fractionated.

	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO	00040_A	05292_A	06097_A	06108_A	07172_A	07183_A					DMSO
B	DMSO	00040_B	05292_B	06097_B	06108_B	07172_B	07183_B					DMSO
C	DMSO	00040_C	05292_C	06097_C	06108_C	07172_C	07183_C					DMSO
D	DMSO	00040_D	05292_D	06097_D	06108_D	07172_D	07183_D					DMSO
E	DMSO	00040_E	05292_E	06097_E	06108_E	07172_E	07183_E					DMSO
F	DMSO	00040_F	05292_F	06097_F	06108_F	07172_F	07183_F					DMSO
G	DMSO	00040_G	05292_G	06097_G	06108_G	07172_G	07183_G					DMSO
H	DMSO	00040_H	05292_H	06097_H	06108_H	07172_H	07183_H					DMSO

Table XIII. Schematic representation of mother plaque 3 retrieved from the liquid LEGE-NPL.

	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO	06100_A	06105_A	06122_A	06150_A	07177_A	07189_A	11386_A	13413_A	06123_A	11439_A	DMSO
B	DMSO	06100_B	06105_B	06122_B	06150_B	07177_B	07189_B	11386_B	13413_B	06123_B	11439_B	DMSO
C	DMSO	06100_C	06105_C	06122_C	06150_C	07177_C	07189_C	11386_C	13413_C	06123_C	11439_C	DMSO
D	DMSO	06100_D	06105_D	06122_D	06150_D	07177_D	07189_D	11386_D	13413_D	06123_D	11439_D	DMSO
E	DMSO	06100_E	06105_E	06122_E	06150_E	07177_E	07189_E	11386_E	13413_E	06123_E	11439_E	DMSO
F	DMSO	06100_F	06105_F	06122_F	06150_F	07177_F	07189_F	11386_F	13413_F	06123_F	11439_F	DMSO
G	DMSO	06100_G	06105_G	06122_G	06150_G	07177_G	07189_G	11386_G	13413_G	06123_G	11439_G	DMSO
H	DMSO	06100_H	06105_H	06122_H	06150_H	07177_H	07189_H	11386_H	13413_H	06123_H	11439_H	DMSO

Appendix V. Summary of cell viability results

Table XIV. Summary of the cell viability results obtained with the MTT assay for the SH-SY5Y, hCMEC/D3 and 3T3-L1 cell line after exposure to the 96 fractions obtained by HPLC fractionation (24h and 48h). ++++ represents cell viability higher than 110% +++ represents cell viability of 101-110%; ++ represents cell viability of 81%-100%. + represents cell viability of 71-80% and – represents cell viability of 70% or lower.

	SH-SY5Y		hCMEC/D3		3T3-L1	
	24h	48h	24h	48h	24h	48h
LEGE 00040_A	+++	++	++	++	++	++
LEGE 00040_B	++	++	++	++	++	++
LEGE 00040_C	+++	+++	++	++	++	++
LEGE 00040_D	++	++	++	++	++	++
LEGE 00040_E	++++	++++	++	+	++	–
LEGE 00040_F	++	++	++	++	++	+
LEGE 00040_G	++++	++++	++	+	+	–
LEGE 00040_H	++	++	++	++	+	+
LEGE 05292_A	+++	++	++	+++	++	++
LEGE 05292_B	–	–	–	–	–	–
LEGE 05292_C	–	–	–	–	–	–
LEGE 05292_D	++	++	+	++	++	+
LEGE 05292_E	++++	+++	++	++	++	–
LEGE 05292_F	++	+++	+	+	++	++
LEGE 05292_G	+++	+++	+	+	++	–
LEGE 05292_H	+++	++	++	++	++	++
LEGE 06097_A	+++	+++	++	++	++	++
LEGE 06097_B	++	+	++	++	++	++
LEGE 06097_C	+++	++	++	++	+++	++
LEGE 06097_D	++	++	+	++	+++	+++
LEGE 06097_E	+++	++	++	++	++	+
LEGE 06097_F	++	+++	++	+	++	++
LEGE 06097_G	+++	+++	++	++	++	++
LEGE 06097_H	++	++	++	++	++	+
LEGE 06108_A	++	+++	++	++	++	++
LEGE 06108_B	++	++	++	++	++	++
LEGE 06108_C	+++	++	++	++	++	++
LEGE 06108_D	++	+	++	++	++	+++
LEGE 06108_E	+++	+++	++	+	++	++
LEGE 06108_F	++	++	++	+	+	++
LEGE 06108_G	+++	+++	–	–	++	+

LEGE 06108_H	++	++	++	++	++	++
LEGE 07172_A	++	++	++	++	++	+
LEGE 07172_B	++	++	++	++	++	++
LEGE 07172_C	++	++	++	++	++	++
LEGE 07172_D	+++	++	++	++	+++	++
LEGE 07172_E	++	++	+	-	++	-
LEGE 07172_F	++++	+++	++	++	++	++
LEGE 07172_G	++	+++	++	++	++	-
LEGE 07172_H	+++	++	++	++	++	+
LEGE 07183_A	++	+++	+	+	++	++
LEGE 07183_B	++	++	++	++	++	++
LEGE 07183_C	++	+++	++	++	++	++
LEGE 07183_D	++	+	++	++	++	++
LEGE 07183_E	++	++	++	+	++	-
LEGE 07183_F	++	+++	++	++	++	++
LEGE 07183_G	++	++	++	++	++	+
LEGE 07183_H	++	++	+	++	++	+
LEGE 181159_A	++++	++++	++++	++++	+++	++
LEGE 181159_B	+++	+++	++	++	++	+++
LEGE 181159_C	+++	+++	+++	++	+++	++
LEGE 181159_D	++	++	++	++	+++	+++
LEGE 181159_E	+++	++	++++	+++	++	+++
LEGE 181159_F	++	+++	++	++	++	+++
LEGE 181159_G	+++	+++	++++	+++	++	+
LEGE 181159_H	++	++	++	++	++	+++
VV5_A	+++	+++	+++	+++	++++	++
VV5_B	++	++	++++	+++	+++	+++
VV5_C	+++	++++	++++	++	+++	+++
VV5_D	++	++	++	++	++	+++
VV5_E	++	++	++++	++++	++	++
VV5_F	++	+	++	++	++	++
VV5_G	++	++	++++	++	++	+
VV5_H	++	++	++	++	++	+++
LEGE 181150_A	+++	+++	+++	+++	+++	+++
LEGE 181150_B	+++	+++	+++	++	++++	++++
LEGE 181150_C	+++	++	+++	++	++++	+++
LEGE 181150_D	+++	++++	++	++	+++	+++
LEGE 181150_E	++	+++	++++	++	++	++

LEGE 181150_F	++	++	++	++	++	++
LEGE 181150_G	++	+	+++	++	++	++
LEGE 181150_H	++	++	++	++	++	+++
LEGE 181151_A	++++	+++	+++	++	++	++
LEGE 181151_B	++++	+++	++	++	+++	++
LEGE 181151_C	+++	++++	++++	+++	++++	++++
LEGE 181151_D	++	++	++	++	++	+++
LEGE 181151_E	++	++	+++	++	++	++
LEGE 181151_F	++	++	++	++	++	++
LEGE 181151_G	-	-	++	++	-	-
LEGE 181151_H	+	-	+	++	-	-
LEGE 181153_A	+++	++	+++	+++	++	++
LEGE 181153_B	+++	+++	+++	++	++++	++
LEGE 181153_C	+++	+++	+++	++	++	++
LEGE 181153_D	+++	+++	++	++	++++	++
LEGE 181153_E	++	++	++	++	++	+
LEGE 181153_F	++	++	++	++	++	+
LEGE 181153_G	++	+	+	++	-	+
LEGE 181153_H	++	++	+	+	+	+
LEGE 181156_A	+++	+++	+++	++	++	++
LEGE 181156_B	+++	+++	++	++	+++	++
LEGE 181156_C	+++	+++	++	++	+++	+++
LEGE 181156_D	+++	+++	++	++	++++	++++
LEGE 181156_E	++	+++	+++	++	++	++
LEGE 181156_F	+++	++	+	++	++	++
LEGE 181156_G	++	+	+	++	-	-
LEGE 181156_H	++	+	+	+	-	-

Cytotoxic fractions.

Fractions that induce cell viability above 110%.

Appendix VI. Summary of cell viability data combined with AChE inhibition results

Table XV. Combination of the results of cell viability and the correspondent percentage of AChE inhibition of fractions that did not show cytotoxicity and inhibited AChE by over 10%.

	SH-SY5Y		hCMEC/D3		3T3-L1		AChE inhibition (%)
	24h	48h	24h	48h	24h	48h	
LEGE 00040_A	101.79	89.61	100.07	82.10	94.55	88.78	12.14
LEGE 00040_B	94.00	97.93	94.28	91.08	87.71	92.34	
LEGE 00040_C	106.92	105.66	96.65	93.57	99.00	97.13	
LEGE 00040_D	91.53	86.75	92.29	89.74	95.04	98.37	10.89
LEGE 00040_F	92.44	96.68	85.95	89.48	88.87	76.34	
LEGE 00040_H	94.69	89.10	83.84	81.00	77.81	72.51	
LEGE 05292_A	108.79	99.85	93.23	101.13	89.74	82.95	17.43
LEGE 05292_D	90.16	84.42	78.50	82.97	90.08	77.00	18.47
LEGE 05292_F	97.67	102.39	75.99	70.98	88.47	81.21	
LEGE 05292_H	101.84	90.31	88.36	81.99	87.81	85.03	
LEGE 06097_A	109.94	101.94	94.34	92.26	86.69	89.49	
LEGE 06097_B	82.53	78.06	88.50	83.89	90.46	90.73	16.49
LEGE 06097_C	101.37	96.57	96.52	96.19	106.97	84.55	
LEGE 06097_D	97.69	91.30	80.42	85.31	103.39	102.69	
LEGE 06097_E	105.79	99.05	94.27	82.37	89.98	79.86	
LEGE 06097_F	99.58	102.48	84.99	75.14	87.03	81.14	
LEGE 06097_G	107.20	107.99	83.76	87.91	94.64	82.47	
LEGE 06097_H	95.99	95.01	90.22	82.67	90.67	79.97	13.50
LEGE 06108_A	98.52	102.26	91.82	88.54	82.71	81.04	15.44
LEGE 06108_B	92.76	84.66	91.44	88.41	87.04	98.50	
LEGE 06108_C	103.47	94.42	87.62	98.25	99.63	93.83	
LEGE 06108_D	87.16	75.24	90.78	86.85	94.58	103.28	
LEGE 06108_E	106.78	101.70	82.11	75.24	83.69	81.04	
LEGE 06108_F	90.55	97.23	84.27	78.10	78.10	81.60	
LEGE 06108_H	87.88	84.7	86.15	82.74	85.15	83.63	
LEGE 07172_A	96.67	94.35	85.99	81.02	84.58	80.57	
LEGE 07172_B	99.04	84.24	92.08	87.11	95.56	94.26	
LEGE 07172_C	92.60	90.81	88.55	97.58	91.37	87.30	
LEGE 07172_D	103.86	82.87	92.97	91.82	109.85	94.99	
LEGE 07172_F	114.2	107.01	84.88	86.54	82.53	70.78	
LEGE 07172_H	103.83	83.4	87.18	91.91	95.52	80.80	
LEGE 07183_A	97.98	102.57	80.63	80.33	86.94	89.00	
LEGE 07183_B	96.77	80.96	84.47	86.82	96.30	99.12	
LEGE 07183_C	100.04	101.20	90.35	89.76	94.29	84.15	
LEGE 07183_D	93.40	76.67	83.12	86.09	95.90	87.11	
LEGE 07183_F	94.23	102.86	86.76	89.14	86.31	91.66	11.11

LEGE 07183_G	100.60	98.70	88.95	94.19	97.89	79.59	
LEGE 07183_H	84.66	86.44	80.03	88.65	89.05	77.20	
LEGE 181159_A	118.51	115.49	112.74	117.71	109.96	97.47	13.98
LEGE 181159_B	106.56	109.03	95.54	95.00	97.84	104.46	
LEGE 181159_C	103.00	103.24	108.14	98.97	108.05	95.74	
LEGE 181159_D	94.09	97.75	96.22	81.73	101.77	106.74	
LEGE 181159_E	103.19	95.98	129.61	109.68	98.40	101.24	11.63
LEGE 181159_F	96.09	102.06	96.58	87.88	81.18	107.58	
LEGE 181159_G	104.73	102.97	112.87	102.08	91.42	76.31	
LEGE 181159_H	92.53	96.38	84.49	84.33	83.26	103.10	
VV5_A	105.58	107.36	108.21	106.85	112.59	95.84	
VV5_B	95.14	96.92	114.53	103.71	108.42	104.56	
VV5_C	106.81	111.87	115.60	95.85	101.63	103.62	
VV5_D	94.02	95.17	100.64	88.96	99.11	108.90	
VV5_E	100.24	99.69	122.29	113.54	95.20	81.95	
VV5_F	90.44	75.43	100.77	90.11	88.40	100.73	
VV5_G	99.94	100.07	122.70	95.98	84.23	80.57	
VV5_H	87.56	86.56	89.25	86.21	93.26	101.24	
LEGE 181150_A	106.37	101.54	105.17	104.02	105.38	108.30	
LEGE 181150_B	106.69	106.36	101.39	93.06	111.28	112.18	
LEGE 181150_C	108.14	99.09	101.41	93.90	110.28	101.36	
LEGE 181150_D	105.71	114.95	92.59	87.39	102.12	106.14	
LEGE 181150_E	96.02	101.40	118.83	97.54	85.29	91.50	
LEGE 181150_F	100.31	96.96	96.08	82.10	89.59	98.68	
LEGE 181150_G	100.24	79.05	104.26	93.26	84.17	82.17	
LEGE 181150_H	94.94	95.05	94.81	90.85	86.43	102.87	
LEGE 181151_A	114.36	102.10	103.86	95.17	98.06	99.64	
LEGE 181151_B	111.34	108.44	94.77	97.75	103.74	92.19	
LEGE 181151_C	104.39	113.57	120.00	96.97	112.93	112.52	
LEGE 181151_D	97.61	92.13	98.12	91.87	97.44	107.39	
LEGE 181151_E	93.63	85.71	104.10	99.47	82.40	83.87	
LEGE 181151_F	95.66	93.36	93.54	84.39	87.38	99.89	
LEGE 181153_A	102.88	90.52	105.03	102.70	95.41	85.58	
LEGE 181153_B	109.40	108.74	103.96	97.98	113.93	85.32	
LEGE 181153_C	101.80	105.37	105.13	96.94	100.70	84.03	
LEGE 181153_D	102.48	106.96	91.49	81.67	111.94	82.17	
LEGE 181153_E	95.45	88.34	99.53	91.95	81.50	78.97	
LEGE 181153_F	96.65	93.92	91.36	82.96	87.00	77.29	
LEGE 181153_H	97.49	92.34	70.81	77.50	79.56	77.39	
LEGE 181156_A	109.62	107.24	106.23	99.64	100.04	94.10	11.50
LEGE 181156_B	108.42	106.21	98.58	88.93	101.81	97.87	12.02
LEGE 181156_C	103.32	104.49	92.79	86.10	104.68	104.05	
LEGE 181156_D	106.56	101.64	89.88	94.02	116.44	110.48	

LEGE 181156_E	99.49	104.58	101.62	92.43	85.07	89.24	13.10
LEGE 181156_F	101.12	92.81	79.26	85.36	95.02	97.79	11.19

Non-cytotoxic fractions with AChE inhibition potential above 10%

Appendix VII. Review paper

Stalling the course of neurodegenerative diseases – could cyanobacteria constitute a new approach toward therapy?

Abstract

Neurodegenerative diseases (NDs) are characterized by progressive and irreversible neuronal loss, accompanied by a range of pathological pathways, including aberrant protein aggregation, altered energy metabolism, excitotoxicity, inflammation, and oxidative stress. Some of the most common NDs include Alzheimer's Disease (AD), Parkinson's Disease (PD), Multiple Sclerosis (MS), Amyotrophic Lateral Sclerosis (ALS), and Huntington's Disease (HD). There are currently no available cures; there are only therapeutic approaches that ameliorate the progression of symptoms, which makes the search for new drugs and therapeutic targets a constant battle. Cyanobacteria are ancient prokaryotic oxygenic phototrophs whose long evolutionary history has resulted in the production of a plethora of biomedically relevant compounds with anti-inflammatory, antioxidant, immunomodulatory, and neuroprotective properties, that can be valuable in this field. This review summarizes the major NDs and their pathophysiology, with a focus on the anti-neurodegenerative properties of cyanobacterial compounds and their main effects.

Keywords: cyanobacteria; spirulina; neurodegenerative diseases; natural products.

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