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Exploring the potential of cyanobacteria against neurodegenerative diseases with focus on Alzheimer's disease

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Resumo

As doenças neurodegenerativas (DN), nomeadamente a doença de Alzheimer (DA), afetam milhões de pessoas por todo o mundo. Apesar da sua elevada incidência, não existe cura para estas doenças. A principal linha de tratamento passa pela utilização de inibidores da acetilcolinesterase (AChE) e da butirilcolinesterase (BuChE), como o donepezil, a galantamina e a rivastigmina. As cianobactérias têm sido alvo de procura de terapias contra as DN. Vários estudos in sílico, in vitro e in vivo têm comprovado o potencial neuroprotetor dos produtos naturais derivados de cianobactérias, particularmente na DA. Diversas estirpes de cianobactérias presentes na Blue Biotechnology and Ecotoxicology Culture Colletion (LEGE-CC) do CIIMAR foram avaliadas quanto à sua citotoxicidade em células de neuroblastoma (SH-SY5Y) e fibroblastos (3T3-L1) e quanto ao seu potencial inibidor das enzimas AChE e BuChE.

A abordagem seguida passou pela análise de frações de cianobactérias presentes na biblioteca de produtos naturais (LEGE-NPL) obtidas por HPLC e cultivo de algumas estirpes para produção de biomassa. Por cada estirpe foram obtidas 8 frações, tendo no total sido analisadas 176 frações. Foram também testados três compostos extraídos de cianobactérias (compostos A4, B2D e C3). Os resultados dos ensaios de viabilidade celular, através da utilização do ensaio MTT, mostraram que a grande maioria das frações não apresenta toxicidade a uma concentração de 25 μg/mL. Quanto aos ensaios relativos à inibição enzimática, tendo por base o método de Ellman, os resultados mostraram não ocorrer inibição com a maior parte das frações analisadas. De uma forma geral, os resultados mais promissores quer para os ensaios celulares quer para os ensaios enzimáticos, foram obtidos com as frações LEGE 07175_E, LEGE 11439_A e LEGE 181150_B. Assim, mais testes devem ser realizados utilizando estas frações no sentido de elucidar a composição química das mesmas. Relativamente aos ensaios com os compostos, nos ensaios de viabilidade celular apenas o composto C3 não se mostrou tóxico nas concentrações estudadas. Quanto aos ensaios enzimáticos, nenhum dos compostos inibiu as enzimas na gama de concentrações testadas.

Em suma, mais estudos devem ser realizados de forma a elucidar a capacidade de utilização das cianobactérias no tratamento de DNs, assim como elucidar os perfis químicos das frações mais promissoras.

Palavras-chave: Doenças neurodegenerativas; Cianobactérias; Doença de Alzheimer; Acetilcolinesterase; Butirilcolinesterase; SH-SY5Y

Abstract

Neurodegenerative diseases (ND), particularly Alzheimer's disease (AD), affect millions of people around the world. Despite their high incidence, there is no cure for these diseases. The main line of treatment involves the use of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitors, such as donepezil, galantamine and rivastigmine. Cyanobacteria have been the target for new therapies against DN. Several in silico, in vitro and in vivo studies have demonstrated the neuroprotective potential of natural products derived from cyanobacteria, particularly in AD. Several strains of cyanobacteria present in CIIMAR's Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC) were evaluated for their cytotoxicity in neuroblastoma cells (SH-SY5Y) and fibroblasts (3T3-L1) and for their potential to inhibit the enzymes AChE and BuChE.

The followed approach involves analyzing cyanobacterial fractions from the natural products library (LEGE-NPL) obtained by HPLC and cultivating some strains to produce biomass. For each strain, 8 fractions were obtained, with a total of 176 fractions analyzed. Three pure compounds isolated from cyanobacteria were also tested (compounds A4, B2D and C3). The results of the cell viability test, using the MTT assay, showed that the vast majority of the fractions were not toxic. As for the enzyme inhibition tests, based on the Ellman method, the results show no inhibition for most analyzed fractions.

In general, the most promising results for were obtained for the LEGE 07175_E, LEGE 11439_A and LEGE 181150_B fractions. Further tests should be carried out using these fractions in order to elucidate their chemical composition. With regard to the analyses of the compounds, in the cellular viability assay only compound C3 did not demonstrate cytotoxicity at the studied concentrations. As for the enzymatic assays, none of the compounds inhibit the enzymes in the range of concentrations tested.

In summary, further studies should be carried out in order to elucidate the capability of cyanobacteria as treatment against NDs, as well as to clarify the chemical profiles of the most promising fractions.

Keywords: Neurodegenerative diseases; Cyanobacteria; Alzheimer's disease; Acetylcholinesterase; Butyrylcholinesterase; SH-SY5Y

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List of Abbreviations

ABTS- 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)

ACh- Acetylcholine

AChE- Acetylcholinesterase

AChE- I- Acetylcholinesterase inhibitors

AD- Alzheimer's disease

ADAS-cog- Alzheimer's Disease Assessment Scale—cognitive subscale

Aer-865- Aeruginosin-865

AGS- Gastric adenocarcinoma cell-line

ALS- Amyotrophic lateral sclerosis

APP- Amyloid precursor protein

ARE- antioxidant response element

ATCI- Acetylcholine iodine

Aβ- Amyloid beta protein

BACE-I- Beta amyloid cleaving enzyme I

BBB- Blood-brain-barrier

BCh- Butyrylcholine

BDNF- Brain-derived neurotrophic factor

BOGA- Bioterium of Aquatic Organisms

BTCI- Butyrylthiocholine iodine

BuChE- Butyrylcholinesterase

BuChE- I- Butyrilcholinesterase inhibitors

C. elegans - *Caenorhabditis elegans*

CAG- Triplet cytosine, adenine and guanine

CAT- Catalase

CDR-SB- Clinical Dementia Rating Scale—Sums of Boxes

CHI- Chlorophyl

CIIMAR- Interdisciplinary Centre of Marine and Environmental Research

CNS- Central nervous system

COX- Cyclooxygenase

dH_2O- Deionized water

DHA- Docosahexaenoic acid

DMEM– Dulbecco's Modified Eagle Medium

DMSO– Dimethyl sulfoxide

DNA– Deoxyribonucleic acid

DOX– Doxorubicin

DPPH– 2,2–Diphenyl–1–picrylhydrazyl

DTNB– 5,5′–dithiobis–(2–nitrobenzoic acid)

EC_{50}– Median effective concentration

EO– Essential oils

EOAD– Early onset Alzheimer's disease

EPA– Eicosapentaenoic acid

fALS– Familial amyotrophic lateral sclerosis

FDA– Food and drugs administration

FRA –Ferric Reducing Ability

FRAP–Ferric ion reducing ability of plasma

GBE– Ginkgo biloba extract

G–CSF–Granulocyte–colony stimulating factor

GLP– Ganoderma lucidum polysaccharides

GLUT– Glucose transporter

GSH– Glutathione

H_2O_2– Hydrogen peroxide

HD– Huntington's disease

HEK293 ARE–luc–human embryonic kidney cells stably transfected with firefly luciferase reporter gene

HI– Hexane: isopropanol

HLMVECs– Stimulated human lung microvascular endothelial cells

$HO^{\cdot}$– Hydroxyl radical

HPLC– High performance liquid chromatography

hPRP– Human platelet–rich plasma

HRBC– Human red blood cell

hTNF–α– Human tumor necrosis factor–alpha

HTT– Huntingtin gene

IC_{50}– Half maximal inhibitory concentration

ICAM-1- Intercellular adhesion molecule-1

ICR- Institute of cancer research

IL1-α- Interleukin 1 alpha

IL-8- Interleukin-8

LDH- Lactate dehydrogenase

LEGE-CC- Blue Biotechnology and Ecotoxicology Culture Collection

LEGE-NPL- Cyanobacterial Natural Products Library

LOAD- Late-onset Alzheimer´s disease

LPS- Lipopolysaccharides

MAO-A- Monoamine oxidase A

MAO-B- Monoamine oxidase B

MAOs- Monoamine oxidases

MCP-5-Murine monocyte chemoattractant protein-5

MDA- Malondialdehyde

MeCN- Acetonitrile

MIP3-α-Macrophage inflammatory protein-3-alpha

MMSE- Mini-Mental State Examination

MTT-3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

MWM- Morris water maze

NADH- Nicotinamide adenine dinucleotide (NAD) + Hydrogen

NADPH- Nicotinamide adenine dinucleotide phosphate

ND- Neurodegenerative diseases

NF-kβ- Nuclear factor kappa-light-chain-enhancer of activated B cells

NMDA- N-methyl-D-aspartate

•NO- Nitric oxide

Nrf2- Nuclear factor erythroid 2

NSAIDs- Nonsteroidal anti-inflammatory drugs

$O_2^{\bullet -}$ - Superoxide anion

PAF- Platelet-activating factor.

PARP- Poly (ADP-ribose) polymerase

PBPs- Phycobiliproteins

PB-TURSO- Sodium phenylbutyrate and taurursodiol

PC 12- Pheochromocytoma

PC- Phycocyanin

PD- Parkinson's disease

PE- Phycoerythrin

PNS- Peripheral nervous system

PP- Peroxidation potential

Rd-Dammar-24(25)-ene-3b,12b,20(S)-triol-(20-O-b-D-glucopyranosyl)-3-O-b-D-glucopyranosyl-(1, 2)-b-D-glucopyranoside

RNA- Ribonucleic acid

RNS- Reactive nitrogen species

ROS- Reactive oxygen species

RP- reducing power

sALS- Sporadic amyotrophic lateral sclerosis

SM- *Spirulina maxima*

SM70EE- 70% *Spirulina maxima* ethanol extract

SNpc- Substantia nigra pars compacta of the brain

SOD- Superoxide dismutase

TAC- Total antioxidant capacity

TMT- Thimethyltin

TNB_2^- - Anion 5-thio-2-nitrobenzoate

TNF-α- Tumor necrosis factor alpha

TPA- 12-O-Tetradecanoylphorbol13-acetate

TSPO- 18-kDa translocator protein

TUNEL- Terminal deoxynucleotidyl transferase dUTP nick-end labeling

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1. Introduction

1.1. General Introduction

Neurodegenerative diseases (ND) affect millions of people worldwide. This group of disorders affect the nervous system leading to progressive deterioration and loss of nerve cells (1,2). Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and Amyotrophic lateral sclerosis (ALS) are some of the most common ND (3). Memory loss, involuntary movements, difficulty in speaking and walking are some of the most common symptoms associated with these neurological conditions (1).

A major challenge towards ND is the search for efficient therapies. In fact, despite their high incidence there is still no effective medicinal therapy (1). Presently, most of the research is focused on strategies to discover new targets for ND, and new drugs for therapy. The application of marine-derived bioactive compounds is among the new features for therapies against ND, as described in a significant number of reviews in the field (4–7) . Marine organisms are recognized as an important source of bioactive compounds with a broad range of structures and bioactive applications. Due to the inhospitable conditions of marine life and the long evolutionary stages, marine organisms evolved a remarkable secondary metabolism ensuing in the production of a chemical and biochemical arsenal of compounds, which are found to be active in pathologies such as cancer and pain (8).

The neurodegenerative process or neurodegeneration is believed to be caused by a set of interconnected mechanisms that include hereditary factors, synaptic loss, misfolded or overexpressed proteins, excessive amounts of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and inflammation, as shown in Figure 1 (2).

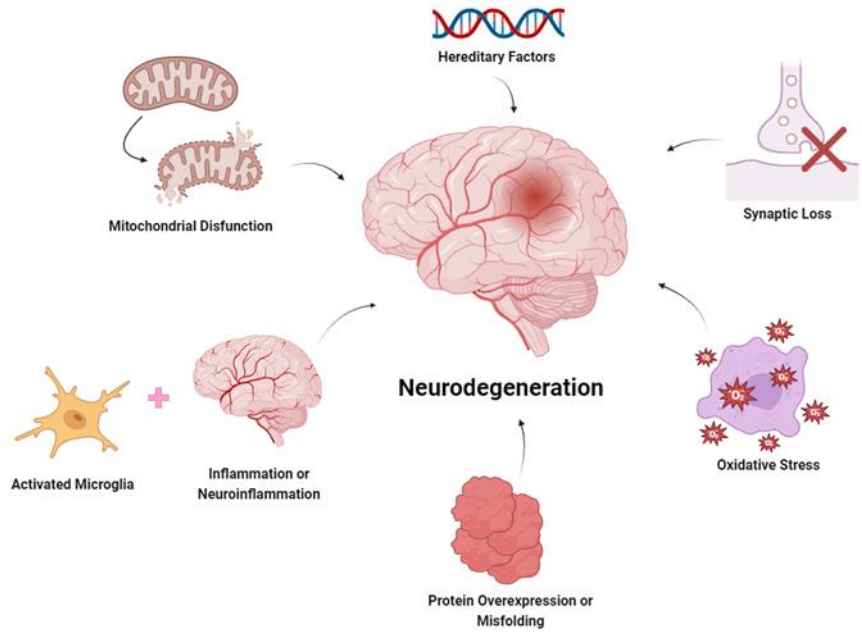


Figure 1. Principal causes related to the neurodegenerative process.
*Created with https://www.biorender.com/

Bioactive features of marine compounds towards neurodegenerative disorders such as AD encompass anti-inflammatory and antioxidant properties, acetylcholinesterase inhibitors (AChE-I), butyrylcholinesterase inhibitors (BuChE-I) and beta amyloid cleaving enzyme I (BACE I) inhibitors, among others. Oxidative stress, caused by unregulated ROS production, and inflammation have been pointed out as two important process in the development of neuroinflammation and subsequent neurodegeneration (9). Cholinesterase inhibitors are used to alleviate the symptoms of neurological disorders such as AD, including the approved drugs donepezil, galantamine and rivastigmine. Since ND have a multifactorial basis, it is difficult to find effective treatments and therapies. In addition, the crossing of drugs through the blood-brain barrier (BBB) consists in another difficulty since not all substances can cross this membrane. Therefore, it is important to study and identify new pharmaceuticals or targets that can be used to fight these mechanisms (10,11).

Marine organisms ranging from microorganisms, invertebrates and macro and microalgae were found to be prolific in the production of such type of enzyme inhibitors such as alkaloids, plastoquinones and farnesylacetones (12,13). Although marine natural products are not yet being used for ND treatment, the success stories in marine drugs make pertinent the effort to research in this field, namely on less exploited organisms such as cyanobacteria. Cyanobacteria are also known to produce structurally diverse natural products, including terpenes, glycosides

polyketides, peptides, and lipopeptides with various pro-health properties, such as anti-inflammatory, antioxidant and neuroprotective activity (14).

As recently reviewed, cyanobacteria reveal potential in the treatment or prevention of neurodegenerative diseases, particularly in AD (15,16).

In line with this assumption, the collection of cyanobacteria from the Interdisciplinary Center for Marine and Environmental Research (CIIMAR) - Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC) - has been studied in order to identify strains with the potential for ND treatment or prevention. The collection comprises more than 1000 strains of cyanobacteria, some of which have already been studied in previous dissertations (17,18). In this dissertation, our main objective was to continue the study of strains aimed at inhibiting enzymes involved in the pathology and cellular toxicity. In addition to the general introduction presented above, which aims to give a general approach to the topic of the dissertation, the introduction includes:

- a general description of cyanobacteria;
- a general description of the main ND;
- a general description of many factors involved in neurodegeneration mainly directed to AD, inflammation and oxidative stress;
- a linkage between cyanobacteria and ND.

1.2. Cyanobacteria

Cyanobacteria, also known as, blue-green algae, are Gram-negative photoautotrophic prokaryotes and are considered the most primitive organisms on Earth (19). These are particularly resistant organisms that can survive in a wide range of environments, even in extreme conditions such as hot springs, frozen systems and extremely saline environments (19). Cyanobacteria taxonomy has undergone some changes mostly related to the advances in 16S rRNA gene sequencing. In the last years, more than 270 species in 140 genera were newly described with an actual number of 19 orders (20). These microorganisms can be found in a variety of colors, such as green, red, and yellow (21) due to the presence of natural pigments such as chlorophylls, phycobiliproteins (PBPs) and carotenoids (22). Cyanobacteria present different morphologies, from unicellular and colonial to multicellular filamentous forms (19). Unicellular cyanobacteria have round, oval, or cylindrical cells that can aggregate into irregular or regular colonies bound. The number of cells in each colony deviate from two to several thousand according to the specie

(19,23). In the filamentous cyanobacteria forms, cells are connected to each other forming a chain called "trichomes". Branches and/or "pseudobranches" are formed when trichomes break or fragment (19). Aquatic cyanobacteria can be divided into planktonic and benthic cyanobacteria. Planktonic cyanobacteria float freely in water columns as opposed to benthic cyanobacteria that adhere to submerged solid surfaces like sediments, stones, and aquatic plants (14,24).

Cyanobacteria are a major source of compounds and products of interest, especially in the areas of pharmacology, cosmetics and nutrition (24,25). These include lipids, alkaloids, polyketides, peptides (lipopeptides, depsipeptides), vitamins, phenolic compounds, amino acids, polysaccharides, terpenes and PBPs. Due to their rich biochemical content, cyanobacteria possess great pro-health properties such as antioxidant, anti-inflammatory, antibacterial, antifungal, antidiabetic, anticancer, and neuroprotective activities (14,25).

1.3. Neurodegenerative diseases

Neurodegenerative diseases are related with the progressive loss of neurons in the central nervous system (CNS) or peripheral nervous system (PNS). Neurodegeneration is being related to metabolic and toxic disorders and very linked to inflammation and accumulation of free radicals which lead to oxidative stress (1). Main ND include dementia in which AD is the most prevalent form, monogenic neurodegenerative disease in which HD is included and motor neuron diseases in which are included PD and ALS.

1.3.1. Alzheimer's Disease

AD is characterized by a slow decline in cognitive faculties, memory loss, and behavioral changes (26). Although the precise etiology behind AD is unknown, it is thought to be a result of a confluence of hereditary, environmental, and lifestyle factors (26). AD tends to get worse over time and its symptoms and progression vary from patient to patient. Age is considered the primary risk factor for development of this disease, around half of all population over the age of 85 can be affected by this sickness (27). It is expected that by the year 2050 more than 100 million people will be affected with AD (27).

AD can be classified into two types, sporadic and familial Alzheimer. The sporadic form, known as late-onset AD (LOAD), affects individuals over 65 years old. Early onset AD (EOAD), or familial AD can affect people between the ages of 30 and 65 and is related to hereditary and biological

factors. It is also related to mutations in Amyloid precursor protein (APP), Presenilin 1, and Presenilin 2 genes (28).

Brain atrophy in AD is caused by degradation of synapses, neuronal death, deposition of amyloid–β (Aβ) plaques in the brain, tau protein aggregation and deposition, and inflammatory and oxidative processes (29). In patients with AD, there is a deficiency in the neurotransmitters acetylcholine (ACh) and butyrylcholine (BCh) and alterations on the expression of the enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), which is related to the loss of communications between neurons (15,30).

Therapeutics used in AD usually contain neuroprotective, anti-inflammatory, and antioxidant characteristics (31). Other treatments include inhibitors of AChE such as donepezil, rivastigmine and galantamine all approved from the Food and Drugs Administration (FDA) (32). Memantine is another FDA approved drug used for moderate to severe AD treatment as its act as an N-methyl-D-aspartate (NMDA) receptor antagonist. This drug inhibits glutamate hyperactivation which causes excitotoxicity and neuronal vulnerability (33).

1.3.2. Parkinson's Disease

PD is the second most common ND. Despite age being the main risk factor for this pathology, 5-10% of PD cases are caused by genetic factor, that include mutations in the PARKIN and α-synuclein genes (34). Patients with this disease suffer from bradykinesia (slowness of movement), tremor (typically at rest and absent during movement), rigidity (resistance to movement), and balance changes (34).

This pathology is the result of dopaminergic neuron loss in the substantia nigra pars compacta of the brain (SNpc). The loss of these neurons is believed to be associated with an increase in ROS production due to inflammation and loss of mitochondrial functions. In PD is also observed an unusual aggregation of α-synuclein in Lewy bodies (35).

Currently the treatment for PD focuses mainly on the dopaminergic mechanism. Levodopa is the preferred drug used in PD treatment; this medication is converted into dopamine in the SNpc. Monoamine oxidase B (MAO-B) inhibitors, which decrease dopamine metabolism, are also used as a therapy (36).

1.3.3. Huntington's disease

HD is characterized by progressive physical weakening, mental symptoms, and cognitive decline (37). HD is an autosomal dominant inherited disease, caused by an overexpression of the triplet cytosine, adenine and guanine (CAG) in the huntingtin gene (HTT) in the chromosome 4 (4p16.3) (37). With this mutation HTT proteins become more susceptible to misfolding leading to formation of aggregates and consequent deposition, affecting several areas of the brain specially the striatum (38). Furthermore, abnormal HTT mediate mitochondrial dysfunction, leading to increase in production of ROS and RNS and their accumulation (9).

Patients suffering from HD show reduce levels of the glucose transporter (GLUT) 1 and 3, increase in the amount of lactate, and alterations in the respiratory chain being affected the activity of complexes II, III, and IV (9,39).

Eastern countries such as Japan, Korea and Hong Kong show a lower prevalence of this condition compared to countries like Canada, United States and United Kingdom, which may be related to low mutation rates in these East Asian countries (40).

1.3.4. Amyotrophic lateral sclerosis

ALS, also known as Lou Gehrig's disease, is a ND that affects upper and lower motor neurons and other brain neurons such as the frontotemporal region (41). This ND is characterized by weakness, muscle degenerate and fasciculations. People suffering from this disease normally have a survival time of up to 50 months after diagnosis, and usually die from respiratory failure (2,41).

Currently ALS is divided into sporadic ALS (sALS) or familial ALS (fALS). The majority of patients with this disease are included in sALS (42). In fALS a large percentage of the cases is caused by mutations in SOD1, C9orf72, TARDBP and FUS genes (42). The SOD1 gene, for instance, is important for modulation of cellular respiration and scavenging excessive superoxide ($O_2^{\cdot-}$) radicals (43). ALS leads to various alterations at the cellular level, such as alterations in RNA (ribonucleic acid) metabolism, mitochondrial dysfunction, oxidative stress, excitotoxicity and neuroinflammation (44).

There is no cure for ALS, however there a few drugs that can slowdown the progression of this illness and prolong survival, such as riluzole, edavarone, and sodium phenylbutyrate and taurursodiol (PB-TURSO) (45–47). New treatments might include the use of stem cells, antibodies, RNA-interventions, and small molecules related to oxidative stress and inflammation (47).

1.3.5. Inflammation

Inflammation is the body's natural response to an infection, exposure to pathological agents, chronic and autoimmune diseases and an unhealthy lifestyle (48). This defense mechanism can have two stages: acute or chronic. Acute inflammation is the first response of the immune system and is usually temporary. When this defense mechanism fails, chronic and persistent inflammation occurs leading to diseases such as diabetes, ND, and cancer (48). In an inflammatory condition several cellular components and biochemical mediators, including cytokines (interleukin-1 (IL-1), interleukin-6(IL-6)), tumor necrosis factor-α (TNF-α), kinases and transcription factors (NF-kβ) are activated (49).

Nowadays, therapy for inflammation and inflammatory diseases is based on steroidal and non-steroidal anti-inflammatory drugs (NSAIDs). These NSAIDs act as prostaglandins inhibitors by inhibiting the cyclooxygenase (COX) enzymes. Despite having multiple benefits, the prolonged use of steroidal and non-steroidal drugs is associated with hypertension, hyperglycemia, osteoporosis, cardiovascular, gastrointestinal and renal toxicity (49). Therefore, search for new therapies and drugs for this disease has become essential. In this sense, natural compounds have been seen as an alternative therapy (50).

Inflammation in the CNS is typically named "neuroinflammation". This process occurs as a response to endogenous or exogenous stimuli, for example, toxins and pathogens, protein misfolding and changes in mitochondria function (51). During this defense mechanism, microglia and astrocytes are activated, leading to the production of inflammatory mediators such as cytokines and chemokines (52). When there is an overproduction of these pro-inflammatory elements, severe neurological damage can occur and ultimately neuronal death (53).

The use of neuroinflammation biomarkers is extremely important for disease prognosis, however there are no specific biomarkers for various neurological diseases. Nevertheless, biomarkers such as 18-kDa translocator protein (TSPO) and MAO-B are usually used as biomarkers in neuroinflammation (51).

TSPO is a transmembrane protein located in the outer mitochondrial membrane (54). TPSO is highly expressed by the brain defense cells in response to a stimulus associated with inflammation. In the CNS, this biomarker is involved in several function such as apoptosis regulation (55). TSPO ligands have demonstrated to be effective in reducing neuroinflammation and neuronal damage both in in vivo and in vitro models of neurodegenerative diseases.

Neurosteroids' production, cytokine release and metabolism of ROS are believed to be some of the events related to these findings (56).

Monoamine oxidases (MAOs) enzymes regulate the amine levels in the brain (53). These enzymes have two isoforms, monoamine oxidase A (MAO-A) and MAO-B, this last one is predominant in the glial cells in the brain (57). MAO-B inhibitors are used in the treatment of neurodegenerative diseases such as PD and AD (57). MAO-B activity is regulated by astrocytes (glial cells) in this sense it is believed that this enzyme can be used as a biomarker for neuroinflammation (53).

1.3.6. Oxidative stress

Reactive oxygen species are produced from molecular oxygen and are the result of cellular metabolism (58). Superoxide anion, hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\cdot}$) are some examples of this species (59). ROS are mainly produced from the cytoplasmic membrane, nicotinamide–adenine dinucleotide phosphate (NADPH) oxidase and from the mitochondrial respiratory chain (59).

The brain is very susceptible to oxidative stress since it consumes high amounts of molecular oxygen and has elevated quantity of polyunsaturated fatty acid which are very sensitive to peroxidation (59). Most ND are associated with atypical formation of protein aggregates, this event can lead to oxidative stress and inflammation due to mitochondrial dysfunction and ROS production (59).

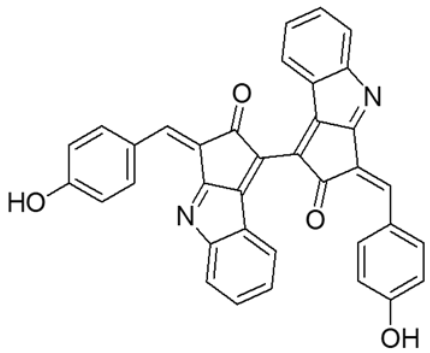
Oxidative stress arises when there is an imbalance between the production of ROS and the capacity of antioxidant molecules to remove these radicals from the system. An antioxidant is a substance or molecule that can eliminate and/or prevent cell damage, caused by ROS, which are produced in the system during physiological processes or can be related to external factors such as smoking, pollution and radiation (60). Excessive quantities of ROS can cause alterations in deoxyribonucleic acid (DNA) molecules, lipids, and proteins. These modifications can lead to the development of diseases such as asthma, chronic inflammation, neurodegenerative and cardiovascular diseases, diabetes, and cancer (60).

In order to be protected against the harmful effects of ROS, human cells depend on their antioxidant capacity. Tissues incorporated antioxidant systems composed of various liposoluble (vitamin E, carotenoids), hydrosoluble (ascorbic acid) and enzymatic components (glutathione peroxidase (GSH), superoxide dismutase (SOD), catalase (CAT)) (61–63).

1.4. Cyanobacteria in neurodegenerative diseases

In June 2021 a review focused on linking cyanobacteria to AD was publish by Castaneda and co-authors (15). In this review around 23 research results were published regarding enzymatic inhibition, namely AChE, BChE and β-secretase (BACE-1) enzymes and Aβ aggregation and toxicity. In August 2023, a new review was published by Vitoria Ramos and co-authors whose focus was the application of cyanobacteria in neurodegenerative diseases (16). In this review, twenty-three works related to AD, thirteen works associated to PD, two works related to HD and other two works associated to ALS were compiled (Table 1). After extensive research, it was found that only two research works related to cyanobacteria in ND focuses on bioactivities other than the antioxidant and anti-inflammatory potential were published last year. These are present in Table 1 highlighted in blue. Since inflammation and oxidative stress are mechanisms highly associated to the neurodegenerative process a compilation of studies related to this topic was performed, being the main results presented in Table 2.

Cyanobacteria's potential against inflammation and oxidative stress has been studied for the last decades, with researchers focusing in the isolation of compounds with these properties (14,16,64). Scytonemin **(1)** (Figure 2) is a yellow-green ultraviolet absorber pigment found in several genera of cyanobacteria (65). This indole alkaloid demonstrated anti-inflammatory properties in both in vivo and in vitro studies (65). In BALB/c mice, topical application of scytonemin (300 ng/ear) inhibited TPA (12-O-Tetradecanoylphorbol13-acetate) -induced ear edema. It was also verified that TNF-α expression and inducible nitric oxide synthase (iNOS) levels were suppressed in animals' model. In addition, in LPS-stimulated RAW 264.7 macrophages, scytonemin (10-20 ng/mL) inhibited TNF- α and NF-kβ expression.

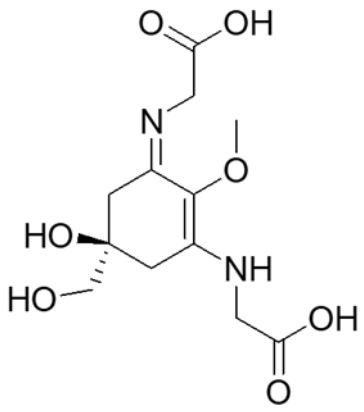


scytonemin **(1)**

Figure 2. Structure of scytonemin (1) a Mycosporine-like amino acid able to inhibit iNOS and suppress the NF-kβ pathway.

Rastogi et al. (2014), reported significant inhibitory effects on ROS production in cyanobacterial cells treated with scytonemin (20.83%), ascorbic acid (AA) (45.75%), and SCY + AA (58.65%), after exposure to UVA, UVB and photosynthetically active radiation (PAR). These results indicate some potential role of scytonemin as a natural anti-oxidant (66).

Mycosporine-like amino acids (MAAs) are metabolites that help cyanobacteria to cope with UV radiation. Aside from this photoprotective effect, these compounds have been investigated as antioxidants and anti-inflammatory agents. Mycosporine-2-glycine **(2)** (Figure 3) extracted from *Aphanothece halophytica* was evaluated for its anti-inflammatory effects in LPS-induced RAW 264.7 macrophages. At a concentrations of 1 and 10 μM this compound was able to inhibit iNOS and suppress the NF-kβ pathway (67).



mycosporine-2-glycine **(2)**

Figure 3. Structure of mycosporine-2-glycine (2) a mycosporine-like amino acid able to inhibit iNOS and suppress the NF-kβ pathway.

Sacran, a sulphated polysaccharide extracted from cyanobacterium *Aphanothece sacrun*, showed anti-inflammatory effects against paw edema induced by carrageenan, kaolin and dextran and ear edema induced by TPA in male Wistar rats and female BALB/c mice (68). During the study, sacran exhibited edema inhibition against all the phlogistic agents, with concentrations of 0.01% and 0.05% (w/v) for kaolin and 0.05% (w/v) for carrageenan, dextran and TPA. Sacran cytotoxicity was studied in HaCaT cell line with no cytotoxicity effects observed. In another study, sacran reduced cell damage induced by sodium lauryl sulfate (SLS) and also restored the ROS levels stimulated by SLS and by IL-1α. The authors suggest that the anti-inflammatory effects of sacran are based on its trapping effect in its matrix (69).

PBPs are colored macromolecules that form part of the light-harvesting system in cyanobacteria. These water-soluble proteins can be classified according to their structure and light absorption

properties as phycoerythrin, phycocyanin, phycoerythrocyanin, and allophycocyanin (70). PBPs extracts from *Cyanobium* sp. had the capacity of inhibit COX-1 and COX-2 in 50% and 40%, respectively. This extract also presented great antioxidant capacity (71).

Phycocyanin from cyanobacteria *Geitlerinema sp.* was investigated for its antioxidant activities (72). Several methods were performed such as Phosphomolybdenum assay, DPPH, H_2O_2 ,FRAP, andanti-lipid peroxidation assay. The pigment revealed a maximum absorbance of 0.49 by Phosphomolybdenum assay, 0.85 absorbance by FRAP assay, 78.75% DPPH scavenging activity, 95.27% H_2O_2 scavenging activity and in the anti-lipid peroxidation assay an activity of 53.65% all in a concentration of 200μg/mL. These results suggest that phycocyanin acts as a promising antioxidant compound and has the potential to be used in diseases related to oxidative stress.

Hexane:isopropanol (3:2) (HI) *Gloeothece* sp. lipidic extracts were studied for their antioxidant (ABTS, DPPH, $^{\bullet}NO$ and $O_2^{\bullet-}$ assays), anti-inflammatory (HRBC (human red blood cell) membrane stabilization and COX-2 screening assays) and antitumor capacity (death by TUNEL and BrdU assays in AGS cancer cells) (73). Several reagents (acetone, ethanol, ethyl lactate and hexane:isopropanol) were selected to extract the lipidic compounds, however the most promising to be used in the nutraceutical industry was the HI. Results showed that HI extracts had a $^{\bullet}NO$ scavenging capacity with a IC_{50} of 1258± 0.353 μg/mL; a 50% COX-2 inhibition with 130.2±7.4 μg/mL; 61.6±9.2% of lysosomes protection from heat damage and induce AGS cell death by 4.2- fold and their proliferation up to 40% in a concentration of 23.2±1.9 μg/mL.

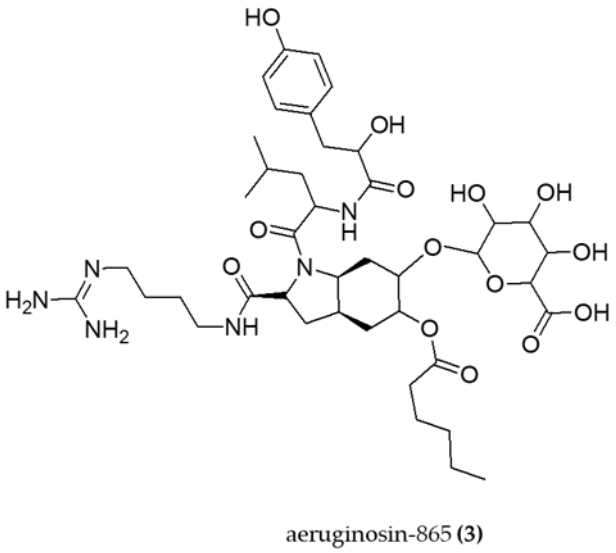
Phycoerythrin (PE) extract from *Halomicronema* sp. R31DM, show great in vitro antioxidant activity and decreased the ROS levels in *Caenorhabditis elegans* (*C. elegans*) (74). The in vitro antioxidant activity of PE was measured by the DPPH, FRAP and RP assays. In the DPPH assay, PE presented a scavenging activity increasement up to 64% in a dose of 100 μg/mL. The positive control, ascorbic acid, show a 100% scavenging activity at 100 μg/mL. The FRAP assay show a direct relation between the absorbance and the increasing of the PE concentration. Regarding to the RP assay, PE showed a dose dependent increasement in the OD at 700 nm. All these assays proved that PE has a great antioxidant activity. In the in vivo tests, *C. elegans* worms were exposed to an atmosphere of high temperature and strong oxidizing agent. PE- feeding worms showed a higher survival (up to 72% with 100 μg/ml PE) rate compared to the control group. Also, the worms were exposed to 10 mM paraquat solution which induces ROS production. ROS levels

were measured with DCFH-DA. Fluorescence was measured, the groups with PE feeding show no fluorescence which indicates PE's ROS-scavenging potential.

Lyngbya sp. and *Oscillatoria* sp. species were characterized for their total phenolic content (TPC), total flavonoid content (TFC), PBPs and other active compounds. *Lyngbya* sp., showed TPC values of 5.02 ± 0.20 mg/g, TFC of 664.07 ± 19.76 mg/g, and total PBPs of 127.01 mg/g. When these two species were compared in terms of antioxidant potential, the FRAP assay was higher in *Oscillatoria* sp. (39.63 ± 7.02 μM Fe[II]/100g). DPPH radical scavenging activity was also highest in *Oscillatoria* sp. (465.31 ± 25.76 mgAA/g). With these results, the authors suggest that these two cyanobacteria strains may be good sources of antioxidants with special use in food and pharmaceutical industrial (75).

Glycolipids are a common type of lipid in cyanobacteria, serving as components of thylakoid membranes and heterocyst cell walls (76). These molecules were discovered to induce anti-inflammatory activity (77). Tena Pérez et al.(2021) (78) studied the effects of glycolipid fractions from *Nodularia harveyana* in LPS-stimulated leukemic monocyte cells (THP-1) for their TNF-α and NF-kβ inhibition. The fraction containing digalactosyldiacyl glycerols was the one with the highest inhibition for both factors, with an IC_{50} of 5.81 ± 0.23 μM, for TNF-α and 3.75 ± 0.63 μM, for NF-kβ. This fraction also exhibited the lowest cytotoxicity (IC_{50} of 31.27 ± 0.12 μM).

Cyanobacteria from the genus *Nostoc* have shown a great interest as antioxidants and anti-inflammatories (79,80). Aeruginosin-865 (Aer-865) **(3)** (Figure 4), isolated from cyanobacteria *Nostoc* sp., has shown to have great anti-inflammatory effects. These findings were observed by Kapuscik et al. (2013), in a study using Human tumor necrosis factor-α (hTNF-α)-stimulated human lung microvascular endothelial cells (HLMVECs). Interleukin-8 (IL-8) and intercellular adhesion molecule-1 (ICAM-1) levels were detected using the AlphaLISA assay. Several concentrations of Aer-865 were tested for its inhibition capacity on IL-8 and ICAM-1. EC_{50} values of (3.5±1.5) μg/mL ((4.0±1.7) μm) and (50.0±13.4) μg/mL ((57.8±15.5) μm) of Aer-865 show the highest inhibition levels of IL-8 and ICAM-1, respectively. The authors also discovered that Aer-865 inhibits, partial or total, translocation of the NF-kβ dimer into the nucleus of endothelial cells (81).



aeruginosin-865 **(3)**

Figure 4. Structure of aeruginosin-865 (3), a nonribosomal linear tetrapeptide that inhibit interleukin-8 (IL-8).

In another study, the antioxidant activity of polysaccharides extracted from cyanobacteria *Nostoc commune* was tested trough several in vitro antioxidant assays (H_2O_2, $O_2^{\bullet -}$, DPPH and reducing power (RP)). The DPPH assay shown the bests results, with an EC_{50} of 2719.1 mg/L (79). C-phycocyanin (CP), isolated from *Nostoc sphaeroides*, was studied in DOX (doxorubicin) + CP induced C57BL/6 male mice in other to investigate its neuroprotective activity. DOX is a commonly use drug in the cancer treatment, however it causes cognitive dysfunctions on patients. CP as shown to have neuroprotective effects mostly due to its antioxidant, anti-inflammatory and mitochondrial properties. After several tests (MWM, quantification of TNF-α, IL-1β, IL-6, malondialdehyde (MDA), GSH and SOD levels), the authors observed that CP treatment (50 mg/Kg) suppressed all the neuronal problems caused by DOX treatment. In this sense, C-phycocyanin proves to be a good option choice for neuroinflammation and oxidative stress attenuation (80).

Exopolysaccharides (EPS) are among the classes of compounds that have demonstrated anti-inflammatory effects in both in vivo and in vitro tests. EPS are extracellular polysaccharides produced by several strains such as *Anabaena* spp., *Calothrix marchica*, *Cyanospira capsulate*, *Cyanothece* sp., *Leptolyngbya* sp., *Plectonema* sp., *Phormidium* sp. and *Nostoc* spp.. These strains can produce in average up to >1 g/L of EPS, with *Nostoc* sp. CCALA H06/21 being the highest producer at around 8 g/L (82). Zampieri et al. (2020) (83) extracted EPS from the cyanobacterium *Phormidium* sp. ETS05 and found that a concentration of 50 μg/mL EPS showed anti-inflammatory properties in chemical and physical injured zebrafish larvae by the

reduction of NF-kB activity. Also using the human skin fibroblast cell line HSF, the authors found that EPS concentrations ranging from 25 to 100 μg/mL increased cell viability.

Spirulina is a blue-green micro-alga with anti-inflammatory, antioxidant, and neuroprotective effects (84). C- phycocyanin from *Spirulina maxima* (SM) was tested for its anti-inflammatory and antiulcerogenic effects in rats with gastric ulcer induced by ethanol (96%, 5 mL/Kg). Ethanol is associated with ROS production, this was confirmed with the MDA elevated activity and decrease in SOD, GSH and CAT activity in the group of rats who received its administration and also by the increasement in TNF-α and NF-kβ in the same group. In contrast, in the group who received CP administration (200 mg/Kg) MDA activity was decreased and increased SOD and CAT activities, which indicates its antioxidant effect (85). Koh et al. (2018) (86)used a 70% *Spirulina maxima* ethanol extract (SM70EE, 100 μg/mL) in order to investigate its effects against Aβ (amyloid beta)$_{1-42}$- induced neurotoxicity in PC12 cells. High levels of Aβ induce oxidative stress in the brain and neuronal cell death. Also, Aβ stimulates the cleavage of poly (ADP-ribose) polymerase (PARP). Overactivation of PARP is related to cell death and neuroinflammation. Neurotoxicity was induced with 4 μg/mL of Aβ$_{1-42}$. The results shown that SM70EE prevented PC12 cells death and reduced PARP cleavage. Oxidative stress was reduced and levels of GSH were restored. In another study, SM70EE was used in trimethyltin (TMT, 10μM)-induced HT-22 cells and in scopolamine (1 mg/kg body weight/day)-induced ICR (institute of cancer research) mice (87). TMT induces neuronal cell apoptosis, is related with oxidative stress and mitochondrial and neurotransmitter dysfunction and increases AChE activity. Once again, PARP cleavage was inhibited, and ROS production was decreased in the presence of the extract (50 and 100 μg/mL). Moreover, AChE activity was blocked with SM70EE. Regarding to the in vivo tests, oral administration of 200 and 400 mg/kg body weight/day of SM70EE prevented learning and memory damages related to scopolamine-induced neurotoxicity.

A non-protein extract from *Spirulina platensis* was examined for its neuroprotective effects using PC12 (pheochromocytoma) cells (88). Abnormal iron accumulation in the brain has been pointed as one of the causes of oxidative damage and neuronal cells. Determination of ferric-reducing antioxidant potency and the free radical scavenging activity were tested. These assays, show good results at concentrations of 5 mg/mL or higher and between 5-50 mg/mL, respectively. The authors conclude that *Spirulina* has properties that can be effective against oxidative damage in neuronal cells. In an in vivo study using DJ-1β$^{\Delta 93}$ flies, a PD model in *Drosophila*, exposed to paraquat to induce oxidative stress, *Spirulina* supplementation (5% or

10%) and CP (1 or 2 μg/mL) reduced cellular stress and show antioxidant effects. Diet with Spirulina increased the lifespan and locomotor activity in the flies and downregulated SOD and CAT activity (89). In another in vivo study, the effects of oral administration of C-phycocyanin (200 mg/kg) from *Spirulina platensis* were studied in EAE (experimental autoimmune encephalomyelitis) induced Lewis rats. This administration, reduced MDA, peroxidation potential (PP) and ferric reducing ability (FRA) levels and prevented myelin integrity. In the same study, treatment with phycocyanobilin (5mg/kg) in EAE-C57BL/6 mice reduced neuroinflammation by lowering the expression of pro-inflammatory cytokines, IL-6 and IFN-γ (90). Tablets of *Spirulina platensis* (1500 mg/kg) showed neuroprotective potential in aluminum chloride ($AlCl_3$) induced Wistar rats. The treatment reduced TNF-α, indicating anti-inflammatory activity and showed high antioxidant potential by restoring GSH levels, thiol content and total antioxidant capacity (TAC) (91).

Lipid factions of *Spirulina subsalsa* exhibited strong anti-inflammatory properties in human platelets. These fractions strongly reduced platelet aggregation induced by PAF, with IC_{50} values between 60-100 μg (92).

In a study conducted by Al-Awadhi et al. (2023) (93), a monounsaturated fatty acid (7(E)-9-keto-hexadec-7-enoic acid) isolated from VPFK21-7 cyanobacterial mat, presented anti-inflammatory and antioxidant activities. A ARE-luciferase reported assay was performed in HEK293 cells, the compound induced Nrf2 activity in a concentration of 10 and 32 μM, which demonstrates its antioxidant activity. The anti-inflammatory activity was tested by measuring the •NO levels in LPS-activated mouse macrophage RAW 264.7 cells. A dose-dependent of the compound (up to 3.2 μM) reduced •NO levels. This reduction was due to reduction in the iNOS transcript levels.

Table 1. Cyanobacteria in Neurodegenerative diseases from (16).

Strain	Compound/Extract	Bioactivity	In Vitro Assays	In Vivo Assays
Anabaena flos-aquae NRC-525-17	Anatoxin-a(s)	AChE and BChE inhibition	AChE and BChE inhibition assay	
Nostoc 78-12A	Nostocarboline	BChE inhibition	AChE and BChE inhibition assay	
Phormidium autumnale	SFE-EtOH extract	AChE and LOX inhibition Antioxidant	AChE inhibition assay. LOX inhibition assay. ORAC assay	
Anabaena variabilis	Methylene chloride/ methanol extract (Fraction 7)	AChE inhibition	AChE inhibition assay	
Oscillatoria sancta	Methylene chloride/ methanol (1:1) extract	AChE inhibition	AChE inhibition assay	
Nostoc sp.	Ethanolic Extract	AChE and BChE inhibition Antioxidant	AChE and BChE inhibition assay DPPH assay	
Spirulina sp.	Phycocyanin	Inhibition of amyloid formation	Fluorometric assay Kinetic analysis Circular dichroism analysis	
Spirulina sp.	Phycocyanin	Inhibition of Aβ40/42 amyloid fibrillation	Fibrillar and amorphous aggregation assays Transmission electron microscopy imaging	
Symploca sp.	Tasiamide B	BACE-1 inhibition	BACE-1 inhibition assay	
Lyngbya sp.	Tasiamide F	BACE-1 inhibition	BACE-1 inhibition assay	

Leptolyngbya sp. N62DM	Phycocyanin	BACE-1 inhibition	Protein–complex interface identification	*Caenorhabditis elegans* CL4176 transgenic AD-model: Paralysis assay
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
Spirulina maxima	70% ethanol extract	Improved cognition AChE inhibition Reduced Aβ, APP, and BACE-1 levels. BDNF/PI3K/Akt pathway activation Antioxidant		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
Aphanizomenon flos-aquae	KlamExtra®	Improved metabolic parameters Protection of neuronal morphology and synapses Reduced Aβ, APP and BACE-1 levels. Anti-inflammatory and anti-gliosis		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

Spirulina platensis	Diet supplementation	Improved metabolic parameters Improved locomotor and cognitive function Decreased Aβ_{1-42}, APP, BACE-1, p-tau, and p-GSK levels Anti-inflammatory Improved microbiota dysbiosis		High-Fat Diet C57BL/6J mice: Barnes Maze test. Morris Water Maze test. ELISA (Aβ_{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)
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)
cf. *Symploca* sp.	Santacruzamate A	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)

Spirulina platensis	*S. platensis*–loaded niosome	Recognition and working memory improvement Protection of neuronal morphology Restored levels of AChE and Ach Gene modulation	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)
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
Spirulina platensis	Phycocyanin	Memory improvement Gene and miRNA modulation Anti–inflammatory Anti–apoptotic	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)
Spirulina platensis	Phycocyanin	Memory improvement AChE inhibition ChAT activity increase Gene modulation Increased PI3K/Akt pathway Anti–inflammatory	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)

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
Spirulina sp.	Phycocyanin	Inhibition of A53Tα-synuclein amyloid fibrillation	Fibrillar and amorphous aggregation assays. Transmission electron microscopy imaging	
Spirulina platensis	Phycocyanin	Reduction of α-synuclein Inclusions Gene modulation Antioxidant Improved proteostasis		BY4741 Yeast transformed with p42FAL-αsyn-GFP: Spot assay. Fluorescence microscopy. Western Blot (α-syn). Flow cytometry. TBARS assay. CAT activity. Total thiols assay. qRT-PCR (SOD1, SOD2, HAP4, LHS1, HRD1, GSH1, GLR1, RPN4, ATG8)
Spirulina maxima	Diet supplementation	Protection of DA and HVA Content Blockage of lipid peroxidation		MPTP-induced parkinsonism in male C-57 rats: HPLC (DA, HVA, 5-HIAA, 5-HT). TBARS Assay

Spirulina maxima	Diet supplementation	Improved locomotion Recovery of mitochondrial activity Protection of DA, DOPAC, and HVA levels Antioxidant	6-OHDA-induced parkinsonism in male Wistar rats: Turn-behavior test. Closed-field test. Cylinder test. Fluorescence ROS determination. Griess reaction. TBARS assay. MTT assay. HPLC (DA, DOPAC, HVA)
Spirulina fusiform	Aqueous freeze-dried extract suspended in olive oil	Improved behavior and Locomotion Protection of DA levels Antioxidant	6-OHDA-induced parkinsonism in male Wistar albino rats: Amphetamine and Apomorphine -induced rotations. Locomotor activity. Rota rod. TBARS assay. Reduced glutathione content assay. HPLC (DA)
Spirulina platensis	Polysaccharide	Increased TH and DAT expression Antioxidant	MPTP-induced parkinsonism in male C57BL/6J mice: Immunohistochemistry and RT-PCR (TH, DAT). SOD and GSH-Px assays
Spirulina	Diet supplementation	Maintenance of extension reflex Anti-inflammatory Neuroprotection against motor neuron degeneration	$SOD1^{G93A}$ mice: Weight and measurement. Extension Reflex test. Ribonuclease Protection Assay (IL-1α, IL-1β, IL-6, TNF-α). Immunohistochemistry (Fluoro-Jade, GFAP)

Oscillatoria Planktothrix sp.	VB3323	TLR4 antagonist Improved motor function tests Anti-inflammatory and anti-gliosis Neuroprotection	Purified microglial cells: Immunocytochemistry (CD11b). Immunoblotting (CD68). Live cell imaging (GFP) Motor neurons/glia co-culture: ELISA (TNF-α, IL-1β and IL-6). Motor neurons/glia coculture and purified motor neurons: Motor Neuron Viability Assay (SMI32)	Wobbler Mice: Paw abnormality and grip strength test. Immunohistochemistry (GFAP, CD11, and TNF-α)
Microcystis aeruginosa (94)	β-cyclocitral	AChE inhibition		*Daphnia sinensis* fed with *M.aeruginosa* and exposed to β-cyclocitral
Synechococcus sp. XM-24 (95)	Several compounds ((E)-octadec-11-enoic acid, tridecanoic acid, 12-methyl-, hexadecanoic acid, indolizine, isoquinoline 3,4-dihydro- and phthalazine)	Anti-AD targets		

Table 2. Cyanobacteria in inflammation and oxidative stress.

Genera/Specie	Compound/Extract	Mechanism/Effect	In Vitro Assays	In Vivo Assays	Reference
–	Scytonemin	Anti-inflammatory	LPS-stimulated RAW 264.7 cells (TNF-α and NF-kβ)	BALC/c mice with TPA-induced ear edema (TNF-α, iNOS)	(65)
Aphanothece halophytica	Mycosporine-2-glycine	Anti-inflammatory Antioxidant	LPS- induced macrophages (RAW 264.7). iNOS, NF-kβ, COX-2, H_2O_2.	–	(67)
Aphanothece sacrum	Sacran	Anti-inflammatory. Antioxidant	HaCaT human keratinocyte cells. WST-1 method	Male Wistar rats and female BALB/c mice with paw edema induced by carrageenan, kaolin and dextran and ear edema induced by TPA. Histological analysis.	(68)
Aphanothece sacrum	Sacran	Anti-inflammatory. Antioxidant	HaCaT keratinocytes cells. ROS induced by SLS and IL-1α.	–	(69)

Cyanobium sp.	Phycobiliproteins	Anti-inflammatory Antioxidant	ABTS, $^{\bullet}NO$, $O_2^{\bullet-}$ scavenging COX inhibition	–	(71)
Geitlerinema sp TRV57	Phycocyanin	Antioxidant	Phosphomolybdenum, DPPH, H_2O_2, FRAP, Antilipid peroxidation assays	–	(72)
Gloethece sp.	Lipidic extracts	Antioxidant Anti-inflammatory Anti-tumor	$ABTS^{\bullet+}$, DPPH, $^{\bullet}NO$, $O_2^{\bullet-}$ HRBC, COX-2 TUNEL in AGS cancer cells	–	(73)
Halomicronema sp. R31DM	Phycoerythrin	Antioxidant	DPPH, FRAP, RP	N2 Bristol wild type *C. elegans* (ROS levels)	(74)
Lyngbya sp. and *Oscillatoria sp.*	Phycobiliproteins, Phenolic and Flavonoids compounds	Antioxidant	TPC, TFC, PBPs, FRAP and DPPH	–	(75)
Nodularia harveyana	Glycolipids	Anti-inflammatory Downregulation of TNF-α and NF- kB	LPS-stimulated leukemic monocyte cells (THP-1)	–	(78)

Nostoc	Aeruginosin-865 **(1)**	Anti-inflammatory NF-kβ inhibition	hTNF-α – stimulated HLMVECs cells (AlphaLISA assay). IL-8 and ICAM-1 levels	–	(81)
Nostoc commune	Nostoc commune Polysaccharides	Antioxidant Antibacterial	H_2O_2, $O_2^{\bullet -}$, DPPH, RP	–	(79)
Nostoc sphaeroides	C-phycocyanin	Anti-inflammatory Antioxidant Mitochondria protection Synapse protection	–	DOX + CP- induced C57BL/6 male mice (MWM, TNF-α, IL-1β, IL-6, MDA, GSH and SOD levels)	(80)
Phormidium sp. ETS05	Exopolysaccharides (EPS)	Anti-inflammatory	Human skin fibroblasts (HSF) exposed to EPS	Chemical (copper and DSS) and injury (amputation) zebrafish larvae	(83)
Scytonema sp R77DM	Scytonemin	Antioxidant	Cyanobacterial cells treated with scytonemin (ROS production)	–	(66)
Spirulina maxima	C-phycocyanin	Anti-inflammatory Anti-ulcerogenic	–	Male Wistar rats with ethanol-induced gastric ulcers (MDA, GSH, SOD, CAT, TNF-α, NF-kβ)	(85)

Spirulina maxima	*Spirulina maxima* 70% ethanol extract (SM70EE)	Neuroprotection Antioxidant	$A\beta_{1-42}$– induced PC12 cells (MTT, PARP, LDH, GSH levels, western blot)	–	(86)
Spirulina maxima	*Spirulina maxima* 70% ethanol extract (SM70EE)	Antioxidant AChE inhibition	TMT– induced HT–22 cells (MTT, PARP, western blotting)	Scopolamine–induced ICR mice (MWM, Passive avoidance test)	(87)
Spirulina platensis	Diet supplementation (5 and 10% w/v)	Antioxidant Reduced cellular stress	–	DJ–1β^{Δ}93 *Drosophila melanogaster* exposed to paraquat: Survival assay. Locomotor assay. Enzymatic assays (SOD and CAT).	(89)
Spirulina platensis	Phycocyanin	Antioxidant Anti–inflammatory Neuronal protection	–	EAE indued male Lewis rats and female C57BL/6 mice: MDA assay. PP assay. FRA assay. ELISA (IL–17, IL–6, IFN–γ).	(90)
Spirulina platensis	Diet supplementation (1500 mg/kg, tablets)	Antioxidant Anti–inflammatory Neuronal morphology protection	–	Wistar rats induced with AlCl3: GSH content assay. Total thiol content assay. TAC assay. ELISA (TNF–α). Histology. Immunofluorescence (Aβ).	(91)

Spirulina subsalsa	*Lipid fractions*	Anti-inflammatory	hPRP (anti-PAF, antohrombotic activities)	-	(92)
VPFK21-7	Monounsaturated fatty acid 7(E)-9-keto-hexadec-7-enoic acid	Anti-inflammatory	HEK293 ARE-luc cells (ARE-Luciferase Reporter Assay) RAW264.7 cells (NO levels)	-	(93)

Abbreviations: NF-kβ- Nuclear factor kappa B; hTNF-α- human tumor necrosis factor alpha; HLMVECs- human lung microvascular endothelial cells; IL-8- Interleukin-8; ICAM-1- intercellular adhesion molecule 1; WST-1-water-soluble tetrazolium salt; TPA- 12-O-Tetradecanoylphorbol13-acetate; HEK293 ARE-luc- human embryonic kidney cells stably transfected with firefly luciferase reporter gene; ARE- antioxidant response element; NO-nitric oxide; ABTS- 2,2 ′ -Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid; DPPH- 2,2-diphenyl-1-picrylhydrazyl; O_2- superoxide; HRBC-Human red blood cell; COX-2- cyclooxygenase-2; TUNEL- Terminal deoxynucleotidyl transferase dUTP nick-end labeling; AGS- gastric adenocarcinoma cell-line; H_2O_2- Hydrogen peroxide , O_2^- - Superoxide anion; RP- reducing power; DOX- Doxorubicin; CP- C-phycocyanin; MWM- Morris water maze; TNF-α- Tumor necrosis factor alpha, IL-1β- Interleukin 1 beta, IL-6- Interleukin-6, MDA-Malondialdehyde; GSH- Glutathione; SOD- Superoxide dismutase; FRAP-Ferric ion reducing ability of plasma; *C.elegans*- Caenorhabditis elegans; PC 12- Pheochromocytoma; MTT-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PARP- poly (ADP-ribose) polymerase; LDH- lactate dehydrogenase; ICR- institute of cancer research; CAT- catalase; hPRP- human platelet-rich plasma; PAF- platelet-activating factor.

2. Aims

Several *in silico*, *in vitro*, and *in vivo* studies have provided support for the neuroactive potential of cyanobacterial natural products, particularly in AD. In this context, the main aim of this work was to screen nineteen cyanobacterial strains from the LEGE-CC for its neuroprotective effects. In addition, three cyanobacterial compounds were also tested.

A bioactivity-guided approach was followed to discover LEGE-CC cyanobacterial strains able to produce compounds with inhibitory activity against the main enzymes linked to AD, namely, AChE and BuChE. In addition, the cytotoxicity of the cyanobacterial fractions was tested on cell lines usually used in AD studies as the neuroblastoma cell line SHSY-5Y and a fibroblast cell line 3T3-L1.

Specifically, this work included:

- Cyanobacteria selection and biomass production;
- Preparation of a crude extract and fractionation into a library of 176 fractions;
- Cytotoxicity evaluation through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using the neuroblastoma cell line SH-SY5Y and the fibroblast cell line 3T3-L1;
- Assessment of the inhibitory potential of fractions towards AChE and BuChE enzymes through the Ellman's method.

3. Materials and Methods

3.1. Cyanobacteria strains

Nineteen cyanobacteria strains, (LEGE 06072, LEGE 06108, LEGE 06111, LEGE 06131, LEGE 06139, LEGE 06144, LEGE 06155, LEGE 06361, LEGE 07168, LEGE 07171, LEGE 07175, LEGE 07177, LEGE 07189, LEGE 11386, LEGE 11394, LEGE 11439, LEGE 16525, LEGE 181150, LEGE 181156), from the LEGE-CC, of CIIMAR were selected for the evaluation of the inhibitory potential against the enzymes AChE and BuChE and cytotoxicity against SH-SY5Y and 3T3-L1 cell lines. Six cyanobacteria strains (LEGE 00040, LEGE 00064, LEGE 06070, LEGE 07085, LEGE 07168, LEGE 07365) were selected for culture to enrich the Cyanobacterial Natural Products Library (LEGE-NPL) (96–98). Three pure compounds (A4, B2D and C3) isolated from cyanobacteria were also evaluated for their inhibitory potential against AChE and cytotoxicity against the cell lines. Compounds A4 and B2D were extracted from LEGE 07167 and compound C3 was extracted from LEGE 15488. In Figure 5 it is presented the scheme representative of the workflow and in Table 3 de strains included in the study. Information in Table 3 was based in the LEGE-CC website (https://lege.ciimar.up.pt/).

When selecting the strains, parameters such as: different morphologies, different genders, strains whose bioactivity had already been described, namely anti-cancer activity, were considered.

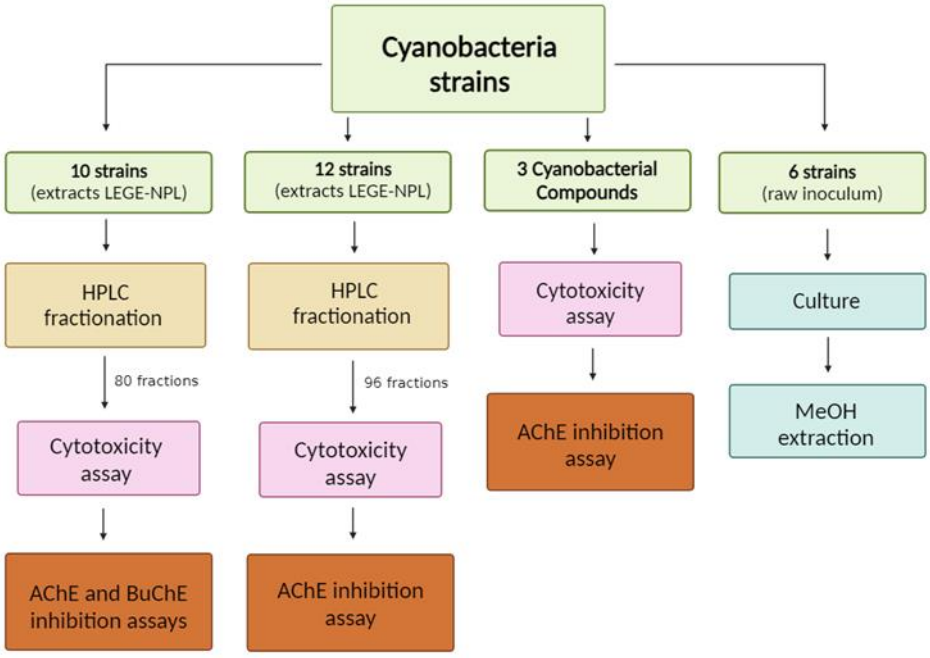
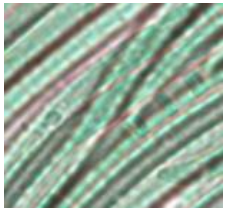
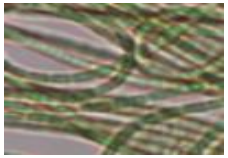
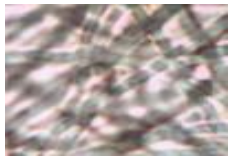
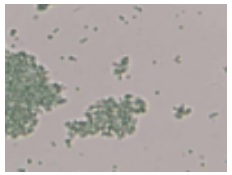
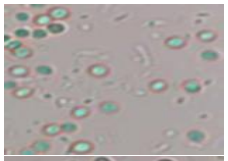
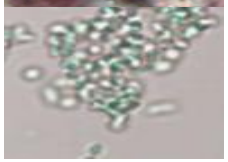
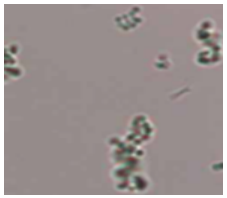
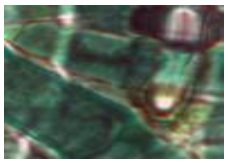
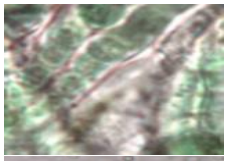
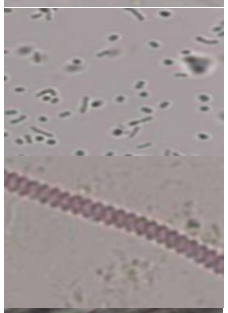


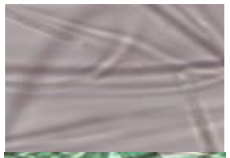
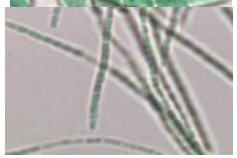
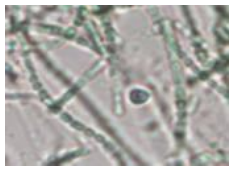
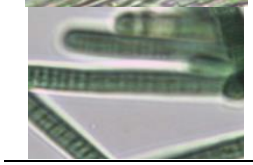
Figure 5. Schematic representation of the workflow followed in this work.
*Created with https://www.biorender.com/

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

Microphotograph	Strain ID	Identification	Sampling	Lifestyle	Activity	Reference
	LEGE 06072	*cf. Oxynema acuminatum*	São Jacinto, Portugal (mesotidal zone, benthic)	Aquatic, brackish	Inhibition of the embryonic development of Paracentrotus lividus	(18,99)
	LEGE 06108	*Toxifilum mysidocida*	Praia da Luz, Portugal (tide puccle, rock surface scraping)	Aquatic, marine	No cytotoxic to cancer cell lines namely SHSY5Y	(17,100)
	LEGE 06111	*Lusitaniella coriacea*	Praia do Martinhal, Portugal (tidal pool, on emersed rock)	Aquatic, marine		
	LEGE 06131	*Leptolyngbya saxicola*	Algarve, Portugal (wave-exposed rock, surface scraping)	Aquatic, marine		
	LEGE 06139	Cyanobium sp.	Praia da Aguda, Portugal (intertidal zone, on a Mytilus sp. shell)	Aquatic, marine	No cytotoxic to cancer cell lines namely SHSY5Y	(100)

	LEGE 06144	unidentified filamentous *Synechococcales*	Praia de Burgau, Portugal (intertidal zone, wave-sheltered zone, sand)	Aquatic, marine	No cytotoxic to cancer cell lines namely SHSY5Y Lipid reducing activity in zebra fish	(18,100)
	LEGE 06155	*Synechocystis salina*	Esposende, Portugal (tide pool, rock surface scraping)	Aquatic, marine	Anti-aging Anti-cancer	(18,100–102)
	LEGE 06361	*Leptolyngbya sp.*	Vila Nova de Gaia, Portugal (biofilm, from a secondary decanter tank bank)	Aquatic, freshwater		
	LEGE 07168	*Chroococcopsis sp.*	Vila Nova de Gaia, Portugal (on a brown macroalga)	Aquatic, marine		
	LEGE 07171	*Synechococcus nidulans*	Praia de Burgau, Portugal (tide puddle, air-exposed rock surface scraping)	Aquatic, marine		(18)
	LEGE 07175	*Cyanobium sp.*	Vila do Bispo, Portugal (sea water sample, coastal, surf zone)	Aquatic, marine	Anti-aging Lipid reducing activity No cytotoxic to cancer cell lines namely SHSY5Y	(18,97,101,103)
	LEGE 07177	*Rivularia sp.*	Praia do Martinhal, Portugal (intertidal zone, on green macroalga)	Aquatic, marine		(17)

	LEGE 07189	*Scytonema sp.*	Caminha, Portugal (rock surface scraping)	Aquatic, marine		(17)
	LEGE 11386	unidentified *Nostocales*	Portugal (intertidal zone, on a marine sponge)	Aquatic, marine		(17)
	LEGE 11394	*Synechococcus sp.*	Rio de Janeiro, Brasil (upper layer of a microbial mat)	Aquatic, hypersaline		
	LEGE 11439	*Spirulina sp.*	Porto, Portugal	Aquatic, marine		(17)
	LEGE 16525	*Microcoleus sp.*	Rio Douro, Portugal (floodgate wall, scraping)			
	LEGE 181150	Synechococcales cyanobacterium	Cape Verde	Aquatic, marine	Hyaluronidase inhibition Antioxidant Anticancer	(17,98,103)
	LEGE 181156	*Leptothoe sp.*	Cape Verde	Marine	Tyrosinase inhibition Antioxidant	(17,103,104)

	LEGE 00040	*Synechocystis* salina	Caminha, Portugal (intertidal zone, on a Patella sp. shell)	Aquatic, marine		
	LEGE 00064	*Phormidium sp.*	Morocco	Aquatic, freshwater		
	LEGE 06070	unidentified filamentous *Synechococcales*	Porto, Portugal (mesotidal zone, benthic)	Aquatic, brackish	Inhibition of the embryonic development of Paracentrotus lividus	(99)
	LEGE 07085	*Nodosilinea sp.*	Porto, Portugal (mesotidal zone, benthic)	Aquatic, brackish	Anti- herpes type 1 activity Inhibition of the embryonic development of Paracentrotus lividus	(99,105)
	LEGE 07365	*Nostoc sp.*	Portugal	Aquatic, brackish		
	LEGE 07167	*Lusitaniella coriacea*	Praia de Lavadores, Portugal (rock surface scraping)	Aquatic, Marine		
	LEGE 15488	*Phormidium sp.*	Macapá, Amazon river, Brazil (water sample)	Aquatic, Freshwater		

3.2. Biomass production

Cyanobacteria culture was carried out at the Bioterium of Aquatic Organisms (BOGA) at CIIMAR. All strains were culture in liquid Z8 medium supplemented with 25% synthetic sea salt and 1% vitamin B12. A portion of each selected strains was removed from the original culture flask (30 ml) and culture into a new flask with 140 mL of medium. Cultures were performed at 25 °C with light/dark cycles of 14/10 h at a light intensity of 10–30 μmol photons m^{-2} s^{-1}. When the strains presented a significant growth, they were transferred to ballons containing 4L of Z8 medium. 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. In order to remove the excess salt, the concentrated biomass was washed with distilled water. Finaly the biomass was 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. Freeze-dried biomass was stored at -20° C.

3.3. High performance liquid chromatography: Preparation of a small library of fractions

Methanolic extracts already available in the extract bank of LEGE-NLP were fractionated as described in Ferreira et al., 2021 (98). Briefly, the extracts were fractionated by reverse-phase High performed liquid chromatography (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) (98). The dry extracts were resuspended in methanol (MeOH), to a final concentration of 40 mg/mL, in 2 mL HPLC glass vials then vortexed and filtered. The eluents used were degassed during 15 minutes in an ultrasonic bath (Vibra Cell, USA). 500 μL of each extract were injected with a 1 mL loop and separated on an ACE 10 C8 column (50x10 mm, ACE, Reading, UK), using a H_2O: MeCN (Water: Acetonitrile) gradient. The extracts were separated into 8 fractions (A-H) and collected in 48-well plates (Riplate, Ritter, Schwabmünchen, Germany). Table 4 describes the conditions of the elution.

Table 4. HPLC chromatographic and collection program for generating the fractions. Adapted from ((1)).

Fractions	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	

To remove the solvent, fractions were dried using a CentriVap Concentrator (LabConco, Kansas City, MO, USA). Then were solubilized in 500 μL of MeOH and transferred to a 96-well plate. A schematic representation of the plates is depicted in Table 5. A general scheme of the LEGE-NPL workflow is represented in Figure 6. To perform the viability assays, 20 μL of each fraction were transferred to a new 96-well plate, then dried and resuspended in 20 μL of DMSO. The remaining 480 μL were used for the enzymatic inhibitory assays.

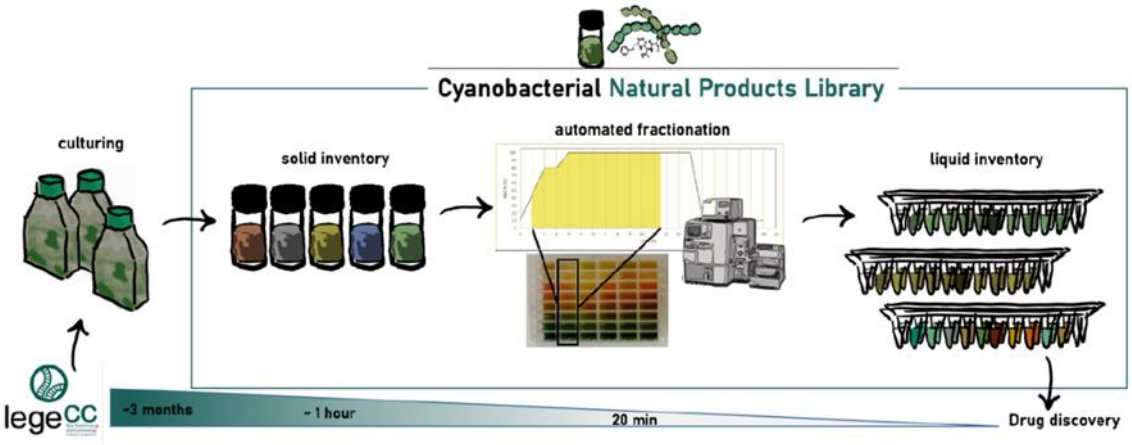


Figure 6. Schematic Representation of the Natural Products Library Workflow.
*Courtesy of the Blue Biotechnology and Ecotoxicology group.

Table 5. Schematic representation of the 96-well plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	MeOH	06072_A	06131_A	06155_A	06361_A	07168_A	07175_A	07189_A	11394_A	11439_A	16525_A	
B	MeOH	06072_B	06131_B	06155_B	06361_B	07168_B	07175_B	07189_B	11394_B	11439_B	16525_B	
C	MeOH	06072_C	06131_C	06155_C	06361_C	07168_C	07175_C	07189_C	11394_C	11439_C	16525_C	
D	MeOH	06072_D	06131_D	06155_D	06361_D	07168_D	07175_D	07189_D	11394_D	11439_D	16525_D	
E	MeOH	06072_E	06131_E	06155_E	06361_E	07168_E	07175_E	07189_E	11394_E	11439_E	16525_E	
F	MeOH	06072_F	06131_F	06155_F	06361_F	07168_F	07175_F	07189_F	11394_F	11439_F	16525_F	
G	MeOH	06072_G	06131_G	06155_G	06361_G	07168_G	07175_G	07189_G	11394_G	11439_G	16525_G	
H	MeOH	06072_H	06131_H	06155_H	06361_H	07168_H	07175_H	07189_H	11394_H	11439_H	16525_H	

3.4. Cell lines and culture

Two cell lines (Table 6) were cultured according to routine procedures and exposed to cyanobacteria's fractions.

Table 6. Information about the selected cell lines.

Cell	Cell line/ Passage (P)	Origin
Neuroblastoma	SH-SY5Y – P1-P10	CLS, Cell Lines Service, Germany
Fibroblasts	3T3-L1 – P32-P42	CLS- Cell Lines Service, Germany

- SH-SY5Y, a neuroblastoma cell line commonly used in AD studies.
- 3T3-L1, fibroblast cells from Swiss albino mice, used as representation of the main cells of connective tissue.

Cells were cultured in DMEM High Glucose basic medium (CLS, Cell Lines Service, Germany), with 10% (v/v) fetal bovine serum (Gibco, USA), 1% Amphotericin B (Gibco), and 1% Penicillin-Streptomycin (Biowest). Cells were maintained at 37 °C in an 5% CO_2 atmosphere. Initially, cells were cultured in 25cm^2 flaks containing 5 mL of medium. After 24 to 48 hours the medium was renewed to ensure optimal growth conditions. When cells reached 80-90% confluence, a cell passage was performed. Briefly, the medium was removed and about 2 mL of phosphate-buffered saline (PBS, Sigma) was added to remove any suspended cells, cell debris and medium that was left. Then, 1.5 mL of TrypLE™ Express (Gibco) was added to detach the cells from the flasks and incubated for a few minutes. When all the cells were detached and individualized, 5 mL or 10 mL (depending on the flask) of medium was added to inactivate the TrypLE™ Express. The cell suspension was transferred to a 15 mL falcon tube and centrifuged for 3 minutes at 20°C and

1500 rpm. Then, the supernatant was carefully removed, not to disturb the pellet, which was resuspended in 2 mL of fresh medium. For cell counting, 20 µL of the cell suspension were mixed with 20 µL of trypan blue dye. Trypan blue enter the cells only if the membrane is damaged coloring the cell in blue, while viable cells will appear colorless. Therefore, this assay allows us to determine the viability of the cells before the cytotoxicity assays. Cellular concentration was determined using a Neubauer Chamber. After the counting, about 50 to 100 µL of cell suspension were transferred to a new culture flask containing medium, according to the dimension, and incubated as previously described.

3.5. MTT assay

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This assay is largely used to screen the cytotoxicity effect of extracts or compounds. This method is based on the metabolization of the reagent by mitochondrial enzymes in viable cells. In this case, purple formazan crystals are formed (106).

Cytotoxicity assays were performed according to Ferreira et al, 2021 (98), with some modification. Briefly, cells were seeded in 96-well plates at a density of $1x10^5$ cell/mL for SH-SY5Y and $3.3x10^4$ cell/mL for 3T3 cells, each well containing 100 µL of culture medium and incubated for 24h for cell adhesion. After this time, medium was replaced with 100 µL of new medium with 0.5 µL of cyanobacteria factions to a final concentration of 25 µg/mL in each well. For the cyanobacteria compounds, a dose response assay was performed with compound concentrations of 0.1 µM, 0.075 µM, 0.05 µM, 0.025µM and 0.0125µM for all assays. The controls consisted of 0.5% DMSO for the negative control and 20% DMSO for the positive control. After 24h and 48h of incubation, the MTT assay was performed. Briefly, 20 µL of MTT, at concentration of 1 mg/mL, were added to each well, to a final concentration of 0.2 mg/mL. After 3h incubation, all the medium was removed and 100 µL of DMSO were added to dissolve the purple-colored formazan crystals, which are insoluble in the medium.

Absorbance was measured at 562 nm using Biochrom EZ Read 800 Plus microplate reader.

Cell viability was calculated according to the equation bellow, where 100% viability is considered in the negative control.

$$\text{Cell viability } (\%) = \frac{Abs_{fractions}}{\overline{X}_{abs\ negative\ control}} \times 100\%$$

3.6. Acetylcholinesterase and Butyrylcholinesterase inhibition assays

Fractions were tested for their AChE and BuChE inhibition potential following Ellman's method (107), with some modifications.

The enzymes AChE, BuChE and the respective subtracts, acetylcholine iodide (ATCI) and S-butyrylthiocholine iodide (BTCI), respectively, were purchase from Sigma. Also, the Ellman's Reagent or 5,5'-bisdithionitrobenzoic acid (DTNB, Chemical Co, St. Louis, MO, USA) was from Sigma. For buffers, Buffers A (50mM 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 MgCl2.6H20), were used.

Initially, all cyanobacteria fractions were dried and resuspended in buffer A with 10% MeOH to a concentration of 2.5 mg/mL and then all content transferred to individual Eppendorf's. Then, 25 µL of the previously dissolved fractions were added to a 96-well plate with 125 µL of DTNB reagent (3 mM), 50 µL of buffer B, 25 µL of ATCI/BTCI (15 mM), and 25 µL of AChE/ BuChE (0.20 U/mL), to a final concentration of 250 µg/mL in the wells. The composition of the testing samples, the blank of the samples, the negative control, and the blank of the negative control is exhibited in Table 7. Each assay was carried out three times in triplicates. A schematic representation of the plates is shown in Table 8.

Table 7. Composition of the samples, blank samples, negative control, and blank negative control used in the AChE and BuChE inhibition assays.

Samples (S)	Blank samples (BS)	Negative control (NC)	Blank of the Negative control (BNC)
25 µL of fraction	25 µL of fraction	25 µL of Buffer A + 10% MeOH	25 µL of Buffer A+ 10% MeOH
125 µL of DTNB	125 µL of DTNB	125 µL of DTNB	125 µL of DTNB
50 µL of Buffer B	75 µL of Buffer B	50 µL of Buffer B	75 µL of Buffer B
25 µL of substrate	25 µL of substrate	25 µL of substrate	25 µL of substrate
25 µL of enzyme		25 µL of enzyme	

Table 8. Schematic representation of each 96-well plates used in the AChE and BuChE inhibition assays.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S	S	S	BS	S	S	S	BS	S	S	S	BS
B	S	S	S	BS	S	S	S	BS	S	S	S	BS
C	S	S	S	BS	S	S	S	BS	S	S	S	BS
D	S	S	S	BS	S	S	S	BS	S	S	S	BS
E	S	S	S	BS	S	S	S	BS	S	S	S	BS
F	S	S	S	BS	S	S	S	BS	S	S	S	BS
G	S	S	S	BS	S	S	S	BS	S	S	S	BS
H	NC	NC	NC	BNC	NC	NC	NC	BNC	NC	NC	NC	BNC

The hydrolysis of ATCI by AChE and BTCI by BuChE produces thiocholine which reacts with the DTNB releasing the anion 5-thio-2-nitrobenzoate (TNB_2^-), that has a yellow color. Readings were performed at 405 nm during 2 min using a Synergy HT Multi-detection Microplate Reader running GEN5™ software.

The percentage of inhibition was determined using the equation below.

$$\%\ \text{inhibition} = \frac{(\text{slope}_{\text{negative control-blank of negative control}}) - (\text{slope}_{\text{sample-blank of sample}})}{(\text{slope}_{\text{negative control-blank of negative control}})} \times 100\%$$

4. Results and Discussion

4.1. Cytotoxicity assays

Cell viability assays are performed in screening assays as they allow the evaluation of harmful effects of an extract or compound. These assays secondarily also act as indicators of cell proliferation. In vitro cytotoxicity and/or cell viability assays are advantageous as they are fast, easy, economic and with high reproducibility (106). The MTT assay, is a colorimetric assay that evaluate cytotoxicity or cell viability through evaluation of mitochondrial function. In viable cells the MTT is reduced by the NADH to purple formazan crystals that can be quantified through spectrophotometry (106).

The neuroblastoma cell line SH-SY5Y is commonly used as a model of AD research as they serve as model of neuronal cells (108,109). This cell line has a high proliferation rate and has ability to differentiate into neurons. It is also easy to handle and affordable (108,109). For ND diseases it is

fundamental that extracts, fractions or compounds do not exhibit cytotoxicity against this cell line.

Due to the limited amount of fractions available, only a concentration of 25 μg/mL was tested. Cells were exposed to the fractions for 24 and 48 hours and were considered toxic when cell viability was lower or equal to 70%. The negative control (0.5% DMSO) represents 100% viability. For positive control 20% DMSO was used, this percentage is considering toxic to cells leading to their death.

Cell viability results for SH-SY5Y after fractions exposure are presented in Figure 7. As observed, most fractions were not toxic towards this cell line, with their viability being higher than 70%. Fractions of the strain LEGE 11439, except faction H, presented a cell viability higher than 110%, with focus on fraction B that presented a viability over than 180% for both 24 and 48 hours. Also fraction F from LEGE 11394 exhibited a viability superior to 130% for both incubation times.

After 24h of exposure, fractions A, B, C and D from LEGE 06072, showed some toxicity (60.52%, 61.53%, 62.32%, and 67.8% respectively). Fraction D from LEGE 06144 was toxic at both 24h and 48h markers with 66.06% and 41.21% cell viability, respectively. LEGE 06361 fractions C, E and G showed toxicity at the first 24h (68.49%, 62.11% and 70.41% cell viability, respectively). Also fraction B from this strain, was toxic this time for both time markers (57.14% and 67.006%, respectively). Fraction A from LEGE 07168 was toxic at 24h with cell viability around 68%. At 48h fraction D (LEGE 06139), C (LEGE 07171) and D and F (LEGE 1386) show some cytotoxicity, 69.19%, 69.79%, 66.44% and 67.7% respectively.

In this cell line, LEGE 06131 was the strain where most fractions were considered toxic. Fractions showed cytotoxicity values around 38.02%-69.35% for the first 24 hours. However, in the 48h marker, every fraction presented a cell viability higher than 89%.

The viability results for the 3T3-L1 cell line are presented in Figure 8. The majority of fractions were not toxic to this cell line. However, five of the eight fractions from LEGE 07168 were found toxic at 24h and 48h, with values between 31.14% and 57.18%. Also fractions F and G from this strain were cytotoxic (68.54% and 57.17%, respectively) at 24h. Fraction F (LEGE 06072) and fraction D (LEGE 06139) were toxic at 48h, with cytotoxicity value of 70.81% and 63.53%, respectively.

Fractions B, C and D from LEGE 06144 also showed some toxicity. The first two fractions presented values of cell viability of 70.28% and 43.52%, respectively, at 48h. Fraction D from the same strain was toxic at both time markers with values of 70.04% and 34.26% for 24h and 48h.

LEGE 06131 fractions B, C and D were toxic for both markers (63.45%, 51.89% and 67.80% (24h) and 53.26%, 46.62% and 44.36% (48h), respectively). Relatively to the 48h marker, fractions E and G from this strain presented a cell viability of 58.62% and 67.90%, respectively.

LEGE 06108 show the best results with cell viability superior to 90% for all fractions.

Reviewing the combined results related to the two cell lines, most fractions did not show cytotoxicity. Only 11 fractions presented for both time markers cell viability of 70% and below.

In addition, the three compounds isolated from cyanobacteria strain LEGE 07167 (A4 and B2D) and LEGE 15488 (C3), were also analyzed for cytotoxicity against the SH-SY5Y and 3T3-L1 cell lines (Figure 9). The A4 and B2D compound were considered cytotoxicity in the two cell lines. However, at a concentration of 0.0125 μM compound A4 presented a cell viability superior to 180% at 48h.

Compound C3 show no cytotoxicity in the cell in all the tested concentrations.

A summary of the cell viability results obtained through the MTT assay for the cell lines after exposure to the cyanobacteria fractions is presented in Table 9, where ++++ 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.

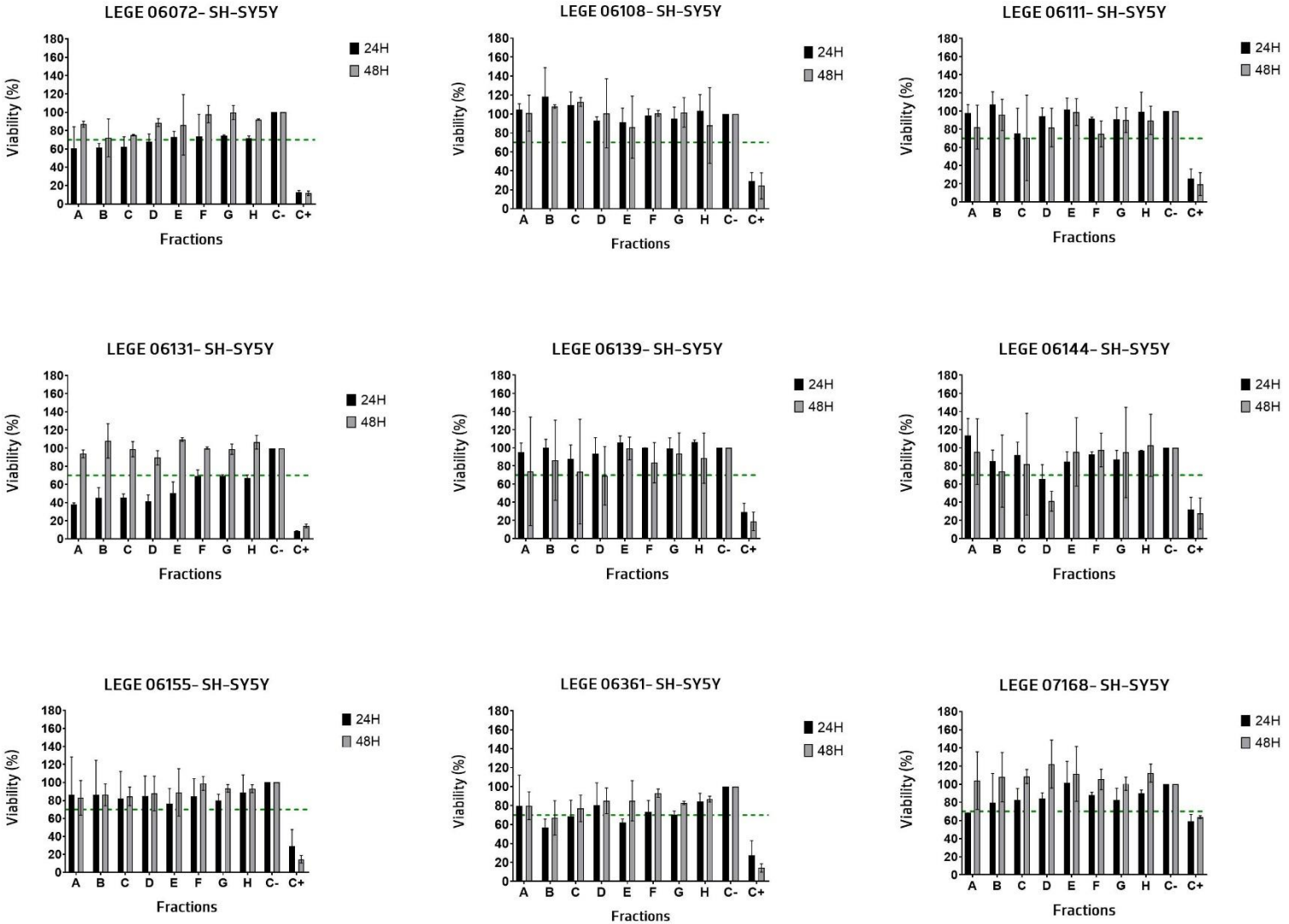
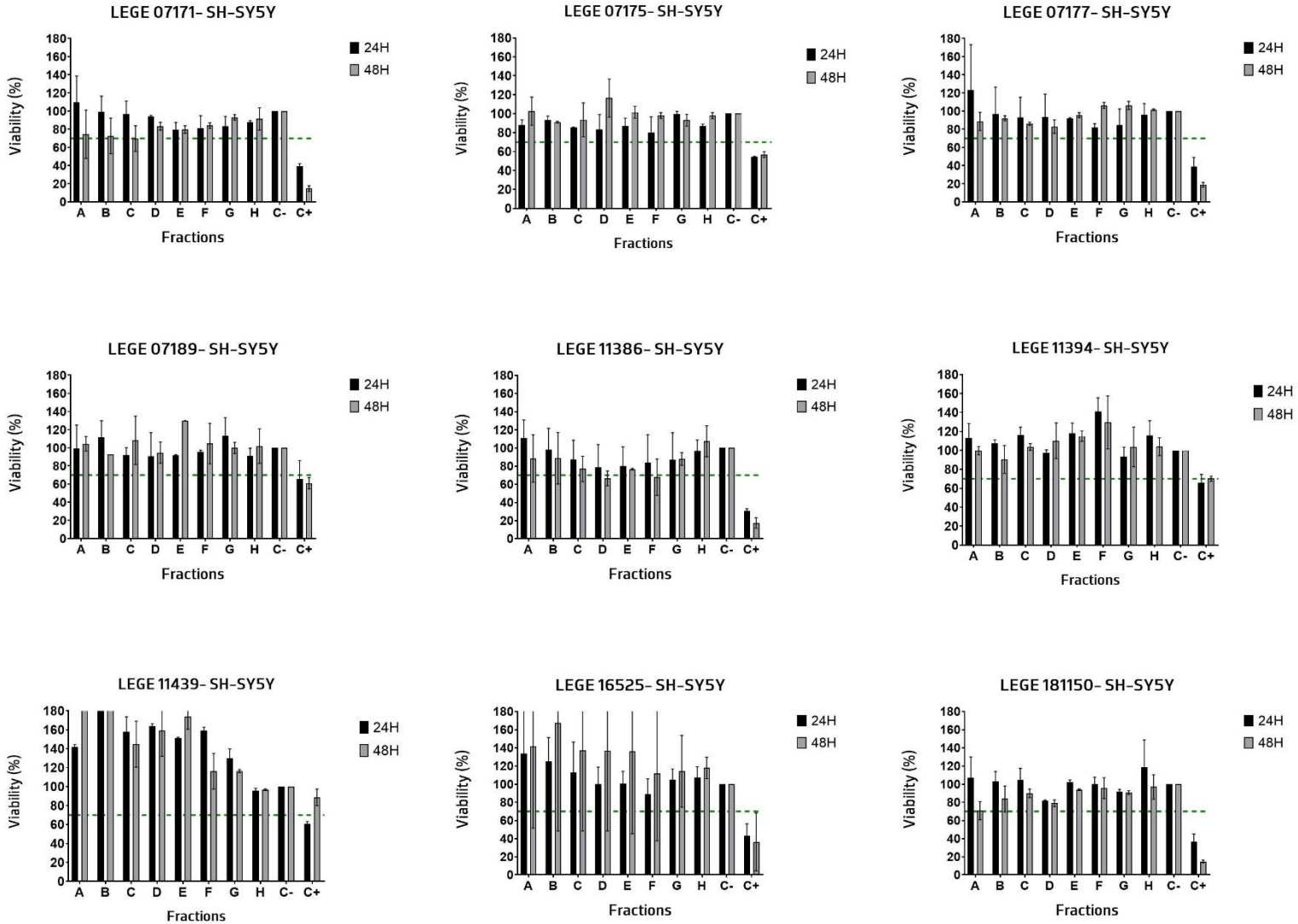


Figure 7. Cell viability (%) at 24h and 48h of the neuroblastoma cell line SH-SY5Y exposed to the 8 fractions (25 µg/mL) . Negative control (C-) was 0.5% DMSO (100% viability) and Positive Control (C+) was 20% DMSO. Results are expressed as mean±SD (n=3).



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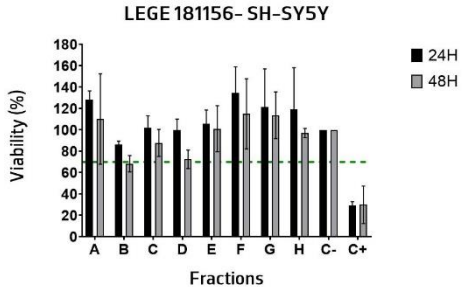

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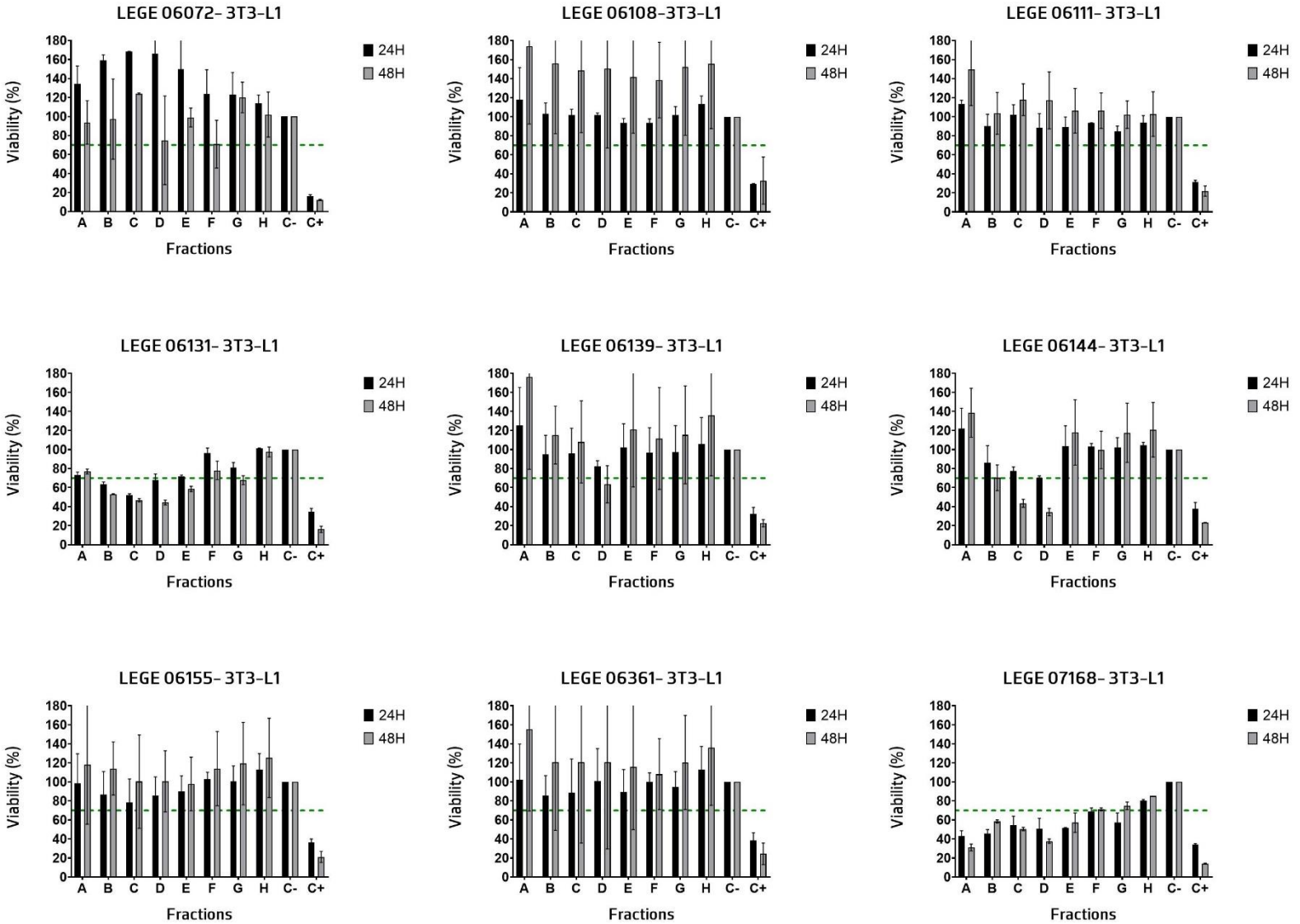
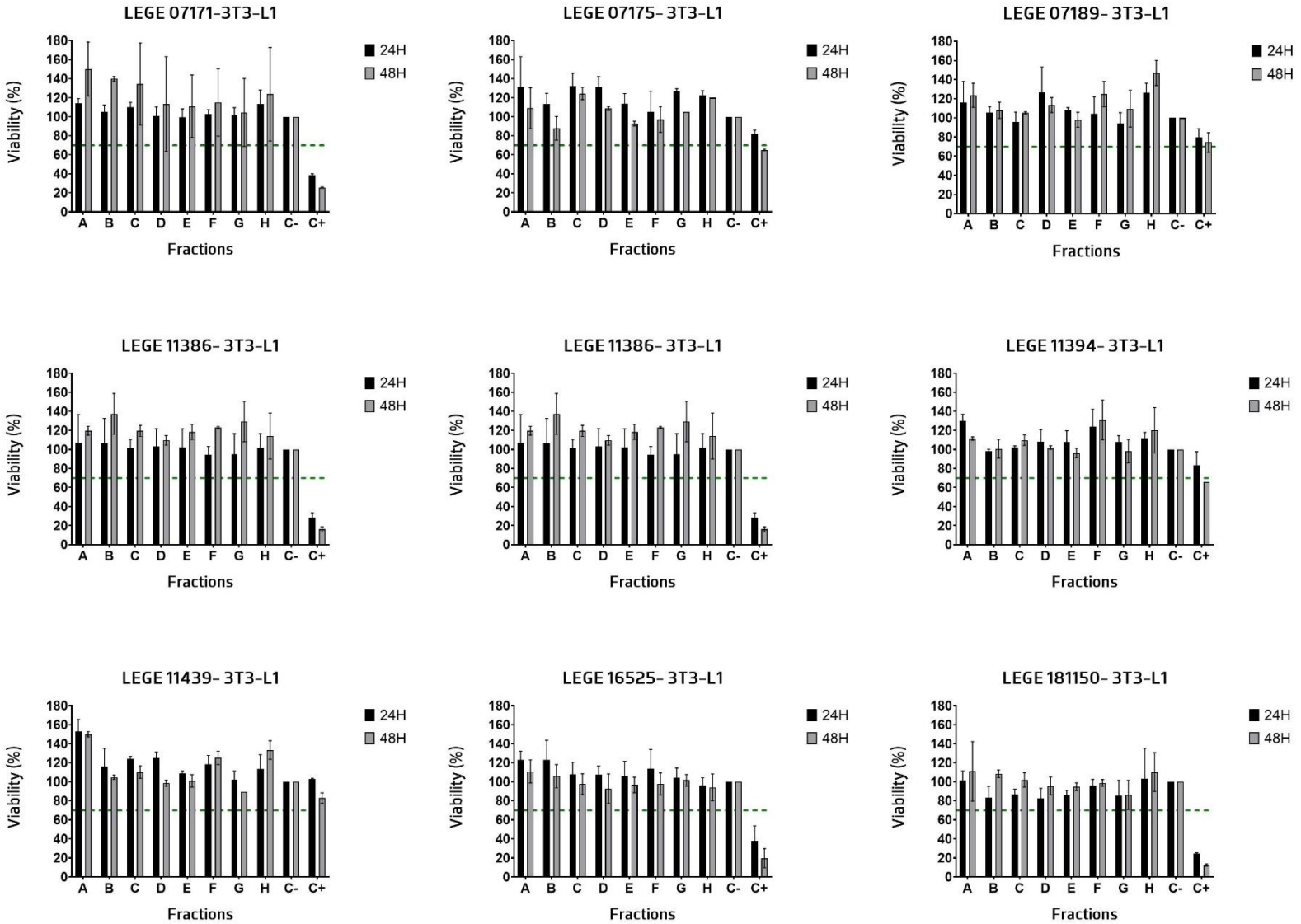


Figure 8. Cell viability (%) at 24h and 48h of the fibroblast cell line 3T3-L1 exposed to the 8 fractions (25 µg/mL) . Negative control (C-) was 0.5% DMSO (100% viability) and Positive Control (C+) was 20% DMSO. Results are expressed as mean±SD (n=3).



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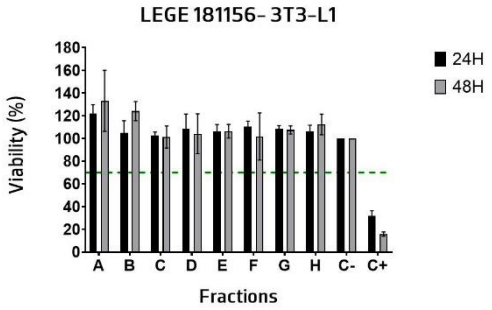


Figure 8. Cell viability (%) at 24h and 48h of the fibroblast cell line 3T3-L1 exposed to the 8 fractions (25 µg/mL) . Negative control (C-) was 0.5% DMSO (100% viability) and Positive Control (C+) was 20% DMSO. Results are expressed as mean±SD (n=3).

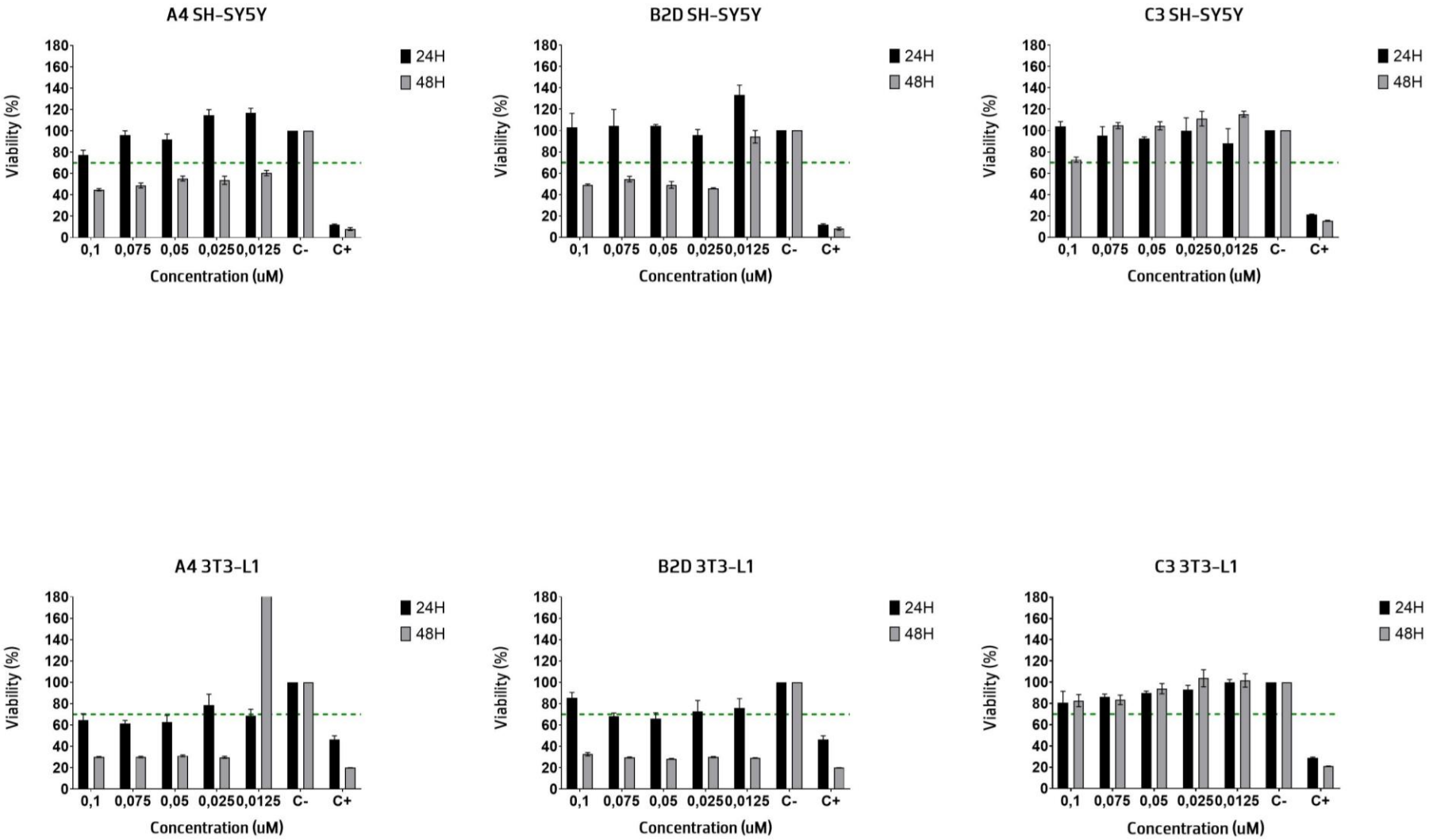


Figure 9. Cell viability (%) at 24h and 48h of the neuroblastoma (SH-SY5Y) and fibroblast (3T3-L1) cell lines exposed to the different concentrations (0.1; 0.075; 0.05; 0.025 and 0.0125 µM) of compound A4, B2D and C3. Negative control (C-) was 0.5% DMSO (100% viability) and Positive Control (C+) was 20% DMSO. Results are expressed as mean±SD (n=3).

Table 9. Summary of the cell viability results obtained through the MTT assay for the cell lines after exposure to the cyanobacteria fractions. ++++ 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		3T3-L1	
	24h	48h	24h	48h
LEGE 06072_A	–	++	++++	++
LEGE 06072_B	–	+	++++	++
LEGE 06072_C	–	+	++++	++++
LEGE 06072_D	–	++	++++	+
LEGE 06072_E	+	++	++++	++
LEGE 06072_F	+	++	++++	–
LEGE 06072_G	+	++	++++	++++
LEGE 06072_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 06111_A	++	++	++++	++++
LEGE 06111_B	+++	++	++	+++
LEGE 06111_C	+	+	+++	++++
LEGE 06111_D	++	++	++	++++
LEGE 06111_E	+++	++	++	+++
LEGE 06111_F	++	+	++	+++
LEGE 06111_G	++	++	++	+++
LEGE 06111_H	++	++	++	+++

	SH-SY5Y		3T3-L1	
	24h	48h	24h	48h
LEGE 06131_A	–	++	+	+
LEGE 06131_B	–	++	–	–
LEGE 06131_C	–	++	–	–
LEGE 06131_D	–	++	–	–
LEGE 06131_E	–	+++	+	–
LEGE 06131_F	–	++	++	+
LEGE 06131_G	–	++	++	–
LEGE 06131_H	–	+++	+++	++
LEGE 06139_A	++	+	++++	++++
LEGE 06139_B	++	++	++	++++
LEGE 06139_C	++	+	++	+++
LEGE 06139_D	++	–	++	–
LEGE 06139_E	+++	++	+++	++++
LEGE 06139_F	++	++	+++	++++
LEGE 06139_G	++	++	++	++++
LEGE 06139_H	+++	++	+++	++++
LEGE 06144_A	++++	++	++++	++++
LEGE 06144_B	++	+	++	–
LEGE 06144_C	++	++	+	–
LEGE 06144_D	–	–	–	–
LEGE 06144_E	++	++	+++	++++
LEGE 06144_F	++	++	+++	++
LEGE 06144_G	++	++	+++	++++
LEGE 06144_H	++	+++	+++	++++

	SH-SY5Y		3T3-L1	
	24h	48h	24h	48h
LEGE 06155_A	++	++	++	++++
LEGE 06155_B	++	++	++	+++
LEGE 06155_C	++	++	+	++
LEGE 06155_D	++	++	++	++
LEGE 06155_E	+	++	++	++
LEGE 06155_F	++	++	+++	+++
LEGE 06155_G	+	++	++	++++
LEGE 06155_H	++	++	++++	++++
LEGE 06361_A	+	+	+++	++++
LEGE 06361_B	-	-	++	++++
LEGE 06361_C	-	+	++	++++
LEGE 06361_D	+	++	+++	++++
LEGE 06361_E	-	++	++	++++
LEGE 06361_F	+	++	++	+++
LEGE 06361_G	-	++	++	++++
LEGE 06361_H	++	++	++++	++++
LEGE 07168_A	-	+++	-	-
LEGE 07168_B	+	+++	-	-
LEGE 07168_C	++	+++	-	-
LEGE 07168_D	++	++++	-	-
LEGE 07168_E	+++	+++	-	-
LEGE 07168_F	++	+++	-	+
LEGE 07168_G	++	++	-	+
LEGE 07168_H	++	++++	+	++

	SH-SY5Y		3T3-L1	
	24h	48h	24h	48h
LEGE 07171_A	+++	+	++++	++++
LEGE 07171_B	++	+	+++	++++
LEGE 07171_C	++	-	++++	++++
LEGE 07171_D	++	++	++	++++
LEGE 07171_E	+	+	++	++++
LEGE 07171_F	++	++	+++	++++
LEGE 07171_G	++	++	+++	+++
LEGE 07171_H	++	++	++++	++++
LEGE 07175_A	++	+++	++++	+++
LEGE 07175_B	++	++	++++	++
LEGE 07175_C	++	++	++++	++++
LEGE 07175_D	++	++++	++++	+++
LEGE 07175_E	++	+++	++++	++
LEGE 07175_F	++	++	+++	++
LEGE 07175_G	++	++	++++	+++
LEGE 07175_H	++	++	++++	++++
LEGE 07177_A	++++	++	+++	++++
LEGE 07177_B	++	++	++	++++
LEGE 07177_C	++	++	++	+++
LEGE 07177_D	++	++	++++	++++
LEGE 07177_E	++	++	+++	++++
LEGE 07177_F	++	++++	++++	++++
LEGE 07177_G	++	++++	+++	++++
LEGE 07177_H	++	+++	+++	++++

	SH-SY5Y		3T3-L1	
	24h	48h	24h	48h
LEGE 07189_A	++	+++	++++	++++
LEGE 07189_B	++++	++	+++	+++
LEGE 07189_C	++	+++	++	+++
LEGE 07189_D	++	++	++++	++++
LEGE 07189_E	++	++++	+++	++
LEGE 07189_F	++	+++	+++	++++
LEGE 07189_G	++++	++	++	+++
LEGE 07189_H	++	+++	++++	++++
LEGE 11386_A	+++	++	+++	++++
LEGE 11386_B	++	++	+++	++++
LEGE 11386_C	++	+	+++	++++
LEGE 11386_D	+	-	+++	+++
LEGE 11386_E	+	+	+++	++++
LEGE 11386_F	++	-	++	++++
LEGE 11386_G	++	++	++	++++
LEGE 11386_H	++	+++	+++	+++
LEGE 11394_A	++++	++	++++	++++
LEGE 11394_B	+++	++	++	++
LEGE 11394_C	++++	+++	+++	+++
LEGE 11394_D	++	++++	+++	+++
LEGE 11394_E	++++	++++	+++	++
LEGE 11394_F	++++	++++	++++	++++
LEGE 11394_G	++	+++	+++	++
LEGE 11394_H	++++	+++	++++	++++

	SH-SY5Y		3T3-L1	
	24h	48h	24h	48h
LEGE 11439_A	++++	++++	++++	++++
LEGE 11439_B	++++	++++	++++	+++
LEGE 11439_C	++++	++++	++++	++++
LEGE 11439_D	++++	++++	++++	++
LEGE 11439_E	++++	++++	+++	++
LEGE 11439_F	++++	++++	++++	++++
LEGE 11439_G	++++	++++	+++	++
LEGE 11439_H	++	++	++++	++++
LEGE 16525_A	++++	++++	++++	++++
LEGE 16525_B	++++	++++	++++	+++
LEGE 16525_C	+++	++++	+++	++
LEGE 16525_D	++	++++	+++	++
LEGE 16525_E	++	++++	+++	++
LEGE 16525_F	++	++++	++++	++
LEGE 16525_G	+++	++++	+++	+++
LEGE 16525_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	++++	++	+++	++++

	SH-SY5Y		3T3-L1	
	24h	48h	24h	48h
LEGE 181156_A	++++	++++	++++	++++
LEGE 181156_B	++	+	+++	++++
LEGE 181156_C	+++	++	+++	+++
LEGE 181156_D	++	+	+++	+++
LEGE 181156_E	+++	+++	+++	+++
LEGE 181156_F	++++	++++	++++	+++
LEGE 181156_G	++++	++++	+++	+++
LEGE 181156_H	++++	++	+++	++++
A4_0,1 µM	+	–	–	–
A4_0,075 µM	++	–	–	–
A4_0,05 µM	++	–	–	–
A4_0,025 µM	++++	–	+	–
A4_0,0125 µM	++++	–	–	++++
B2D_0,1 µM	+++	-	++	–
B2D_0,075 µM	+++	-	–	–
B2D_0,05 µM	+++	-	–	–
B2D_0,025 µM	++	-	+	–
B2D_0,0125 µM	++++	++	+	–
C3_0,1 µm	+++	+	+	++
C3_0,075 µM	++	+++	++	++
C3_0,05 µM	++	+++	++	++
C3_0,025 µM	++	++++	++	+++
C3_0,0125 µM	++	++++	++	+++

4.2. AChE and BuChE Inhibition assay

Acetylcholine is a neurotransmitter released in the synaptic cleft in response to nerve stimulation, binding to postsynaptic receptors. It is inactivated by cleavage, in a reaction catalyzed by AChE, therefore this enzyme has a significant role in the cholinergic nervous system (110). Decrease in ACh levels is associated with progressive cognitive deterioration, deterioration of neuromuscular functions and, consequently, reduced autonomy (110). In the brain, ACh is also degraded by the BuChE enzyme. Cerebral cortex and hippocampus from patients with AD present several changes in the activity of these two enzymes (111). Therefore, inhibition of AChE and BuChE in order to maintain ACh levels is an important approach for AD (112). As previously mentioned, galantamine, rivastigmine and donepezil are the most common AChE inhibitors (AChEIs) commercial use (15).

Cyanobacteria have been reported as a great natural source of AChEIs, such as anatoxin(s) and nostocarboline (113,114). It has been found that AChEIs in cyanobacteria are involved with the inhibition of colonization of colonies and filaments of other organisms through inhibition of invertebrate larval settlement (115). In this sense, screening cyanobacteria for AChEIs is a good approach.

The Ellman's method is based on the production of the anion TNB_2^- through the reaction of DNTB with thiocholine, the product of the hydrolysis of ATCI by AChE or BTCI by BuChE. The resulting anion has a yellow color that can be measured by spectrophotometry (116).

This method is widely used in AChEIs testing due to its affordability and accessibility, however, it has the disadvantage of producing false-positive results (117).

Due to limit amount of biomass, not all fractions were able to be screened for both AChE and BuChE inhibition. A fraction was considered with inhibitory potential when the inhibition percentage was over 20%.

The results related to the inhibition of this enzyme are presented in Figure 10. The most interesting results are related to Fraction C from LEGE 11394 with an inhibition capacity of 21.09%. Fractions A and F from LEGE 11386 shown an inhibition potential of 22.79% and 23.55%, respectively. Also, fractions A and B from LEGE 07171 shown an inhibition of 22.23% and 26.82%, respectively. LEGE 07175 fraction E presented an inhibition of 24.53%. The best results regarding AChE inhibition were obtained from fraction A (LEGE 11439) and fraction B (LEGE 181150), with values of 27.69% and 31.29%, respectively.

As shown in Figure 11, all the cyanobacteria fractions analyzed for BuChE inhibition, show a percentage of inhibition below 13%. The inhibition capacity of the three cyanobacteria compounds was not tested due to lack of biomass.

The three cyanobacteria compounds were also tested for its AChE inhibition potential in a dose dependent assay. Results are presented in Figure 12. For the studied concentrations, none of the compounds show AChE inhibition potential, , that is, all concentrations tested presented an inhibitory potential below 20%. In regard to the BuChE inhibition, the results were not promising.

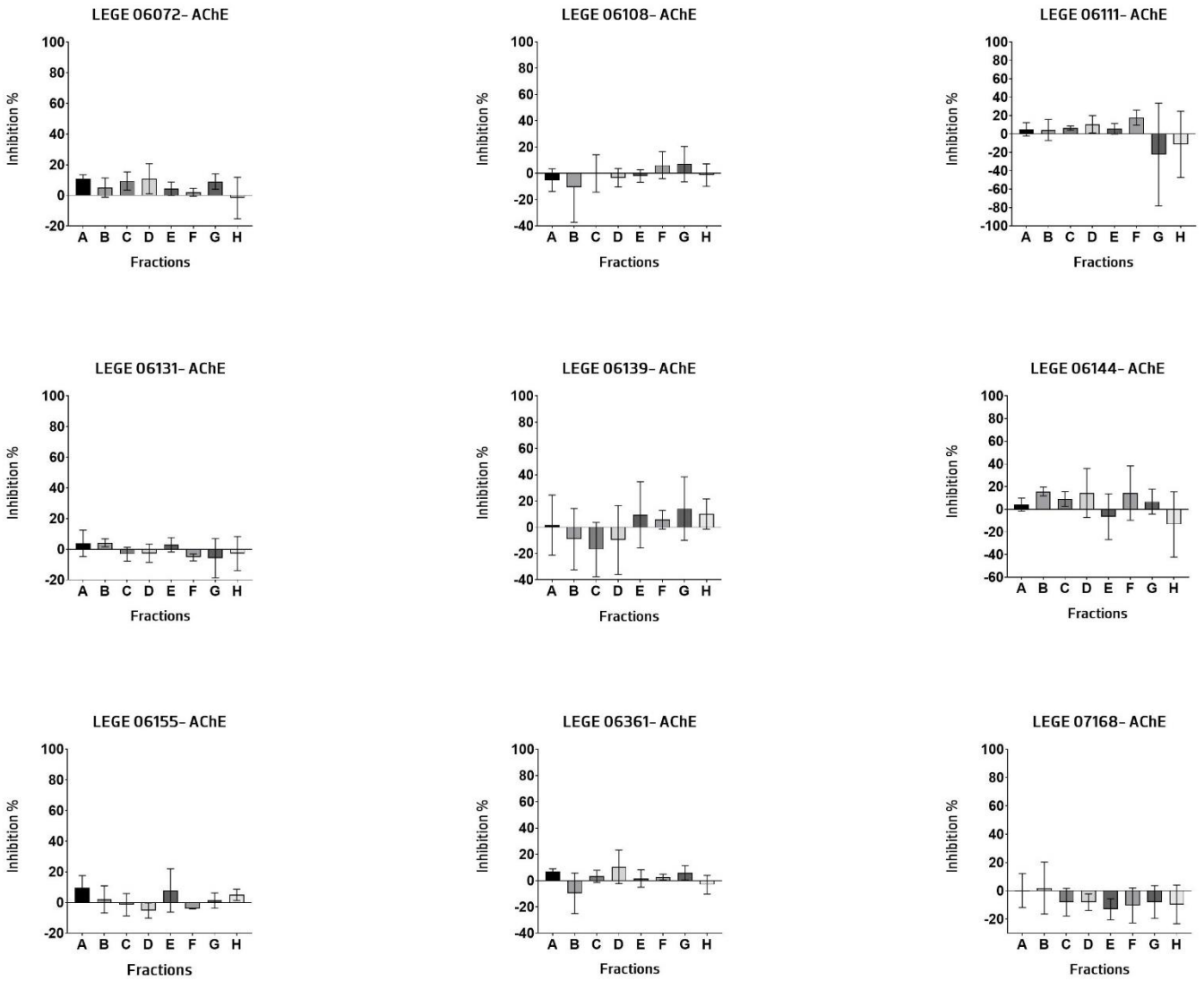
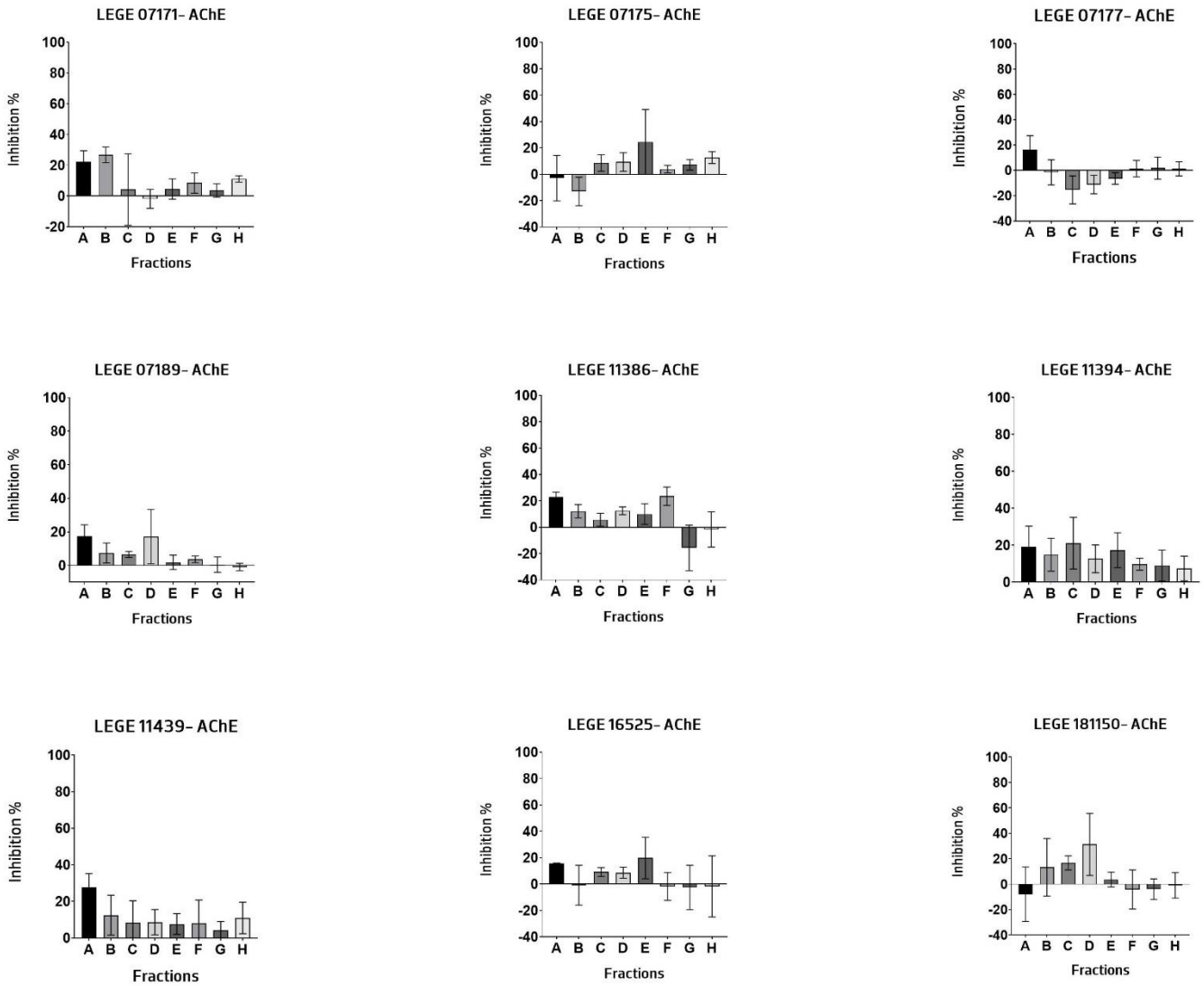


Figure 10. Inhibitory activity (%) against acetylcholinesterase (AChE), determined by the Ellmans method, of cyanobacteria fractions (250 μg/mL). Values are expressed as mean ± SD, of three independent experiments in triplicate.



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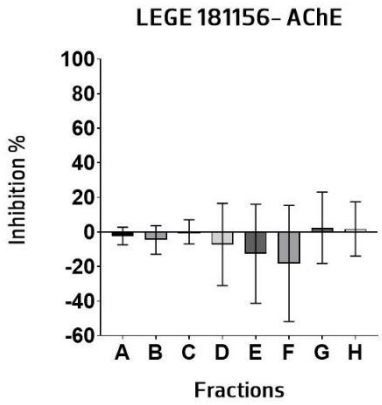


Figure 10. Inhibitory activity (%) against acetylcholinesterase (AChE), determined by the Ellmans method, of cyanobacteria fractions (250 μg/mL). Values are expressed as mean ± SD, of three independent experiments in triplicate.

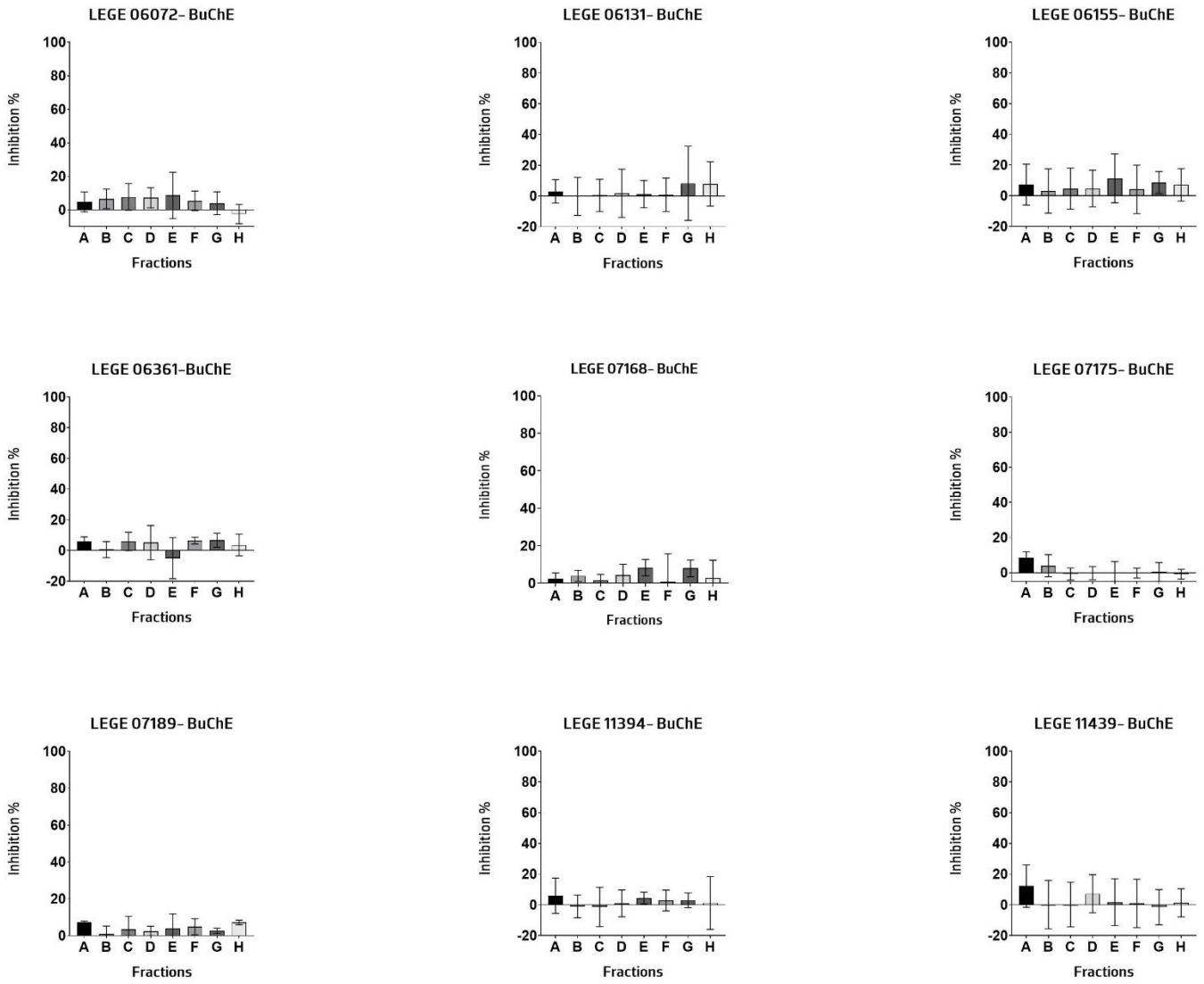
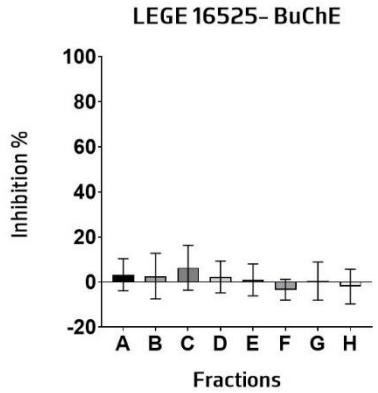


Figure 11. Inhibitory activity (%) against butyrylcholinesterase (BuChE), determined by the Ellmans method, of cyanobacteria fractions (250 µg/mL). Values are expressed as mean ± SD, of three independent experiments in triplicate.



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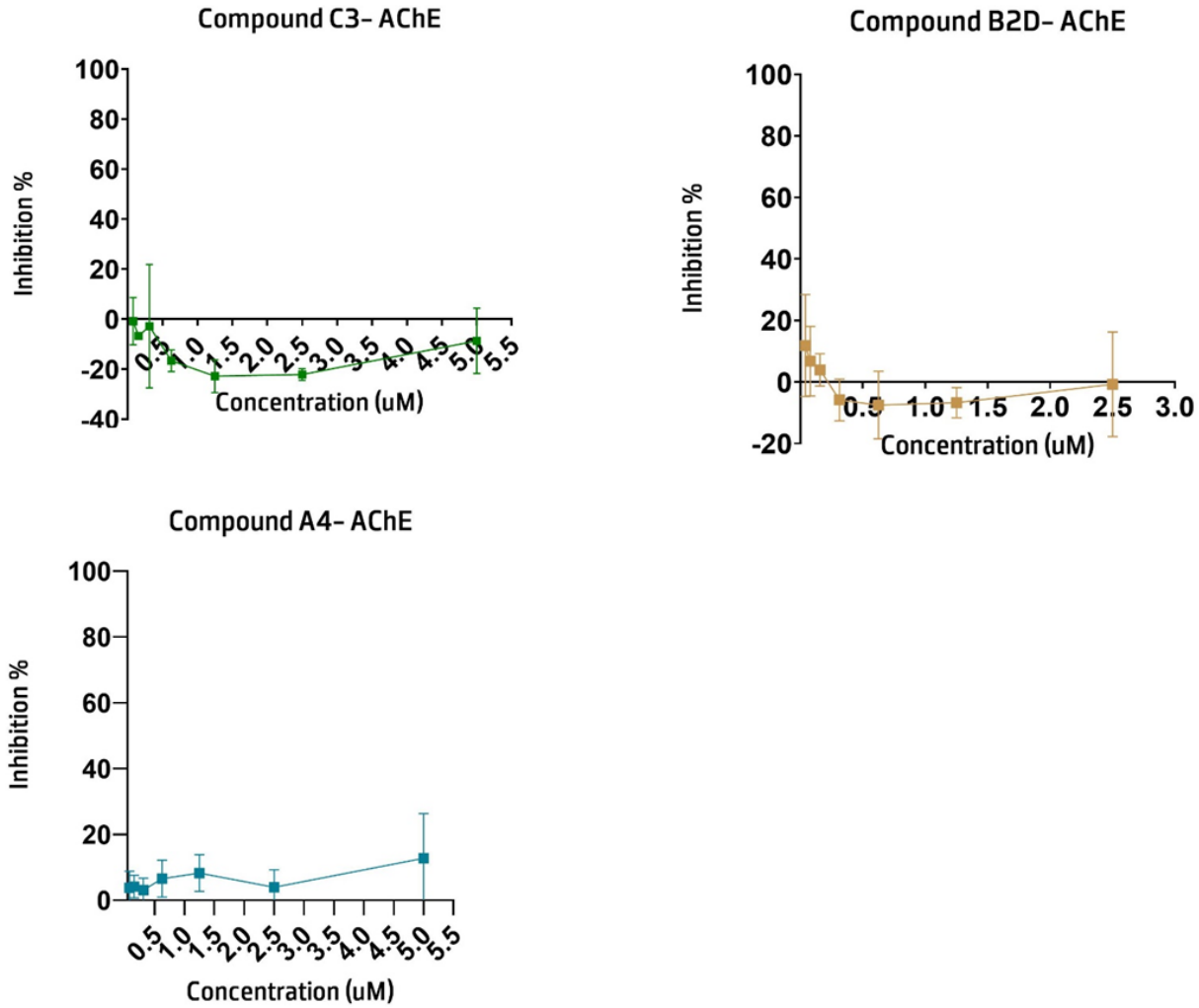

Figure 12. Inhibitory activity (%) against acetylcholinesterase (AChE), determined by the Ellmans method, of the compounds A4, B2D and C3. Values are expressed as mean ± SD, of one independent experiment in triplicate.

When comparing all the results obtained from the cell viability assays with the results regarding the AChE and BuChE inhibition assays it is obvious that fractions who were active against the enzymes did not show cytotoxicity against the cell lines. Table 10 shows a compilation of the best results from all tests performed.

Table 10. Compilation of the best results obtained in the cell viability assays and inhibition assays.

	SH-SY5Y		3T3-L1		AChE Inhibition%	BuChE Inhibition %
	24h	48h	24h	48h		
LEGE 06155_E	76.59 ± 16.67	88.97 ± 26.30	90.07 ± 16.04	97.89 ± 28.16	7.84 ± 14.14	11.34 ± 15.9
LEGE 07171_A	109.72 ± 29.00	74.47 ± 26.72	114.01 ± 4.99	150.12 ± 28.33	22.23 ± 7.24	-
LEGE 07171_B	99.41 ± 17.22	72.76 ± 19.60	105.02 ± 7.39	139.9 ± 2.39	26.82 ± 5.19	-
LEGE 07175_E	87.06 ± 8.05	101.4 ± 6.27	113.89 ± 10.48	92.77 ± 2.49	24.53 ± 24.60	-
LEGE 11386_A	110.59 ± 20.21	88.23 ± 26.22	106.92 ± 29.70	119.7 ± 4.69	22.79 ± 3.80	-
LEGE 11386_F	84.08 ± 30.66	67.7 ± 20.05	94.52 ± 8.70	122.95 ± 1.24	23.56 ± 6.95	-
LEGE 11394_C	116.24 ± 8.28	103.66 ± 3.53	102.33 ± 1.32	109.76 ± 5.52	21.09 ± 14.01	-1.4 ± 12.83
LEGE 11439_A	141.81 ± 2.49	214.7 ± 34.10	152.81 ± 12.71	149.9 ± 2.72	27.69 ± 7.56	12.19 ± 13.83
LEGE 11439_D	164.03 ± 2.26	159.13 ± 26.95	124.94 ± 6.36	98.65 ± 3.27	8.66 ± 6.95	7.2 ± 12.38
LEGE 181150_B	102.78 ± 11.12	83.71 ± 14.20	83.49 ± 11.62	108.4 ± 4.03	31.29 ± 22.61	-
C3_0,025 µM	99.64 ± 12.18	111.15 ± 6.94	93.21 ± 3.86	103.74 ± 8.07	-	-
C3_0,0125 µM	88.2 ± 13.61	115.42 ± 2.76	99.76 ± 2.72	101.71 ± 6.37	-	-
A4_5 µM	-	-	-	-	12.74 ± 13.62	-
B2D_0,0390625 µM	-	-	-	-	11.84 ± 16.58	-

The most active fraction in this work, LEGE 11439_A, comes from the cyanobacteria strain *Spirulina sp.* .Several studies, both in vivo or in vitro, demonstrate that *Spirulina* species exhibit great antioxidant, anti-inflammatory, neuronal protection and AChE inhibition properties (84,118). For these reasons, this cyanobacteria specie is wildly studied for its potential in neurodegenerative diseases, especially AD.

In previous work carried out by the research group, LEGE 11439_A and LEGE 07175_E (*Cyanobium sp.*) showed the same great results regarding the performed assays (17,18). In this sense, these two fractions could be subject for more specific studies related to ND. For example, antioxidant and anti-inflammatory assays and cell viability tests using cell lines related to the

brain-blood-barrier, such as hCMEC/D3 cell line. Also, it would be beneficial testing other concentrations of these fractions in other to figure out their IC_{50}. Study the chemical composition of these fractions, using mass spectrometry, for example, would also be significant. Cyanobacteria LEGE 181150_B (*Synechococcales cyanobacterium* genera) also presented remarkable results. This strain appears to have antioxidant and anticancer properties. Additionally, presents hyaluronidase inhibition highlighting its potential for cosmetic applications (98,103).

Of the three tested compounds only compound C3 presents the best results relative to cell viability, especially at concentrations of 0.0125 and 0.025 μM. These compounds show no AChE inhibition at the tested concentrations, it is possible at in other concentrations the results might be different.

5. Conclusion

Neurodegenerative diseases affect millions of people worldwide, especially AD. Despite being very common diseases, there is still no cure or effective treatment. The demand for new therapies has become more urgent.

Cyanobacteria are ancient prokaryotic organisms with the ability of produce compounds with neuroprotective effects. Nowadays, cyanobacteria based bioactive compounds are commercially available and their market demand will continue to grow due to the increasing demand for natural products in the food industry (119). In particular, C-Phycocyanin is expected to reach a market value of USD 409.8 million within the 2030s (120). *Spirulina* is a protein-rich cyanobacterium (60–70% (w/w)) and an important source for C-Phycocyanin.

In this work several cyanobacteria strains from the LEGE-NPL tool were evaluated for their cytotoxicity and AChE and BuChE inhibition. A total of 176 fractions from 19 different cyanobacteria strains were tested. Three cyanobacterial derived compounds were also tested in the cell lines and enzymes. Most cyanobacteria fractions were not cytotoxic in the SH-SY5Y and 3T3-L1 cell lines. However, compounds A4 and B2D did present toxicity (cell viability less than 70%). Regarding to the inhibitory potential, the best results were LEGE 181150_B, LEGE 11439_A and LEGE 07175_E which presented an AChE inhibition of 31.29%, 27.69% and 24.53%, respectively. These three fractions hold promising expectations in the prevention and treatment of NDs once were the ones with best results in both performed tests. The results related to the cyanobacteria compounds were not promising at the tested concentrations.

In summary, it is possible to see potential in the use of cyanobacteria as a treatment option especially in the three strains mentioned above. However, further screening studies need to be performed to fully understand de use of these microorganisms as treatment option for NDs.

6. Future Perspectives

The awareness in investigating cyanobacteria against neurodegeneration stems from their rich list of compounds, including polysaccharides, lipids, pigments, carotenoids and polyphenols, to the sustainability in the production of biomass and extraction procedures. In fact, the growing awareness of the health benefits from natural compounds allied with the World Health Organization (WHO) Intersectoral global action plan on epilepsy and other neurological disorders emphasizing diets enriched with natural compounds, is an opportunity that drives more research in the biotechnological potential of cyanobacteria (90,91). Moreover, the forthcoming climate crisis has encouraged an interest in cyanobacteria not only due to their potential source of natural compounds but also to the sustainability of biomass production and extraction (92).

Following the results of the present work, the next step would be the investigation of other properties of LEGE 07175 and LEGE 11439, such as anti-inflammatory and antioxidant effects.

The quest for novel anti-inflammatory and antioxidant compounds grips special attention in neurodegeneration research and development. Anti-inflammatories are pivotal in reducing inflammation and antioxidants crucial in neutralizing oxidative stress, both extremely linked to the neurodegenerative process.

The use of other cell lines, such as the hCMEC cell line, would be essential in other to apply these cyanobacteria strains in the neurodegenerative disease field.

It would be also important to repeat the BuChE inhibition assay, especially in the LEGE 07175 once there are no results from this strain due to lack of biomass.

Lastly, the identification and isolation of the chemical compound(s) responsible for the observed activity in these fractions would be essential.

Regarding to the cyanobacteria compounds, it would be important studying other concentrations both in the cell viability and enzymatic inhibition assays.

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Appendix

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

Table I: Composition of the Z8 medium for marine cyanobacteria

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

Table II: Chemical composition of solution A

Solution A	
Reagents	**g/L dH_2O**
Sodium Nitrate (NaNO3)	46.7
Calcium Nitrate Tetrahydrate (Ca(NO3)2.4H2O)	5.9
Magnesium Sulphate Heptahydrate (MgSO4.7H2O)	2.5

Table III: Chemical composition of Solution B

Solution B	
Reagents	**g/L dH_2O**
Potassium Phosphate Dibasic (K2HPO4)	3.1
Sodium Carbonate (Na2CO3)	2.1

Table IV: Composition of Fe-EDTA solution

Fe-EDTA Solution	
Reagents	**g/L dH_2O**
Iron (III) Chloride (FeCl3)	10
Sodium EDTA (EDTA-Na)	9.5

Table VI: 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 (FeCl3.6H20)	2.80	Ethylenediamine tetraacetic acid (EDTA)	3.90

Table VII: Composition of the Micronutrients solution

Micronutrients Solution	
Reagents	**g/L dH_2O**
1 to 12	10
13 to 14	100

Table VIII: Chemical composition of the Micronutrients solution

Micronutrients	
Reagent	**g/L dH_2O**
1- Sodium tungstate dihydrate (Na2W04.2H20)	0.33
2- Ammonium paramolybdate dihydrate ((NH4)6Mo7024.2H20)	0.88
3- Potassium bromide (KBr)	1.2
4- Potassium iodide (KI)	0.83
5- Zinc Sulfate Heptahydrate (ZnS04.7H20)	2.87
6- Cadmium nitrate tetrahydrate (Cd(N03).4H20)	1.55
7- Cobalt (II) Nitrate Hexahydrate (Co(N03)2.6H20)	1.46
8- Copper (II) sulfate pentahydrate (CuS04.5H20)	1.25
9- Nickel (II) ammonium bi-sulfate hexahydrate (NiS04(NH4)2S04.6H20)	1.98
10- Chromium (III) Nitrate Nonahydrate (Cr(N03)3.9H20)	0.41
11- Vanadium pentoxide (V205)	0.089
12 - Aluminum potassium bi-sulfate dodecahydrate (Al2(S04)3K2S04.24H20)	4.74
13- Boric acid (H3B03)	3.1
14- Manganese (II) Sulfate Tetrahydrate (MnS04.4H20)	2.23