Microalgae as a new source of neuroprotective compounds

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Heavenly Father, thank You for Your Love, Mercy and Grace.

Father, thank You for rescuing me from myself through Your begotten Son, Jesus Christ–the precious gift from You that I have in my life; Your kingdom, the LORD, and the Salvation of the world. Miraculously You hid my life with Christ in You! Abba, thank You for giving me Your Holy Spirit, the Interpreter of Your life-giving Word; the Almighty Power, Magnifier and Glorifier of Christ Jesus.

God, Your Love is better than life!

"Surely goodness and mercy shall follow me all the days of my life; and I will dwell in the house of the LORD forever." *Psalms 23: 6*

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My future: *Family* and *students*

My love towards them will remain forever!

DECLARATION

Microalgae as a new source of neuroprotective compounds

Declaration of Authorship

I, Sisay Tesema Uota, hereby declare that this study is my original research work. It has not been submitted to any other university for any award of degree.

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ABSTRACT

Although neurodegenerative diseases (NDD) mostly occur in aged people, its onset can also be triggered by excessive accumulation of metal ions, particularly copper, iron and calcium in the brain which leads to the development of Alzheimer's disease (AD). AD affects cognitive function in the elderly people. Therefore, there is an urgent need to search and identify new sources of anti-AD drugs from natural sources, because available drugs are aggressive and have side-effects. According to a neurotoxicology study, chelating or capturing those metals is a therapeutic solution. Interestingly, marine microalgae (MMA) are rich in several bioactive compounds with neuroprotective, anti-inflammatory and others. Recently, MMA have been featured in neuronal-related diseases. This research shows bioactive compounds produced by different microalgae as a source of neuroprotective agents, in terms of copper (Cu^{2+}CA), iron (Fe^{2+}CA) and calcium chelating activities (Ca^{2+}CA). Hence, nine different microalgae were procured from Necton S.A, Portugal. Three different food graded solvents namely ethyl acetate, ethanol and water were used for extraction. The ethanolic extract of *Porphyridium* sp. and *Skeletonema costatum* were the most significantly bioactive with an IC_{50} of 1.44 mg/mL and 1.95 mg/mL for Cu^{2+}CA, 0.942 mg/mL and 0.883 mg/mL for Fe^{2+}CA and 0.832 mg/mL and 1.02 mg/mL for Ca^{2+}CA respectively. The active crude extracts were further partitioned into hexane, dichloromethane, ethyl acetate and an aqueous alcoholic by liquid-liquid extraction (LLE). Hexane fractions of *Porphyridium* sp. and *S. costatum* presented high metal chelating activity with an IC_{50} of 0.461 mg/mL and 0.361 mg/mL for Cu^{2+}CA, 0.292 mg/mL and 0.263 mg/mL for Fe^{2+}CA and 0.451 mg/mL and 0.181 mg/mL for Ca^{2+}CA respectively. In order to identify the compounds possibly responsible for the activity, the hexane fractions were analyzed by GC-MS. About 19 and 22 of compounds were in *Porphyridium* sp. and *S. costatum* identified, respectively, belonging to 4 classes of metabolites: fatty acids, sterols, alcohols and ethers. The most abundant compounds detected were long chain fatty acids and alcohols including tetrahydropyranyl ether of citronellol and 3,7,11,15-tetramethyl-2-hexadecen-1-ol. The analyzed species exhibited neuroprotective activities in a concentration dependent. Their metal chelating activities proves their potential for future application as natural remedies for Alzheimer's disease.

ABBREVIATIONS

AB = Algal Biomass

AD = Alzheimer's Disease

Aβ = Amyloid Beta

Abs. = Absorbance

APP = Amyloid precursor protein

C^- = Negative Control

C^+ = Positive Control

Ca^{2+}CA = Calcium ion (II) Chelating Activity

CNS = Central Nervous System

CTP4 = *Tetraselmis* sp. CTP4

Cu^{2+}CA = Copper ion (II) Chelating Activity

DCM = Dichloromethane

DFO = Deferoxamine

DMSA = Meso-2,3-dimercaptosuccinic Acid

DMSO = Dimethyl Sulfoxide

DNA = Deoxyribonucleic Acid

DPA = D-Penicillamine

DPPH = 1,1-diphenyl-2-Picryl Hydrazyl

EA = Ethyl Acetate

EDTA = Ethylene Diamine Tetraacetic Acid

EGTA = Ethylene Glycol Tetraacetic Acid

EtOH = Ethanol

FDA = Food and Drug Administration

Fe^{2+}CA = Iron ion (II) Chelating Activity

GC-MS = Gas Chromatography-Mass Spectroscopy

H_2O_2 = Hydrogen Peroxide

Hp = *Haematococcus pluvialis*

IC_{50} = Concentration that chelates the 50% of free metal ion

Isp. = *Isochrysis* species

LLE = Liquid-Liquid Extraction

MCA = Metal Chelating Activity

MeOH = Methanol

MMA = Marine Microalgae

NDD = Neurodegenerative Disease

NFTs = Neurofibrillary Tangles

NIST = National Institute Standard and Technology

NO• = Nitric Oxide radical

NOx = Nitrogen Oxides

Nsp. = Nannochloropsis species

NTA = Nitrilotriacetic Acid

O_2• = Superoxide anion radical

OCPC = o-Cresolphthalein Complexone

OH• = Hydroxyl radicals

ONOO = Peroxynitrite

Psp.aqueous+ethanol = Aqueous ethanol fraction from *Porphyridium* sp.

Psp.C = Crude of ethanolic extracts of *Porphyridium* sp.

Psp.DCM = Dichloromethane fraction from *Porphyridium* sp.

Psp.EtOH = Ethanol extract from *Porphyridium* sp.

Psp.EA = Ethyl acetate fraction from *Porphyridium* sp.

Psp.H = Hexane fraction from *Porphyridium* sp.

Pt = *Phaeodactylum tricornutum*

PI = Polarity Index

PUFAs = Polyunsaturated fatty acids

Psp. = *Porphyridium* species

PV = Pyrocatechol Violet

R^2 = Correlation coefficient

RNS = Reactive Nitrogen Species

ROS = Reactive Oxygen Species

RT = Room Temperature

Sc = *Skeletonema costatum*

Sc aqueous+ethanol = Aqueous ethanol fraction from *Skeletonema costatum*

ScC = Crude of ethanolic extracts of *Skeletonema costatum*

ScDCM = Dichloromethane fraction from *Skeletonema costatum*

ScEtOH = Ethanol extract from *Skeletonema costatum*

ScEA = Ethyl acetate fraction from *Skeletonema costatum*

ScH = Hexane fraction from *Skeletonema costatum*

Pt = *Phaeodactylum tricornutum*

PI = Polarity Index

PUFAs = Polyunsaturated fatty acids

SEM = Standard error of mean

Ssp. = *Spirulina* species

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1. INTRODUCTION

Neuron protection refers to the relative preservation of the structure and function of neurons from neurotoxic substances, among others[1]. Neuron-toxicants can inactivate and damage neurons, resulting in degenerative diseases which are called dementia[2,3]. Dementia is a brain disease that occurs mostly in elderly people due to age related disorder of nerve cell of the brain. It leads to chronic progressive mental sicknesses with main characters of disturbing memory, orientation, thinking, comprehension, calculation and other essential brain functions such as learning capacity, language, problem-solving and judgment that strongly affecting personal daily activities[3].

It has been reported that, over 50 million of people are presently suffering with dementia worldwide and approximately 152 million people will be affected in 2050 globally. Every 3 seconds someone develops dementia somewhere in the world[4], and it is estimated that 9.9 million newly cases are arising each year and from which 2.5 million are detected in Europe[3]. According to the Alzheimer's Disease International Dementia Statistics reports, much of the increase is in developing countries having low and middle income, about where 58% of people are affected with dementia and it will rise to 68% by 2050[5].

The most common cause of dementia is neurodegenerative disease (NDD), which is caused by functional loss and sensory dysfunction in the brain; progressive loss or death of nerve cells or neurons from central or peripheral nervous system in neurons[6]. Also, dysfunction of synaptic function that causes the failure of neurodegenerative networks is due to the accumulation of abnormal proteins. These proteins are toxic, insoluble, misfolded and aggregated that are found either external or internal cellular in the brain[7].

Despite the widespread, prevalence and debilitating effects of dementia, research findings reported that, currently there are no cure and reliable testing kits to detect. The underlying pathophysiology years before the symptom occurrence are missing for the radically growing cases of NDD such as Alzheimer's, Parkinson's, Huntington's and Dementia with Lewy bodies[7,8,9].

1.1. Alzheimer's Disease (AD)

AD was first clinically recognized by a German psychiatrist, Dr. Alois Alzheimer, in 1915[10]. It is an irreversible disease which accounts for 60 to 80% of all progressive degenerative brain disorders or dementia. It affects mostly in aged society i.e., above 65 years and develops during years to decades before clinical symptoms of dementia emerge, by attacking the brain gradually. Thus, the detrimental effects of the aging process are best observed in the brain, where irreversibly damaged cells cannot be replaced, that is involved in the development of AD[11]. Improved understanding of brain aging pathogenic molecular defects that leads to AD is not exceptional[12]. Death usually arises in less than 10 years after of diagnosis. AD affects memory, thinking, calculation, learning, orientation, comprehension, language, judgment abilities, cognitive capacities and ability to carry out the simplest tasks[10,13]. These symptoms are caused by the presence of toxic fibrillar and oligomeric forms of amyloid beta (Aβ) protein plaques and tau protein tangles that contribute to the damage of synapses by killing neurons[14].

Recently, over 30 million people are living with AD worldwide and this number is projected to double every 20 years, to the extent of 66 million in 2030 and will expected to reach 115 million by 2050[15].

Still scientists do not understand when and what exactly causes AD. However, there are leading hypothesis for the pathology that involves in the development of AD such as (i) cholinergic hypothesis (reduction or dis-regulated generation of acetyl and butyl-choline in certain part of brain), (ii) amyloid hypothesis (senile plaques composed of aggregated amyloid beta protein), (iii) tau hypothesis (intracellular neurofibrillary tangles (NFTs) consisting of hyper phosphorylated tau)[16,17,18] and dyshomeostasis of bio-metals hypothesis[19,20]. All hypothesis share similar processes, including: aggregations and misfolding of toxic proteins that causes oxidative stress, mitochondrial dysfunction (a breakdown of energy production within a cell), which contributes to dysfunction, loss, damage and death of neuronal cells in the AD brain. There is an increasing evidence suggesting that dyshomeostasis and neurotoxicity of bio-metals contribute in the pathology of AD[17].

Approximately 100 billion neurons (each with long and branching extensions) are found in a healthy adult's brain. Those extensions allow single neurons to form connections with other neurons called synapses in which information flow in tiny bursts of chemicals that are released by one neuron and detected by a receiving neuron. The human brain contains about 100 trillion

synapses. The synapses enable signals to travel rapidly through the brain's neuronal circuits, creating the cellular basis of memories, thoughts, sensations, emotions, movements and skills. Deposition of Aβ protein plaques outside neurons, contributes to the cells death by interfering with neuron to neuron communication at synapses. Also, deposition of an abnormal protein of tau tangles inside neurons blocks the transport of nutrients and other essential molecules inside neurons in the brain. The presence of toxic amyloid beta and tau proteins activates immune system cells in the brain called microglia. However, microglia, try to clear the toxic proteins from dead and dying cells. An increase and spread of abnormal and toxic aggregation of plaques and tangles of proteins in the brain causes neuronal cell damage and death that leads to brain tissue inflammation and shrinkage. This is due to microglia could not keep up with all the toxic protein aggregation needs to be cleared. Normal brain function is further compromised by the decreased ability of the brain to metabolize glucose, its main fuel. As a consequence, the brain of AD patients shrinks compared to a healthy brain due to the loss and damage of neuronal cells as shown in Figure 1[21].

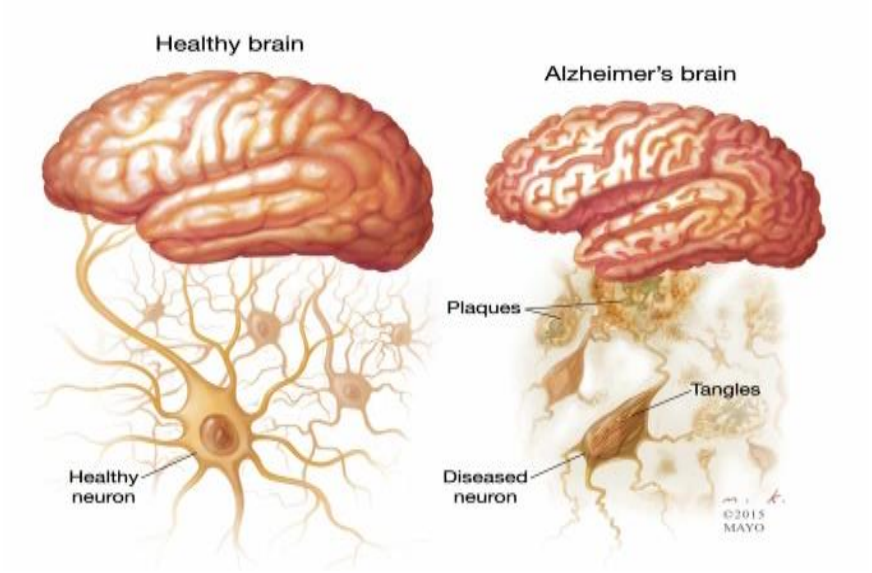


Figure 1: The healthy brain (left) and AD brain (right) (www.newsnetwork.mayoclinic.org).

As neuronal damage increases, the brain can no longer compensate for the changes and then patients display cognitive decline such as memory loss or confusion as to time or place and day to day activity. Treatments to prevent, slow and stop those changes are not yet available, even though many clinical trials are being done[21].

1.2. Bio-metal Homeostasis Hypothesis

Bio-metals are important for the healthy body functioning and have a central role in many bio-chemical pathways, enzymatic activities, mitochondrial function, learning, memory and neurotransmission[22]. Mitochondrial functions are the source of energy for brain cells in which defects result in deficiency in energy production and increases generation of superoxide and hydroxyl radicals. The interaction of superoxide radicals with nitric oxide produces peroxynitrite, which irreversibly inhibits mitochondrial respiration, reacts with proteins, lipids, carbohydrates and deoxyribonucleic acid (DNA), and causes DNA fragmentation and lipid oxidation and, ultimately, radical induced oxidative damage[23]. Imbalanced bio-metals in human brain may have multiple origins such as environmental pollution, radiation, genetic pattern, drinking water and foods (meat and vegetables), dietary supplements and drugs that we take for certain treatments[24,25], age[20] and sleep disorder[26].

Naturally, cells have controlling mechanisms of bio-metal ions homeostasis. However, disproportion or disturbance of those mechanisms of bio-metal ions in their homeostasis result in the accumulation of harmful metals, which lead to neuron-degeneration toxicity, loss, damage and death of cells. From bio-metals, redox transition metals specially: iron, copper and other trace metals are involved in the pathology related neurodegenerative diseases in which their levels in the AD patient brain are found to be high. Notably Cu, Fe, and/or Ca may be linked directly or indirectly to disturbed homeostasis of ions in neurodegenerative diseases, such that new cation chelators can be used as a consecutive therapeutic option[27].

The breakdown in the metals homeostatic mechanisms are closely involved in abnormal processing of amyloid precursor protein (APP) leading to deposition of beta amyloid peptides in the form of senile plaques, along with hyperphosphorylation of tau protein, responsible for the formation of neurofibrillary tangles. Cu^{2+} and Fe^{2+} are able to bind abnormally protein components with AD patient's brain of different affinities[28]. They promote the processing of APP by secretases, senile plaque formation and hyperphosphorylation of tau protein. Thus, abnormal accumulation and distribution of different metals that may stimulate oxidative stress associated with AD[28].

Based on those assumptions, it has been hypothesized that the dysregulation of a specific metal ion leads to the characteristic bio-metal profile. The acceptance and donation of electrons of the bio-metals can also contribute in the reactive radical, nitrogen and oxygen species

formation. Therefore, it might be detected in various accumulation or abnormal interactions with proteins, lipids or nucleic acids oxidative attack of tissue components[20]. Moreover, if such shared bio-metal profiles exist across different neurological disorders, it is possible to correlate with the behavioral impairments from the result of imbalanced metal ion homeostasis[28]. Furthermore, considering altered concentration of calcium in the brain is also related to the development of AD[22]. Gathering all factors together that have a potential to cause AD, Figure 2 shows the brief illustration of how AD disease develops in the brain[29].

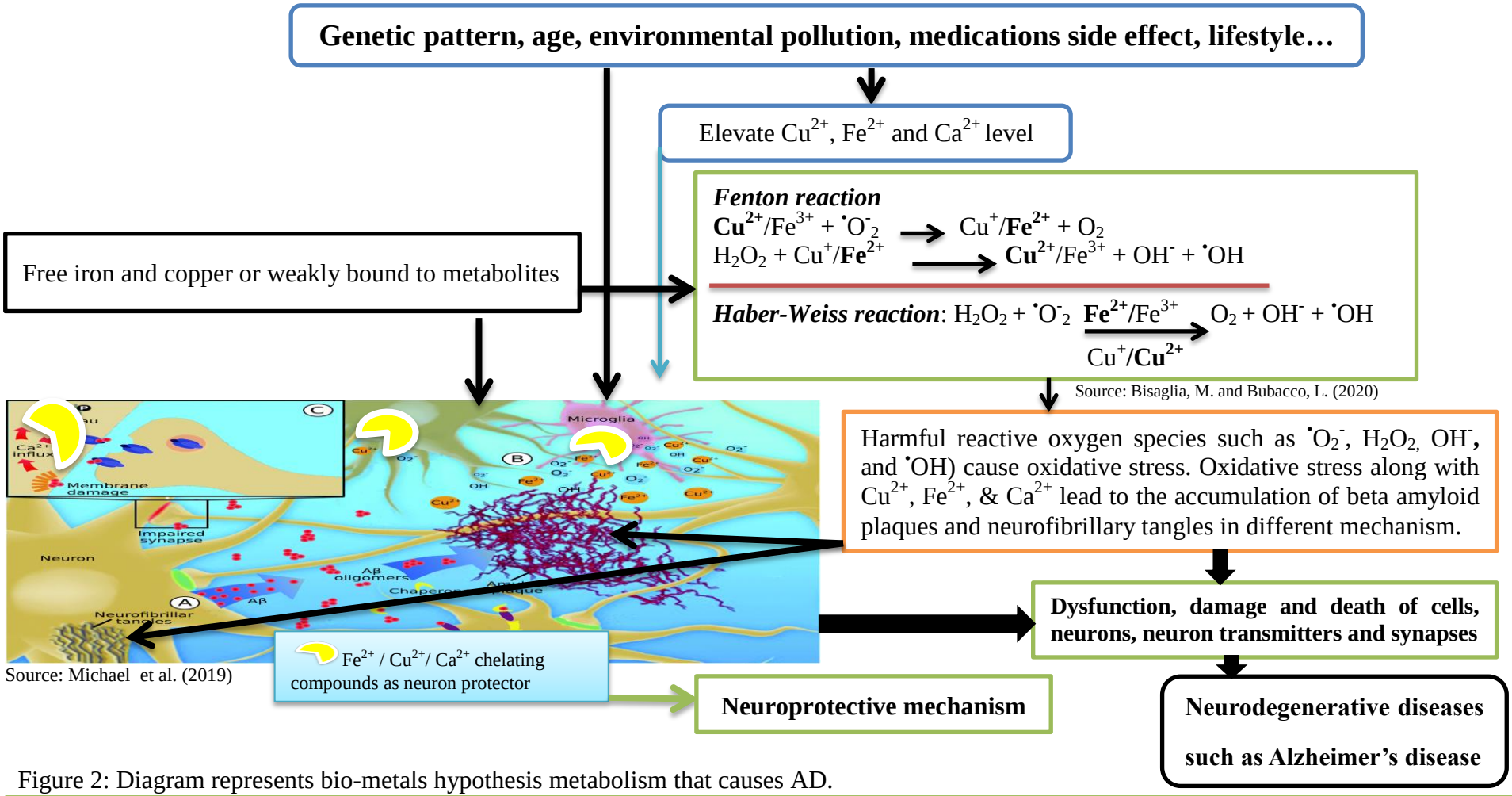


Figure 2: Diagram represents bio-metals hypothesis metabolism that causes AD.

Harmful free radicals interact with amyloid beta proteins that increase reactive oxygen species production, auto oxidation of amyloid beta peptide and plaque formation outside neurons (B), promote phosphorylation of tau that contributes to synaptic pathology and leads to abnormal accumulation of neurofibrillary tangles inside the neuron (A), influx of calcium that damages the membrane (C). Those misfolded beta amyloid protein forms aggregation into oligomers then larger structures of beta amyloid plaques and neurofibrillary tangles, which lead to dysfunction, damage, loss and death of neuron. Those proteins aggregation have an adverse influence in the neuronal processes, such as communication between the nerve cells. Then once after healthy neurons stop functioning, loss and death of connections with other neurons causes AD. Copper and iron, due to their multiple valence states both can react with the molecular oxygen and nitrogen that produce reactive oxygen and nitrogen species. The Fenton and Haber-Weiss reaction have significant role in reactive oxygen species production and AD pathogenesis. Both iron and copper increase oxidative stress by participating in Fenton and Haber–Weiss reactions, which can lead to the formation of harmful reactive species such as super oxide radical, hydrogen peroxide, hydroxyl radical and hydroxyl ion.

1.2.1. Homeostasis of copper (Cu^{2+}) in AD

Copper is a redox-active metal. It is necessary for brain functioning, mitochondrial activity, neurotransmitter biosynthesis, oxidative stress defense and other critical processes for healthy brain[27]. Redox-active copper is well known to be correlated with oxidative stress through formation of reactive oxygen and nitrogen species[27] by means of the Fenton and Haber-Weiss reaction[13] (equation 1). Its toxicity is mainly related with its activity as a catalyst for oxygen free-radical generation producing superoxide, hydrogen peroxide, and highly reactive hydroxyl radical[30].

Fenton reaction

$Cu^{2+}/Fe^{3+} + \bullet O^-_2 \longrightarrow Cu^{+}/Fe^{2+} + O_2$

$H_2O_2 + Cu^{+}/Fe^{2+} \longrightarrow Cu^{2+}/Fe^{3+} + OH^- + \bullet OH$

Haber-Weiss reaction: $H_2O_2 + \bullet O^-_2 \xrightarrow[Cu^{+}/Cu^{2+}]{Fe^{2+}/Fe^{3+}} O_2 + OH^- + {}^{\bullet}OH$ ……..……equation (1)

Reactive oxygen and nitrogen species are originated from the reaction between molecular oxygen and unregulated redox active cations such as copper and iron as well. Free radicals are any chemical species that contain one or more unpaired electrons. Due to their free state, via accepting electrons from other molecules, can easily become a reason to change the structure of molecules leading to change in their normal function in the cell. Normally, not all free radicals are harmful, some of them are useful in the human body by protecting from disease-causing microbes being produced by phagocytes which is a specialized blood cells[24].

Cells have oxidative defense systems as antioxidants and antioxidant enzymes. They maintain and repair the change or harmful effects of the free radicals to keep the equilibrium and flow of molecules in the organism. The equilibrium is maintained between activity of antioxidants to keep down the levels of free radicals, letting them to neutralize useful biological functions without too much damage[31]. When the source of oxidants get high due to malfunctioning of free radical production, the body cells of antioxidant defense may get weak and unable to process biologically, resulting in oxidative and nitrosative stress[32,33]. Both stresses occur due to overproduction of reactive oxygen and nitrogen species in biological systems, or alternatively when the defensive species has shortage of enzymatic and non-enzymatic antioxidants.

Oxidative and nitrosative stress can potentially damage important part of cell proteins, carbohydrates, cellular lipids, deoxyribonucleic acids that can affect the normal biological system[24,33]. Since, human brain due to its high metabolic requirements is known to be an aerobically active organ that constitutes 2% of total body mass and consumes about 20% of total oxygen when comparing to other body part. Oxidative balance directs and controls the brain and this is usually governed by antioxidants that exist in massively higher quantities than in any other organ. Therefore modifications in normal oxidative metabolism as observed in AD brain, provides strong evidence that oxidative stress plays an important role. In AD pathogenesis, redox ion-oxygen radicals are involved in chemical attack, resulting in increased free carbonyls, lipid peroxidation adducts, protein nitration, mitochondrial and nuclear deoxyribonucleic acids oxidation adducts[34].

Copper plays an important part in the formation of extremely toxic beta amyloid protein in senile plaques by facilitating reactive oxygen and nitrogen species causing ultimately neuron inflammation in AD brain. Particularly, Cu concentration is raised in amyloid beta protein, thereby developing amyloid beta protein oligomerization and neurotoxicity. The alteration of copper levels is recognized as an important role in accumulation of tau in the neurons. Cu ions are seen in the redox reactions, existing in the neuron fibular tangles.

Copper is also essential for brain enzymes to control neurotransmitters, for example dopamine, neuropeptides and dietary amines. Disruption of copper oxidation in the brain has been related to several neurodegenerative diseases, including AD. Copper linked proteins are present in the cytosol, nucleus, peroxisome[27], cytochrome, ceruloplasmin and mitochondrial intermembrane space of human cells functioning as enzymatic antioxidant detoxifying superoxide in the brain. Therefore, regulation of copper metal ion homeostasis play an important role in neural activity[27,34].

1.2.2. Homeostasis of iron (Fe^{2+}) in AD

The divalent iron is dominant metal ion in the brain, that is also found in beta amyloid plaques[27,34,35]. Due to its redox activity, as a result of the Fenton and Haber-Weiss reactions, the increase of iron cation in the brain may be harmful and is responsible in increasing the oxidative stress generating tissue and neuronal damage. Usually iron accumulates when the brain gets aged, that is why it has been well explained as the reason for neurological disorders. Iron provokes amyloid-induced neuronal damage through formation of amyloid beta protein

plaque[27,34] and hyperphosphorylated tau protein to induce its aggregation leading to the formation of neurofibrillary tangles in AD brain[36]. As a result a symptom such as cognitive dysfunction in AD patients recognized. Therefore, chelation therapy has been proposed in a situation of iron overload because it can provide constant chelation coverage and is able to recover passivity complexes of the metals. Chelators can eliminate the excess or harmful metal from the system rendering it proximately harmless and decreasing the late effects[37].

1.2.3. Homeostasis of calcium (Ca^{2+}) in AD

Calcium is one of the most important cations in brain function, being involved in neurotransmission, signal transduction, synaptic plasticity, energy production and regulation of proteins network that contributes to synaptic activity[38]. This involves Ca^{2+} signaling pathways in extensive neuronal processing[39]. It has been observed in AD patients that beta amyloid plaques can disrupt calcium homeostasis. The impaired ability of neurons to maintain an adequate energy level may impact Ca^{2+} signaling[38]. The fluctuation of Ca^{2+} signaling may result in the learning and memory deficits that occur early during the mild AD[39]. Therefore, developing calcium chelating compounds can be a better solution to the progressive decline in memory and decreased number of neurons in AD[14,39].

1.3. Risk factors for AD

There are numerous risk factors of AD, namely genetic pattern, brain injury and ageing, environmental pollution, medications, and lifestyle such as dieting, exercising, sleeping habit and others. Those risks for developing AD may differ from person to person.

***Genetic pattern*:** Having one form of the apolipoprotein E (APOE) gene, which exists in three isoforms (such as APOE2, APOE3, and APOE4), namely APOE4 does increase a person's risk[40].

***Ageing*:** Cognitive and memory performance with aging are also considerably related to AD[29].

***Brain injury*:** Brain injury is related to reaction of reactive oxygen species with neuronal macromolecules mainly from mitochondrial origin which are vulnerable to mitochondrial damage by free radicals that may lead to AD[11].

Physical inactivity: Not enough physical exercise is indeed related to enhance oxidative stress, since oxidative damage is thought to be an early event in AD.

Exercise induces functional neuronprotection by increasing, for example, the brain-derived neurotrophic factor[41,42].

***Environmental pollution*:** Air toxins (e.g. ozone, carbon monoxide, particulate matter, lead, sulfur dioxide, and nitrogen oxides) can be a risk factor for AD. Those pollutants speed up the accumulation of amyloid beta peptide plaques and neurofibrillary tangles that impair cognitive function which are highly associated with AD[11,43].

***Lack of enough sleep*:** sleep disorder increases the risk of cognitive impairment[26].

***Medications and supplements side effects*:** The consumption of high trans fats combined with redox metal and mineral supplements can boost cognitive decline[25]. However, there are recommended supplements that prevent AD risk such as omega-3 polyunsaturated fatty acids (PUFAs) and docosahexaenoic acid (DHA), which have valuable effects on brain function and delayed cognitive decline[18].

Tobaccos and alcohols: Smoking tobacco and excess consumption of alcohol may substance abuse be risk factors of AD[29].

Poor diet habit: Diet is essential in biochemical reactions that may harm or benefit brain functioning, including nerve cells. A diet rich in antioxidants such as: vitamin E and vitamin C, vegetables, fruit juices, and other foods that are rich in folic acid, vitamins, omega-3 PUFAs and polyphenols may be helpful in AD prevention[18].

***Lack of sociable, enjoyable activities and awareness about AD*:** AD patients quarantine themselves from social involvement. Community participation and activities enhance their cognitive abilities. Also, systematic cognitive stimulation activities such as puzzles, word games, indoor gardening, discussions of the past present reminiscence therapies are advantageous to upkeep cognitive ability, mood and behavior. Those practices can help to decrease neuropsychiatric symptoms and increase the quality of life of AD patients[44]. Lack of awareness about the disease may be a ground to increase the risk factors of AD[29].

1.4. Metal overload chelators and Alzheimer's disease therapy

1.4.1. Metal overload chelators

Different metal chelating agents have been reported as support treatment against to treat cases of metal toxicity for several diseases. Among cation chelators triethylenetetramine (TETA), nitrilotriacetic acid (NTA) and D-penicillamine (DPA) were used as copper chelators.

Deferoxamine (DFO), deferiprone (L1) and nitrilotriacetic acid (NTA) were mentioned as chelators of iron overload[37,45]. Nitrilotriacetic acid (NTA) was considered to chelate calcium overload[37]. Additionally, ethylene glycol-bis (2-aminoethylether)-N, N, N′, N′-tetraacetic Acid (EGTA) was used in the experimental protocols to chelate calcium[46]. Whereas, ethylene diamine tetraacetic acid (EDTA) and meso-2,3-dimercaptosuccinic acid (DMSA) were used as a heavy metal chelator[37]. Table 1 shows cation chelators, their activities and adverse effects.

Table 1: Currently used cation chelators adapted from Lawson et al. (2017) [45].

Drug names	activities	side effects
DFO	iron chelator	ophthalmic and auditory toxicity gastrointestinal disturbances affects lung, kidney and nervous system causes hair loss, dryness
DMSA	heavy metal chelator lipid peroxidation inhibitor	extracellular distribution gastrointestinal discomfort allergy to skin mild neutropenia
Deferiprone	iron chelator	gastrointestinal symptoms headache
TETA	copper chelator	increase urinary excretion
EDTA	metal ions chelator	nephrotoxicity, hypocalcaemia allergy, thrombophlebitis congestive heart failure intestinal toxicity, tuberculosis
DPA	copper chelator	disrupts immune system anorexia, nausea and vomiting cause neurological problems
NTA	calcium chelator copper chelator iron chelator	damage to vacuolated cells of proximal tubules blood histology change, auditory toxicity cause allergy to skin affects kidneys and nervous system

1.4.2. Chemo-therapeutic agents over AD

There are different approaches that have been used to treat AD; from those approaches chemotherapy and non-chemotherapy are the main ones.

Recently, AD chemo-therapeutic agents used to improve mental functions as cholinesterase inhibitors such as tacrine (Cognex®), donepezil (Aricept®), rivastigmine (Exelon®) and galantamine (Razadyne®) and N-methyl-D-aspartate receptors inhibitor of memantine (Namenda®) also combination of (memantine+donepezil) were approved by the U.S. FDA. Due to hepatotoxicity of tacrine, was excluded from market since 2012[5,44,]. Those chemo-therapeutic agents are sourced from alkaloids, piperidine derivatives, carbamates, pyridine derivatives various chemical classes, organophosphorus and polyphenols compounds[47]. A summary of chemo-therapeutic treatments for AD as cholinesterase inhibitors, are presented in Table 2 that describes indications of the stages of the disease and side effects for each medication. Mentioned chemo-therapeutic treatments are used to regulate neurotransmitters[48], which are chemicals that transmit messages between neurons to neurons. Those help to delay the progression of symptoms of neurocognitive, physical decline and with certain behavioral problems. However, those medications do not change the underlying disease process, do not prevent and cure, are not effective for all people and their effect terminates after limited duration[5,44]. All anti-AD drugs share similar adverse effects such as: dizziness, headache, nausea, vomiting, loss of weight and appetite[44].

Table 2: Summarizes of chemo-therapeutic treatments for AD approved by FDA[44]

Drug names	**indications**	**side effects**
Donepezil	mild to moderate	atrioventricular block
	moderate to severe	hypertension, syncope
Rivastigmine	mild to severe	abdominal pain
Galantamine	mild to moderate	atrioventricular block
Memantine	moderate to severe	confusion, kidney injury
Memantine and donepezil	moderate to severe	heart block

1.4.3. Looking for new treatments for AD

International AD researchers have failed to develop a way to cure or prevent the disease. Under study are drug therapies, targeting formation of amyloid beta and tau proteins, loss of synapses, and specific neurotransmitters, and non-chemotrophic approach such as physical activity, social involvements, dieting, cognitive trainings, in a combined plan. For the treatment of AD disease, current pharmacotherapy has troubling side effects, therefore, effective and non-toxic new agents are desperately needed[49]. Therapeutics that prevent biological damage from intra and extra-cellular metals, must be from safe natural origins, effective, forming stable-complexes with the metal, have gained attention from most drug designers[37,45].

1.4.3.1. *Promising natural sources to treat AD*

Ever since ancient time medicinal plants play a major role in the controlling of several neurological disorders. They are plants providing a huge and energetic source of bioactive compounds, some of which can be effective against Alzheimer's disease[48]. For example, water extract from the seeds of *Celastrus paniculatus* have been used for the stimulation of the cognitive function and improving of learning and memory also considered as antioxidant[50]. Sehgal et al. (2012) reported that chloroform-methanol extract from roots of *Withania somnifera* reversed behavioral failures and amyloid beta plaque pathology being the major constituents of alkaloids and steroidal lactones as therapeutic compounds[51]. Study shows that ethanol extracts of seed and root from *P. harmala* and *A. citriodora* have exhibited iron chelating activity (63% and 50%) respectively. Those plants were used as traditional oxidative stress reducing medicines for symptom improvements in AD[35].

Table 3 and Table 4 show the most safe herbs that are used to treat inflammation and forgetfulness, have been believed as a way of AD natural therapy and related to improve their quality of life[5].

Table 3: Safe herbs that are used to treat inflammation, forgetfulness and related diseases[5]

Name of herbs	part of herbs	extracts	neuroprotective agents	traditional usages
Cistanche tubulosa	whole	water	echinacoside, acteoside	treat forgetfulness
Polygala tenuifolia	aerial	water ethanol	tenuigenin	enhance memory antipsychotic agent
Azadirachta indica	leave	water	unknown	anti-inflammatory anti-stress
Coriandrum sativum L.	seed	water	unknown	antioxidant anti-inflammatory
Phyllanthus acidus	fruit	methanol	unknown	anti-fever anti-inflammatory anti-diabetic
Terminalia chebula	fruit	methanol	tannic acid chebulagic acid	enhance memory

Table 4: List of drugs that possess chelating properties adapted from Cuajungco et al. (2006)[52]

Drugs	chelation activities	usages
Aspirin, indomethacin	copper and iron	anti-inflammatory
Ibuprofen, d-penicillamine	copper and iron	anti-inflammatory
Bleomycin, ethambutol, thalidomide	copper and iron	anti-sedative
Lipoic acid	copper and iron	antioxidant
Valproate sodium, phenytoin	copper	anticonvulsant

1.4.3.2. *Bioactive compounds from natural sources*

Polyphenols

More than 8,000 compounds of natural polyphenols have been identified as compounds to prevent AD from natural beverages. Polyphenols reveals their antioxidant effect by removing free radical species. Thus, the antioxidant properties contribute to neuroprotective effect[29].

Flavonoids

Flavonoids are naturally occurring polyphenolic compounds from plants such as: cocoa, fruits, vegetables, cereals, tea, and wine. Flavonoids have been extensively used as alternative compounds protecting against AD by reducing aggregation of neurotoxic amyloid beta peptides into oligomeric[53]. Flavonoids can be classified in to six chemical families such as flavonols found in onions, leeks, and broccoli; isoflavones mainly present in soy and soy products; flavones present in parsley and celery; flavanols abundant in green tea, red wine, and chocolate; flavanones primarily found in citrus fruit and tomato; and anthocyanidins. Plants that are rich in flavonoids enhance memory, cognition and spatial learning[53].

Aldehydes and alcohols

Aldehydes (e.g.octadecenyl[13] and 4-hydroxybenzyl[54]) and alcohols (e.g.dihydroactinidiolide[13] and 4-hydroxybenzyl[54]) found in some higher plants having positive effects in human health[13]. Both protect the cell from oxidative stress, excitotoxicity, neuronal death, are scavengers of hydroxyl radical and inhibitors of lipid peroxidation[13,54].

1.5. Marine environment as promising source of new drug leads

The marine environment encompasses a considerable variety of organisms such as bacteria, micro- and macro-algae, vertebrate and invertebrate animals which are a source of new bioactive molecules and are still under-exploited. Marine microorganisms produce bioactive secondary metabolites whose unique structure is not found in terrestrial organisms[55]. These metabolites therefore potentially represent new molecules of interest and include isoprenoids, polyketides, peptides, and macromolecules such as nucleic acids, carbohydrates, proteins, and lipids. The level of chemical diversity associated with the huge number of environment inhabited by algae and cyanobacteria, exposed to biological and chemical diversity[56].

Algae are unicellular, microscopic and phylogenetic diverse group of photosynthetic microorganisms including species from different phyla such as Cyanophyta (bluegreen algae, cyanoprokaryotes, and cyanobacteria), Chlorophyta (green algae), Rhodophyta (red algae), Cryptophyta, Haptophyta, Pyrrophyta, Streptophyta and Heterokontophyta. Microalgae are one of the most dominant groups of photosynthetic living organisms. Not only living in aquatic medium but also on terrestrial region. Represent an important group of microscopic organisms that have the ability to perform photosynthesis, and producing about half of the atmospheric oxygen. They vary greatly in their metabolic capabilities and environmental adaptations (light, salinity, and temperature)[56,57,58]. Microalgae do not require arable lands for growth; can be cultivated in liquid media with high growth rate and productivity. They produce a biomass enriched in lipids (60 to 65%), carbohydrates, proteins and fibers (33 to 50%)[59]. Their revelations of compounds with new and unique structures make them attractive in terms of biological functions for different food, animal feed and aquaculture. They produced by industries in various pharmaceutical formulations such as cosmetics, textiles, biofuels and biofertilizers[53,56]. Table 5 illustrates isolated bioactive compounds from microalgae, and their promising effects in human health and applications.

Table 5: Bioactive compounds isolated from microalgae

Microalgae	bioactive compounds	health promising effects	applications	ref.
Haematococcus pluvialis	astaxanthin, lutein, zeaxanthin canthaxanthin, β-carotene, oleic acid	antioxidant	food pharmaceuticals	[56,60]
***Spirulina* sp.**	phenols, vitamin E docosahexaenoic acid, linolenic acid	antioxidant anti-inflammatory	food cosmetics	[56,59,60]
***Chlorella* sp.**	carotenoids, sulfated polysaccharides sterols, fatty acids	antioxidant	food pharmaceuticals	[56,60]
***Porphyridium* sp.**	polysaccharides sulphated polysaccharides phycobiliproteins	anti-inflammatory antioxidant neuroprotective	food cosmetics pharmaceuticals	[56,60]
***Nannochloropsis* sp.**	eicosapentaenoic acid	antioxidant brain development for children	pharmaceuticals nutrition	[56,61]
Phaeodactylum tricornutum	lipids, fatty acids, phytol	neuroprotective	pharmaceuticals	[56,59,60]
***Isochrysis* sp.**	lipids, fatty acids, carotenoids	antioxidant brain development for children cation chelator	pharmaceuticals feed nutrition	[56,60,61]
***Tetraselmis* sp.**	hexadecatrienoic acid	antioxidant copper and iron chelator	pharmaceuticals	[61]
S. costatum	long chain-fatty acids, alcohols	antibacterial	pharmaceuticals	[62]

1.6. Search for new neuroprotective compounds from microalgae

It was already mentioned in section 1.2.1 and section 1.2.2 that, redox metals act as catalysts for reactive oxygen and nitrogen species production. Due to their loosely bound of electrons of their outer layer and capacity to exist in more than one valence, can contribute to the pathogenesis of AD. Several reports have established that free radical scavenging mechanisms via metal chelation in the brain are therapeutic targets for the management of AD[63]. Extracts of microalgae like *H. pluvialis, Nannochloropsis* sp., *Neochloris oleoabundans, T. chui, P. tricornutum, Chlorella minutissima* and *Stigmatophora, Rhodomonas salina* and *Chaetoceros calcitrans* exhibited ferric iron, copper, lipid peroxidation, and neuronal damage reducing antioxidant activity. Antioxidant activities of marine algae have been determined by various methods such as 1, 1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging, 2, 2′-azinobis-3-ethylbenzo thizoline-6-sulphonate (ABTS) radical scavenging, singlet oxygen reducing activity, lipid peroxide inhibition, superoxide and hydroxyl radical scavenging assays. Having PUFAs, flavonoids, sterols, carotenoids and phenolics as potent metal chelators, scavengers of free radicals, inhibitors of Fe^{2+}- induced lipid peroxidation and protein oxidation[53]. These chelators are useful for treating AD by protecting the cells from oxidative and nitrosative damage[63]. Compounds feature such as: branches, molecular weight, conjugational exposure of the rings and π bonds in the metal chelators make them more capable in capturing and inhibiting reactive oxygen and nitrogen species formation. For example, it has been stated that the number of hydroxyl groups on the ring structure is associated with the effects of reactive oxygen and nitrogen species dominance[37]. Thus, indicates ways of developing new compounds from marine algae of various organisms that prove to be useful candidates for protecting the brain cell against any oxidative and nitrosative stresses degradation. It may lead to the development of effective neuroprotective agents in order to slow down the progression of neurodegenerative diseases such as Alzheimer's[53,63].

Omega-3 polyunsaturated fatty acids (ω-3 PUFAs)

Omega-3 PUFAs such as α-linolenic acid, eicosapentaenoic acid, docosahexaenoic acid (DHA) have been publicized to be effective in preventing Alzheimer's disease and inflammation. Those are essential for growth of the retina and brain, development and functioning of the nervous system[64].

Sterols

Sterols are organic compounds known as steroid alcohols (have hydroxyl group) and subgroup of the steroids that contain four fused cycloalkane rings[13]. Sterols and phytosterols have antioxidant and hypocholesterolemic properties[64]. Sterols provide protection against oxygen free radicals and amyloid beta linked neurotoxicity[13], anti-inflammatory effects[64] and regulate membrane fluidity of the cells[59]. Microalgae, *Skeletonema* sp. have sterols in which cholesterol being the major form[56] and campesterol, stigmasterol, ergosterol and fucosterol as minor[13] that are capable to reduce the risk of heart and coronary diseases[56].

Long-chain halogenated compounds

Volatile long-chain halogenated compounds have shown biological activities such as anti-inflammatory and neuron protection[59]. Exploring compounds from microalgae with metal chelating activity and other bioactive capability needs deep attention[53].

2. OBJECTIVES

The main objective of this study was to search for new compounds with neuroprotective capacity in natural extracts from marine microalgae. To achieve this, several specific objectives were underlined:

- To select amongst different extracts prepared from several microalgae species (*Haematococcus pluvialis*, *Isochrysis* sp., *Nannochloropsis* sp., *Phaeodactylum tricornutum*, *Porphyridium* sp., *Skeletonema costatum*, *Spirulina* sp., *Tetraselmis chuii* and *Tetraselmis* sp. CTP4) the one's with highest capacity to chelate metals such as iron, copper and calcium relevant for neurodegenerative diseases;
- To obtain an active fraction with a more reduced number of compounds via bio-guided fractionation of the selected extract;
- To chemically characterize and tentatively identify bioactive compounds in the fractions as potential drug leads with neuroprotective ability.

3. MATERIALS AND METHODS

3.1. Chemicals and reagents

All solvents and chemicals used for extraction, preparation, fractionation, derivatization and metal chelating activity assays were of analytical grade. These included: ethanol (EtOH), n-hexane, ethyl acetate (EA), dichloromethane (DCM), methanol (MetOH), acetyl chloride and dimethyl sulfoxide (DMSO) were purchased from VWR International (Leuven, Belgium). Ultrapure, Type 1 water was obtained using a Milli-Q® Water Purification System and sodium sulphate anhydrous (Na_2SO_4) was from (Darmstadt, Germany). For characterization by GC-MS, n-hexane GC grade was used (VWR International, Leuven, Belgium).

For copper chelating activity assay, sodium acetate ($C_2H_3NaO_2$), ethylenedinitrilotetraacetic acid disodium salt dehydrate ($C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$) (EDTA), pyrocatechol violet ($C_{19}H_{14}O_7S$) and acetic acid (CH_3CO_2H) were from Sigma (Steinheim, Germany) while copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) was obtained from VWR International (Leuven, Belgium).

For iron chelating activity assay, iron (II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$) was from VWR International (Leuven, Belgium) and ferrozine ($C_{20}H_{12}N_4Na_2O_6S_2$) was obtained from Acros (Geel, Belgium). For calcium chelating activity assay, ammonium chloride (NH_4Cl), ammonia (NH_3) and calcium carbonate ($CaCO_3$) were purchased from Merck (Darmstadt, Germany). Ethylene glycol-bis (2-aminoethylether)-N, N, N′, N′-tetraacetic acid ($C_{14}H_{24}N_2O_{10}$) (EGTA) and o-cresolphthalein complexone ($C_{32}H_{32}N_2O_{12}$) were from Sigma (Steinheim, Germany) and hydrochloric acid (HCl) was from Fischer Scientific (Loughborough, UK).

3.2. Experimental design

For the evaluation of the neuroprotective capacity, nine species were selected: *Haematococcus pluvialis* (Chlorophyta); *Isochrysis* sp. (Haptophyta); *Nannochloropsis* sp. (Eustigmatophyta); *Phaeodactylum tricornutum* (Bacillariophyta); *Porphyridium* sp. (Rhodophyta); *Skeletonema costatum* (Bacillariophyta); *Spirulina* sp. (Cyanobacteria); *Tetraselmis chui* (Chlorophyta) and *Tetraselmis* sp. CTP4 (Chlorophyta). Those species are industrially cultivated meaning that abundant amounts of biomass can be made available from the producers upon request. This can be a main advantage when high amounts of pure active compounds are needed for structure characterization, for example, as secondary metabolites are usually produced at very low

concentrations. Species of microalgae and extraction solvent have great effects on the yield and type of extracted bioactive compounds. We selected water, ethanol and ethyl acetate as extracting-solvents.

Water was selected because of its high polarity (PI = 10.2)[65] availability, being non-toxic, non-flammable, environmentally friendly and generally a greenest solvent than any other[66]. It has the potential to extract polar compounds such as peptides, carbohydrates and others from microalgae[67]. Ethanol was selected as the extracting-solvent for its low toxicity, being also environmental friendly. It is less polar (PI = 5.50)[65] than water and is expected to provide high yields of polar compounds and some non-polar bioactive compounds such as polyphenols, vitamins and some carotenoids with highest metal chelating activities. Ethyl acetate (EA) is widely used in food industry as artificial aroma or flavoring for making cakes and ice creams and is a source of fragrance in perfumes industries[68]. EA is also classified as environmentally friendly, renewable and less-toxic. It is less polar (PI = 4.40)[69] than water and ethanol. Therefore, extracts of EA are expected to extract less polar compounds from algal biomass. After extraction, their residue can be used to formulate a source of active ingredients with potential applications in feed, food or dietary supplements, fertilizer, bio-colorant for food drinks[70], textile and paper industries[71], and used for biofuel generation[72] due to safety for environment[73]. Dimethyl sulfoxide (DMSO) was selected as a solvent to dissolve extracts (both polar and non-polar compounds) due to its high solubility (103.7 mg/mL in water) at room temperature, less-toxic and it does not interfere in most biological assays when used at low concentration[74,75].

Extracts were firstly tested at a concentration of 10 mg/mL as preliminary screening to identify the most active extracts in terms of metal (Cu^{2+}, Fe^{2+} and Ca^{2+}) chelating activity.

Values of metal chelating activity, MCA > 60% were considered worthy enough for further analysis to determine the mean chelation concentration IC_{50}. Logically, if the extract's activity at 10 mg/mL is less than 50% it could not be expected to keep active when the concentration decreased, rather the activity decreased or remained the same, however, the activity could not be enhanced, otherwise, it had to be active at least more than 50% of total activity at 10 mg/mL. IC_{50} means the concentration of chelator required to chelate 50% of free metal ions[76]. In this study, extracts with less IC_{50} values (IC_{50} < 2 mg/mL) were considered active enough for bio-guided fractionation. Fractions with the lowest IC_{50} are called the highest in activity[76].

To identify the bioactive compounds in the fractions, Gas Chromatography coupled with Mass Spectroscopy (GC-MS) was used.

3.3. Algal biomass (AB)

Freeze dried powdered algal biomass was kindly provided by NECTON S.A.; (Olhão, Portugal), a company established in 1997 that develops its activities in the marine biotechnology field having specialized in the production and commercialization of microalgae. Before conducting all experiments, samples were stored in the dark inside a desiccator at room temperature.

Figure 3 presents micrographs of microalgae species used in this study.

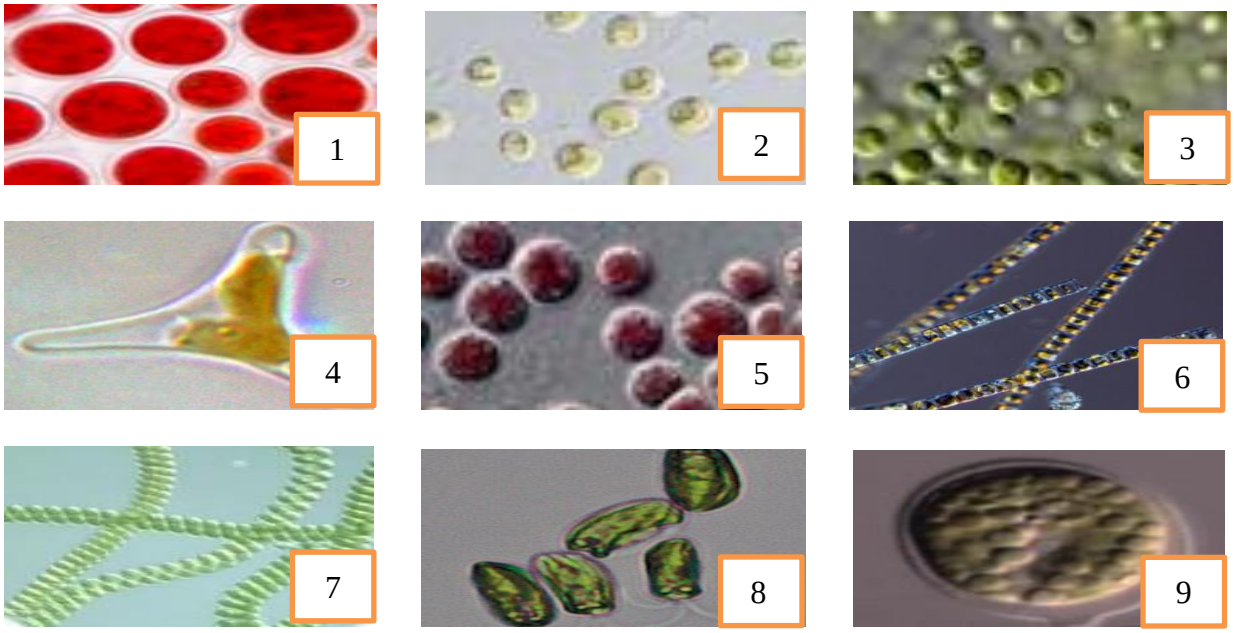


Figure 3: Micrographs of microalgae species used in this study

Haematococcus pluvialis (Hp) (1) (source: Kavitha et al. (2015));

Isochrysis sp. (Isp.) (2) (source: https://algaeresearchsupply.com);

Nannochloropsis sp. (Nsp.) (3) (source: https://algaeresearchsupply.com);

Phaeodactylum tricornutum (Pt) (4) (source: https://mycocosm.jgi.doe.gov);

Porphyridium sp. (Psp.) (5) (source: https://algaeresearchsupply.com);

Skeletonema costatum (Sc) (6) (source: www.eoas.ubc.ca);

Spirulina (Ssp.) (7) (source: https://vnibioscience.en);

Tetraselmis chui (Tc) (8) (source: https://ars.els-cdn.com);

Tetraselmis sp. CTP4 (CTP4) (9) (source: Pereira et al. (2015)).

3.4. Preparation of extracts

One gram of dry powder algal was mixed with 40 ml of solvent (ethanol, ethyl acetate or water) (1:40, w/v) and stirred (200 rpm) at room temperature (RT) under contentious string using a platform shaker (IKA, VMS-A, VWR, Leuven, Belgium) overnight (18 hours). The mixture was centrifuged at 2,500 rpm, for 10 minutes at RT (Thermo Scientific™ ST16R TX-400 Centrifuge, Portugal). The pellet was re-extracted twice more with the same solvent following the same procedure. Supernatants were combined and filtered through Whatman no. 4 filter paper pore size (10-12 µm) followed by a 0.45 µm syringe filter (Prat Dumas, France). Later, filtrates were concentrated using a rotary evaporator (IKA, RV10 digital, Staufen, Germany) at 40°C under reduced pressure (Table 6) and solvents evaporated under a gaseous nitrogen stream flow until complete dryness[77]. Table 7 shows dry yield of extracts from algal biomass. The dried extracts were dissolved in dimethyl sulfoxide (DMSO)[78] at a concentration of 20 mg/mL as a stock solution and stored in amber glass vials[67] in order to protect them from light exposure (Ossila enabling materials science Ltd, Sheffield, UK) and were stored at -20°c[79]. A summarized flow of extract preparation from algal biomass is presented in Figure 4.

Table 6: Properties of organic solvents

Extracting-solvents	**chemical formulas**	**molar mass (g/mol)**	**pressure (mbar)**	**polarity index**	**ref.**
Ethanol	C_2H_6O	46.0	175	5.20	[65,80]
Ethyl acetate	$C_4H_8O_2$	88.1	240	4.40	[69,81]
Water	H_2O	18.0	75	10.2	[65,80]

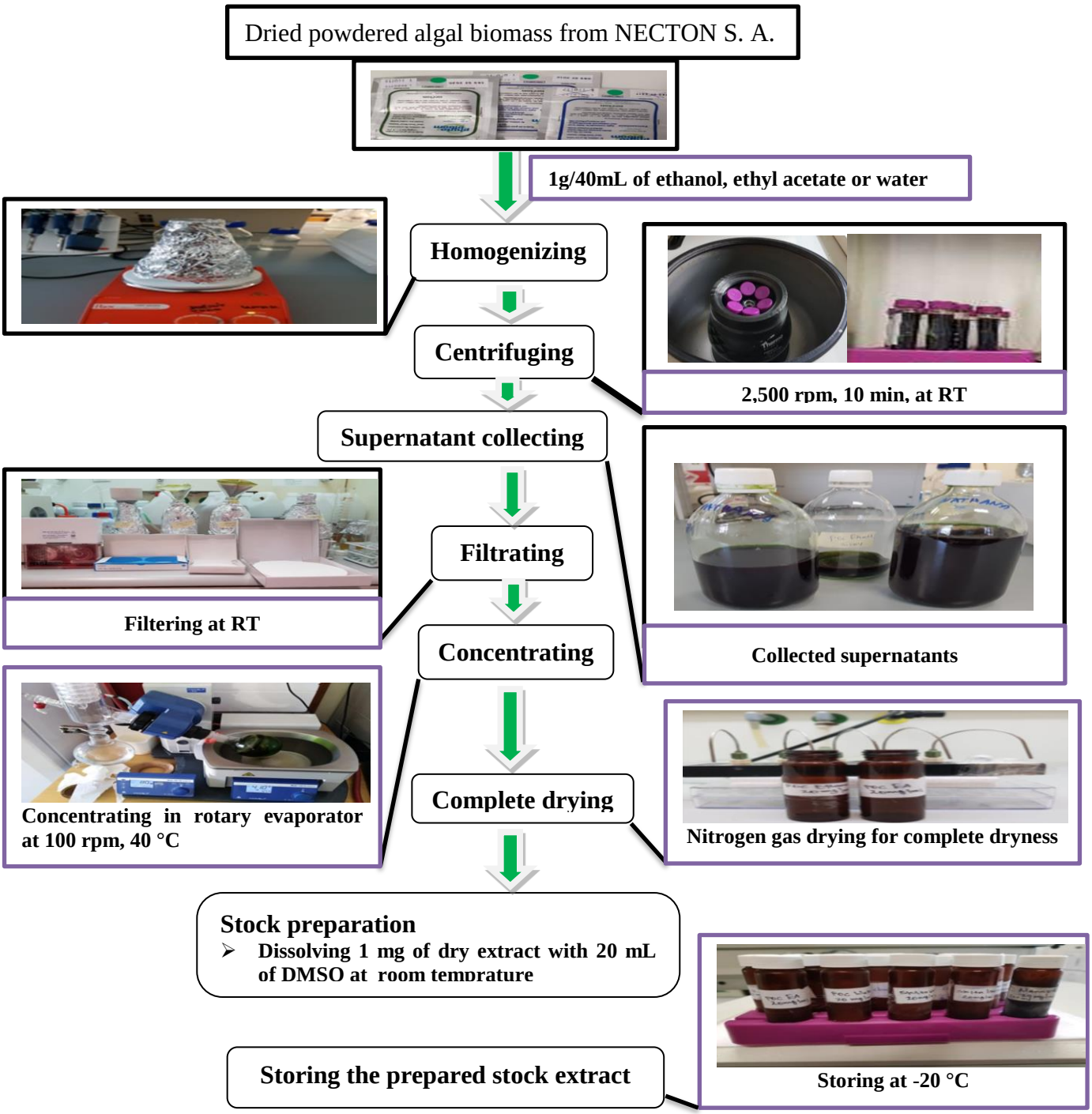


Figure 4: Flow chart of extract preparation from algal biomass

3.4.1. Yield of extracts

The yield was expressed as percentile yield of dried extract (%) with respect to total mass of algal biomass used and multiplied by 100[82,83] (equation 2).

$$Yiel\ of\ dried\ extract\ (\%) = \frac{mass\ of\ dried\ extract}{total\ mass\ of\ algal\ biomass} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots \text{ equation (2)}$$

Table 7: Dry yield extracts from algal biomass

Algal biomass	extracting-solvents	yield of dried extracts (%)
H. pluvialis	ethanol	3.19
	ethyl acetate	3.33
	water	2.23
***Isochrysis* sp.**	ethanol	1.01
	ethyl acetate	1.72
	water	4.57
***Nannochloropsis* sp.**	ethanol	7.32
	ethyl acetate	2.62
	water	11.6
P. tricornutum	ethanol	1.54
	ethyl acetate	1.39
	water	1.51
***Porphyridium* sp.**	ethanol	1.17
	ethyl acetate	1.01
	water	1.49
S. costatum	ethanol	1.09
	ethyl acetate	1.22
	water	5.89
***Spirulina* sp.**	ethanol	11.8
	ethyl acetate	1.71
	water	2.12
T. chui	ethanol	2.01
	ethyl acetate	1.02
	water	11.7
***Tetraselmis* sp. CTP4**	ethanol	3.01
	ethyl acetate	9.8
	water	2.97

3.5. Assays for metal chelating activity

Prior to calculate percentage of metal chelating activity of extracts, the average absorbance of color control was recorded to correct the absorbance of the extract. Since, the extracts are colored and their absorbance could interfere at the same wavelength[84], their absorbance was subtracted from the absorbance of the chromogenic complex formed between cation and chromogenic reagent, the corrected absorbance was calculated by subtracting the absorbance of color interference due to average algal (color/pigment) from absorbance of algae's color, and disturbance of color due to cation-chromogenic reagent complex and presence of chelating agents (equation 3).

$$\text{Corrected absorbance} = Abs._{(of\ extract\ and\ reagents} - Abs._{of\ color\ of\ extract} \quad \text{.....equation (3)}$$

Metal chelating activity of algal extracts was calculated using corrected absorbance with respect to the average Abs. (absorbance) of negative control.

Percentage of metal chelating activity (%) is expressed following the formula[78,83] of equation (4).

$$\text{Metal chelating activity (MCA) } \mathbf{(\%)} = \frac{\mathbf{100} - (\ Abs._{corrected} \times \mathbf{100})}{Abs._{negative\ control}} \quad \text{.....equation (4)}$$

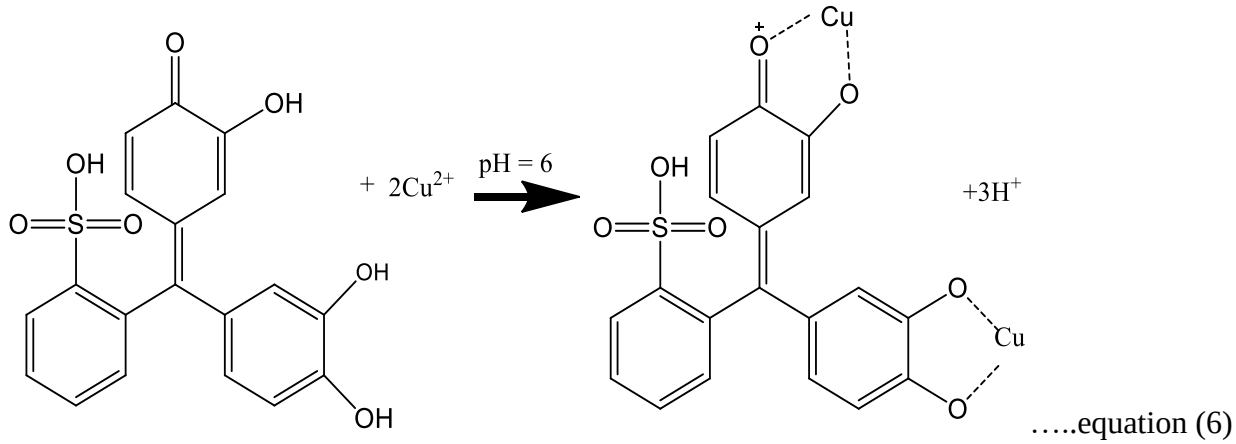
Where, metal chelating activity is expressed in percentage (%), $Abs._{corrected}$ corresponds to the corrected absorbance of each extract obtained having in mind the above (equation 3**),** and $Abs._{negative\ control}$ is the average absorbance of negative control.

It was decided that values obtained using (equation 4) having percentage of metal chelating activity (MCA) greater than 60%, (MCA > 60%) would be selected to determine IC_{50} (mg/mL). The IC_{50} (mg/mL) values were then analyzed using Graph Pad Prism software version 7[85]. A dose-response inhibition curve was obtained by means of non-linear regression, then fitting to a normalized response model with variable slope. The x-intercept of the plot stands for log (IC_{50}), IC_{50} values are calculated from antilog of the x-intercept and y-intercept of the plot stands for metal chelating activity in percentage. Further, the correlation coefficient (R^2) measures the linear association how good the model fits with experimental data. The closer to the $R^2 = 1$ implies greater correlation between the model and experimental data of the IC_{50}[85].

3.5.1. Copper chelating activity (Cu^{2+}CA) assay

The ability of extracts to chelate free Cu^{2+} was determined using pyrocatechol violet (PV) as chromogen according to Ismael et al. (2016)[78] with some modifications. In a flat-bottom 96-well polystyrene microplate (300 µL/well), 164 µL of 50 mM aqueous sodium acetate a slightly acidic (pH = 6.0) buffer solution and (30 µL) of (10 mg/mL in DMSO) of extracts were mixed with 100 µL of $CuSO_4 \cdot 5H_20$ (50 µg/mL, w/v in ultrapure water) and 6 µL of 4 mM PV dissolved in the buffer. In the absence of copper chelating compounds Cu^{2+} reacts with pyrocatechol violet forming a dark-colored complex of Cu-PV with a maximum of absorbance at 632 nm[86]. In the presence of copper chelating compounds Cu-PV complex formation is inhibited, and the solution turns yellowish (Figure 5). Therefore, Cu^{2+} chelating compounds can be estimated by the measurement of color at 632 nm. EDTA (1mg/mL) was used as a positive control; ($\mathbf{C}^{+}$). Cu^{2+}CA was calculated as percentage of chelation and as IC_{50} values (mg/mL) relative to a blank (negative control, $\mathbf{C}^{-}$), which contained 30 µL of DMSO in place of the extract. The absorbance was corrected using color controls containing just extract and buffer, to eliminate interferences due to the color of the extracts.

Cu^{2+} chelating compounds + Cu^{2+} $\xrightarrow{pH = 6.0}$ Cu^{2+}chelating compounds-Cu + $2H^{+}$.....equation (5)



Pyrocatecol violet (PV) Cu-PV complex .....equation (6)

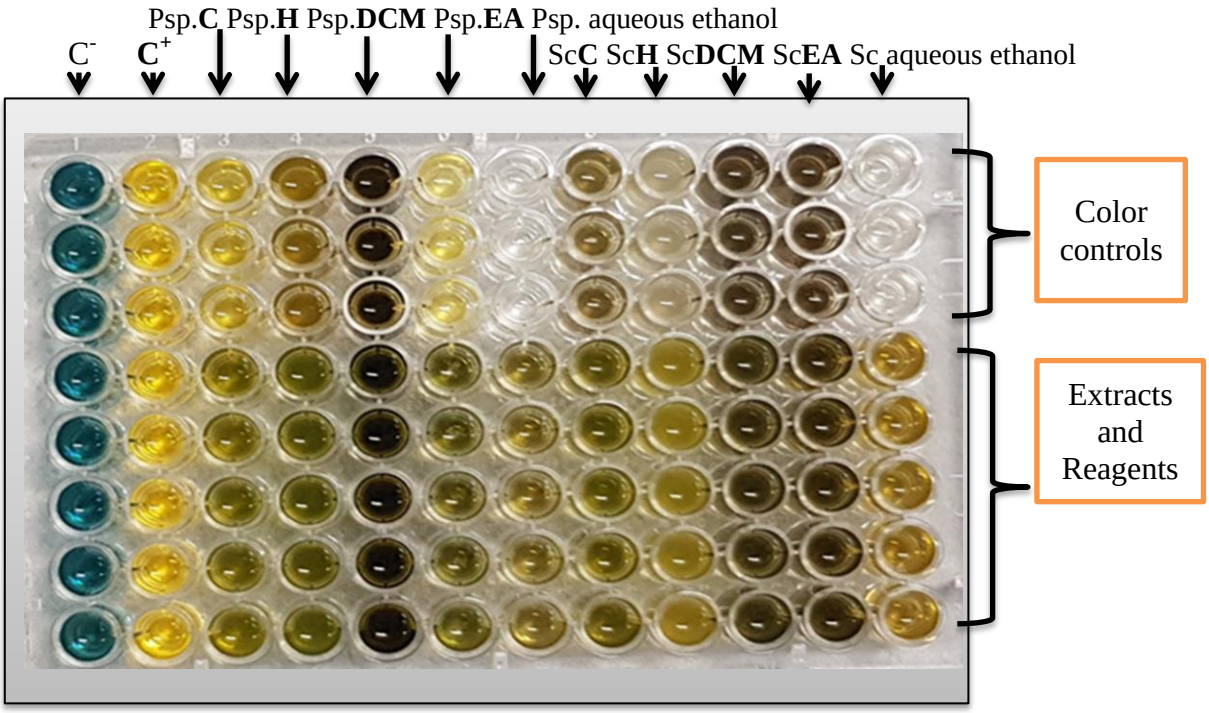


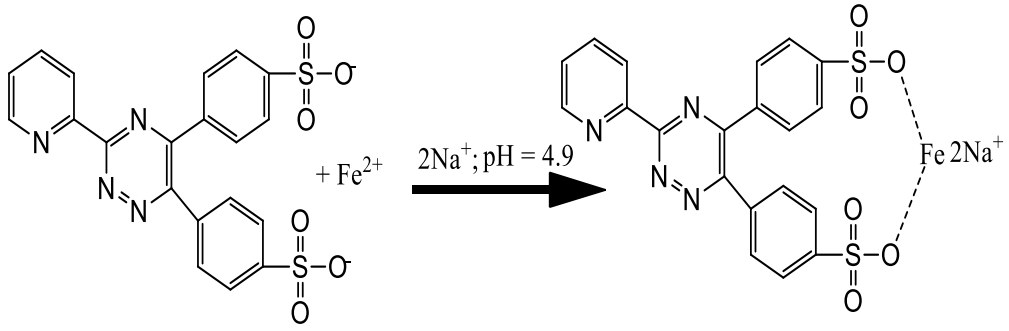
Figure 5: Color change due copper chelating compounds

See after section 4.1. In the copper-binding assay using pyrocatechol-violet as chromogen agent, virtually, copper chelating compounds react with Cu^{2+} (equation 5) forming complex that inhibits the complex between PV and Cu^{2+} (equation 6). The 96-well micro plate contains the crude of ethanolic extract of *Porphyridium* sp. (Psp.C) and S. *costatum* (ScC) and their fractions of (hexane, dichloromethane, ethyl acetate, aqueous ethanol) at 10 mg/mL. The first 2 columns of the plate are contains solution of negative (C^{-}) and positive (C^{+}) controls.

3.5.2. Iron chelating activity (Fe^{2+}CA) assay

The capacity to chelate Fe^{2+} from the extract was determined by measuring the formation of the iron-ferrozine complex as designated in the litrature[13,78] with slight modifications. A 30 μL extract were mixed with 200 μL of sodium acetate buffer (pH = 4.9) in 96-well micro plates. Then, 30 μL of $FeCl_2 \cdot 4H_2O$ (0.1 mg/mL, in ultrapure water) solution was added and incubated after stirring at room temperature[87] for 20 min. A 12.5 μL solution of ferrozine (40 mM) was then added and the mixture was stirred and incubated for 10 min at room temperature. In slightly acidic medium, iron reacts with ferrozine making Fe-ferrozine pink-colored complex (Figure 6). However, in the presence of iron chelating compounds, the formation of the Fe-ferrozine is disrupted which leads to a decrease in the color and absorbance[86]. The estimated binding ability of the extracts was detected spectrophotometrically at λ = 562 nm using a 96-well micro plate reader at room temperature. Therefore, the higher absorbance at λ = 562 nm is due to the Fe-ferrozine complex, while the lower absorbance corresponds to an increase in iron chelating compounds that existed in the reaction mixtures[88]. DMSO (30 μL) replaced the extract as a negative control (C^-) to express the percentage of Fe^{2+} chelation ability of extract using (equation 4) that was mentioned in section 3.5 and as IC_{50} value (mg/mL). Color control (30 μL and 270 μL of buffer) was used to correct the absorbance for unequal color between extracts and complex formations. These activities were compared with the chelating activity of synthetic metal chelator which was EDTA (30 μL, 1mg/mL) in place of extract to refer as positive control.

Fe^{2+} chelating compounds + Fe^{2+} $\xrightarrow{p^H = 4.9}$ Fe^{2+} chelating compounds-Fe + $2H^+$ ….equation (7)



……equation (8)

Ferrozine Fe-ferrozine complex

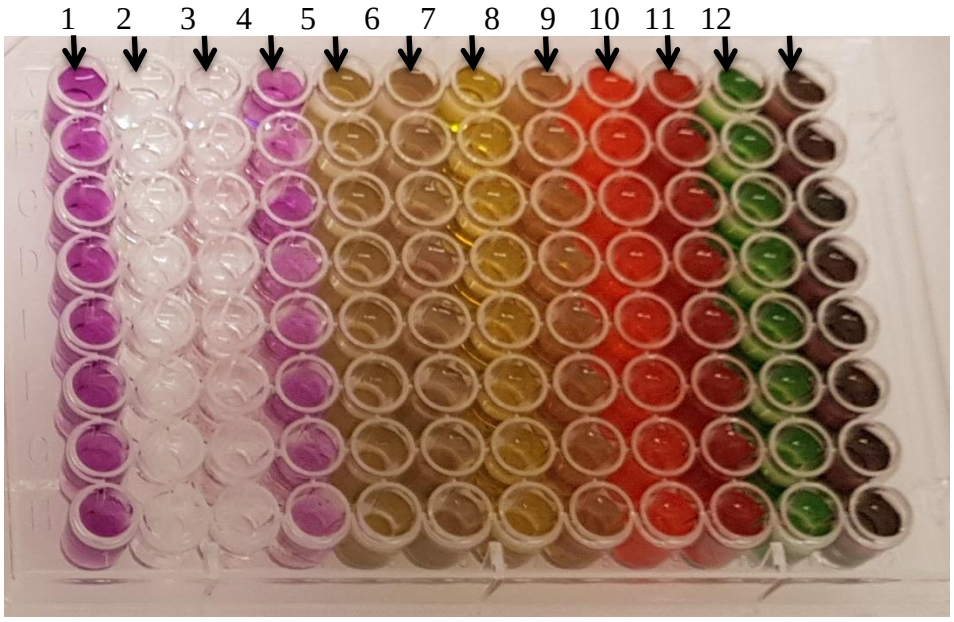


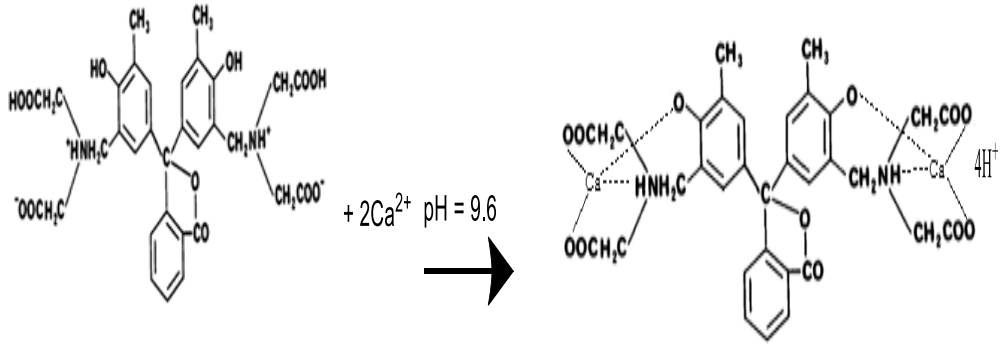
Figure 6: Color change due to iron chelating compounds

See after section 4.2. Iron chelating compounds bind with Fe^{2+} (equation 7) and ferrozine bind with Fe^{2+} (equation 8). A 96-well micro plate of column 1 and column 2 referring (C^- showing pink color due to Fe-ferrozine complex and C^+ viewing colorless is due to Fe^{2+} was already chelated by synthetic chelator (EDTA, 1 mg/mL), resulting no Fe^{2+} leftover to bind with ferrozine) respectively. Column 3,5 and 7 are solutions of color control and 4,6,8 contain reagents + extract of *Porphyridium* sp. of water, ethanol and ethyl acetate respectively; columns 9 and 11 are solution of color control and 10 and 12 reagents + ethanolic extracts of *H. pluvialis* and *Spirulina* sp. respectively, n=8.

3.5.3. Calcium chelating activity (Ca^{2+}CA) assay

The ability of algal extracts to chelate excess Ca^{2+} was performed by measuring the formation of the Ca^{2+} with o-cresolphthalein complexone (OCPC)[78,89]. In 96-well micro plates, 50 µL of extract and 75 µL ammonia-ammonium chloride buffer (pH = 9.96) were diluted with 125 µL ultrapure water. Then 30 µL of prepared Ca^{2+} standard (aqueous of 0.5 mM $CaCO_3$ and 1mL 1M HCl) and 20 µL of 0.47 mM o-cresolphthalein complexone were homogenized with the aid of a 300 µL micropipette and the change in color due to Ca-OCPC complex formation was observed. When calcium reacts with OCPC, acidity of the mixture increases by the presence of ionizable protons causing their ionization to develop color. It is due to calcium ion is bounded by the imino diacetate, two carboxylic oxygen atoms, imino nitrogen and the phenolic oxygen with in o-cresolphthalein complexone that forms calcium-OCPC eight co-ordinate which is pale pink in color[89] (Figure 7). In the presence of calcium chelating compounds, Ca^{2+} reaction with OCPC is inhibited as well as the pink complex color[90] (Figure 7) gets disrupted. The presence of calcium chelating compounds thus decreases absorption at wave-length of λ = 575 nm. The change in color was measured on a micro plate reader (Biotek Synergy 4). A 50 µL of 1 mg/mL of EGTA was used as the positive control due to the ability of higher affinity for Ca^{2+} than other cations[78]. Absorbance due to the extracts' color was adjusted by color control solution. Results were expressed as percentage of chelation ability of extract relative to a negative control 50 µL DMSO in place of the extract, and as IC_{50} values (mg/mL) using (equation 4) mentioned in section 3.5.

Ca^{2+} chelating compounds + $2Ca^{2+}$ $\xrightarrow{pH = 9.6}$ Ca^{2+}chelating compounds-Ca + $2H^{+}$ ....equation (9)



.....equation (10)

o-cresolphthalein complexone (OCPC) Ca-OCPC complex

Figure 7: Color change due to calcium chelating compounds

See after section 4.3, calcium chelating compounds react with calcium which form a complex (equation 9) and assay using o-cresolphthalein (OCPC) complexone as chromogen which forms OCPC-Ca complex (equation 10) as could be observed as pink color, and a 96-well micro plate containing the IC_{50} assay for Psp.EtOH.

3.6. Partition of active crude extracts by LLE

Researchers have been using polar organic solvents such as methanol, ethanol, acetone, ethyl acetate[91], dichloromethane[62], dichloroethane, isopropanol, propanol, and water[92] and non-polar solvents namely n-hexane[93], cyclohexane[62], isooctane benzene, toluene, chloroform, diethyl ether[91] and dimethyl ether[92] to extract and purify active compounds from different extracts. Usually liquid-liquid extraction (LLE) uses less expensive equipment, small solvent consumption, resulting in pollution prevention and high extraction yield, is fast, and easy to operate[91,92]. There are different families of high-value biochemical compounds present in the algal biomass identified from algal extracts using LLE as fractionating techniques such as fatty acids, carotenoids[93], alkanes, terpenes, alcohols, aldehydes[94] and others have been also identified that are responsible in decreasing the exposure of neurological disorder disease, mental illness and inflammation[94,95]. Therefore, in order to fractionate extracts with neuroprotective ability, LLE was chosen over other known techniques such as column chromatography with silica gel (silicon oxide) and/or alumina (aluminum oxide) as a stationary phase and organic solvents as eluent (mobile phase)[96] to fractionate extracts. Because chromatography is time consuming, LLE is preferred as a fractionation method.

The typical apparatus used for LLE is a separatory funnel. During the LLE extraction, hydrophilic components are more soluble in aqueous whereas hydrophobic compounds can be extracted from aqueous phase using organic solvents with different polarity having the principle of like-dissolve-like[91,97]. Position of the phases in the separating funnel depends on their density. The aqueous phase is usually on the bottom as water is denser than most organic solvents such as ethyl acetate and n-hexane whereas halogenated solvents for instance dichloromethane (DCM) and chloroform at the bottom of water (Figure 7C and Figure 7D). In this study, two active ethanol extracts of *Porphyridium* sp. and *S. costatum* were selected for fractionation. Selected extracts were fractionated by LLE using three solvents by increasing polarity such as n-hexane (PI=0.10)[98], dichloromethane (PI = 3.10)[99] and ethyl acetate (PI = 4.40)[69]. Four active fractions were obtained to be further tested for neuroprotective capacity.

For the fractionation, 3.50 g of crude extract was dissolved in 150 mL of ultrapure water+ethanol (80:20; ultrapure water+ethanol, v/v) and poured in to a clean 500 mL sized of separatory funnel. The mixture of the ethanol extract with the water increases the polarity of the mixture making it relatively immiscible with the organic solvents selected[96]. After that,

consecutive extractions were performed using 50 mL of solvents with increasing polarity, starting with hexane (a non-polar solvent). The mixture was gently shaken to avoid emulsions and the vapor pressure of the solvents was released after each round of shaking by opening the stopcock[100]. The funnel was rested until complete separation of the two phases. The bottom phase (layer) was removed into a clean reagent bottle and the top layer poured out from the top of the funnel in a clean labeled reagent bottle[100,101]. The aqueous layer was introduced again into the funnel and then two more consecutive extractions with hexane (50 mL) were obtained. The process was repeated with dichloromethane, ethyl acetate then finally ultrapure water-ethanol fraction. Four obtained fractions of (hexane, dichloromethane, ethyl acetate and ultrapure water-ethanol) were collected carefully (Figure 8D). Hexane, dichloromethane and ethyl acetate fractions were dried over anhydrous sodium sulfate in order to remove remaining moisture solvent and filtered with by Whatman no. 4 filter paper pore size (10-12 µm) and afterwards by 0.45-µm syringe. All fractions were then concentrated under reduced pressure at 40 °C using a vacuum rotary evaporator (Figure 9A) then transferred to pre-weighed and labeled dark-brown vials then dried under a gentle nitrogen flow (Figure 9B). Table 8 represents values of dry yield of fractions in percent after fractionation. All fractions were then dissolved in dimethyl sulfoxide (DMSO) to obtain a stock solution at a concentration of 20 mg/mL. Crude extract and portioned fractions namely hexane, dichloromethane, ethyl acetate and alcoholic aqueous were re-tested for neuroprotective capacity in terms of metal chelating activity as described in section 3.5.1, 3.5.2 and 3.5.3. The most active fraction was chemically characterized by Gas Chromatography-Mass Spectrometry **(**GC-MS) to tentatively identify the major compounds present. The overall stepwise fractionation was summarized as a flow chart in Figure 10.

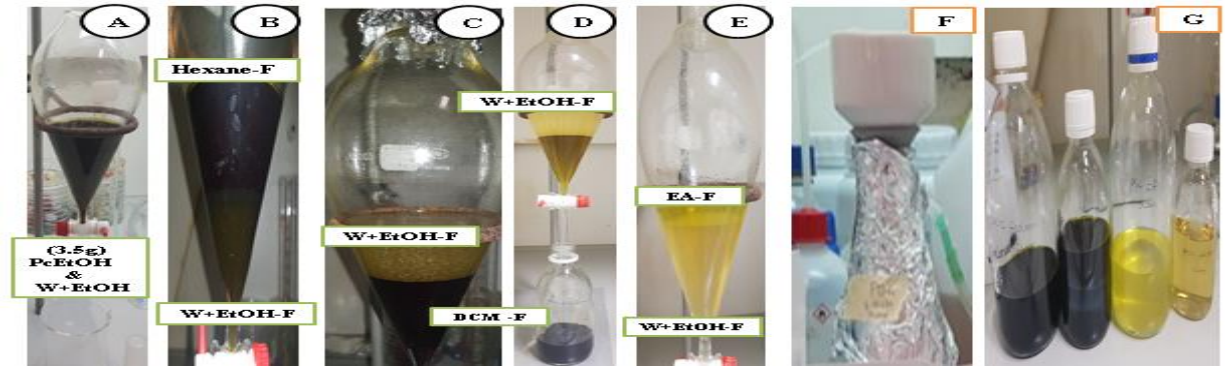


Figure 8: Partitioning of active crude extracts by LLE

Through the LLE, solvents with increasing polarity were used; dried extract was first dissolved in ethanol and water (20:80, v/v) and transferred in to a separating funnel then rested for 15 min (A). Next, hexane was added, funnel was gently shaken and two phases were formed rested in the ring for an hour to settle, hexane (organic) fraction in the upper part forming organic phase and aqueous fraction (water and ethanol) in the bottom part due to the water density (B). Then, aqueous fraction was introduced again into the funnel and extracted with dichloromethane, mixed well and settled for an hour. Then stopper removed and covered by aluminum foil (C). Since dichloromethane has higher density than water, it rested to the bottom, while aqueous fraction formed upper layer (C and D; D the second round after dichloromethane addition and showing the tip of funnel nestled in the reagent bottle). Lastly, extraction of aqueous phase was done with ethyl acetate, producing two fractions, ethyl acetate (organic phase) in the upper layer and water with ethanol (ethanolic aqueous phase) in the bottom layer (E). In order to lower the loss of fractions the procedure was repeated three times[96]. Afterwards, all obtained fractions were filtered through anhydrous Na_2SO_4 to remove water remaining from organic fractions (F). Finally, all fractions were collected (G) and solvent was removed (through rotary evaporating and nitrogen gas stream flow).

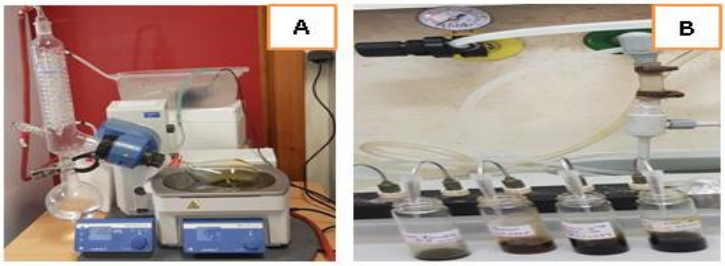


Figure 9: Solvent removal process

After LLE, solvents of obtained organic fractions were concentrated in a rotary evaporator at 40℃, 100 rpm and under controlled pressure (A); afterwards remained solvent was evaporated using nitrogen gas stream for complete dryness of fractions in pre-weighted dark vessels at room temperature (B).

Table 8: Dry yield of fractions (%)

Algal species	**fractionating-solvents**	**dry yield of fractions (%)**
***Porphyridium* sp.**	hexane	6.66
	dichloromethane	4.67
	ethyl acetate	5.91
	aqueous ethanol	10.1
S. costatum	hexane	11.2
	dichloromethane	20.7
	ethyl acetate	9.51
	aqueous ethanol	31.2

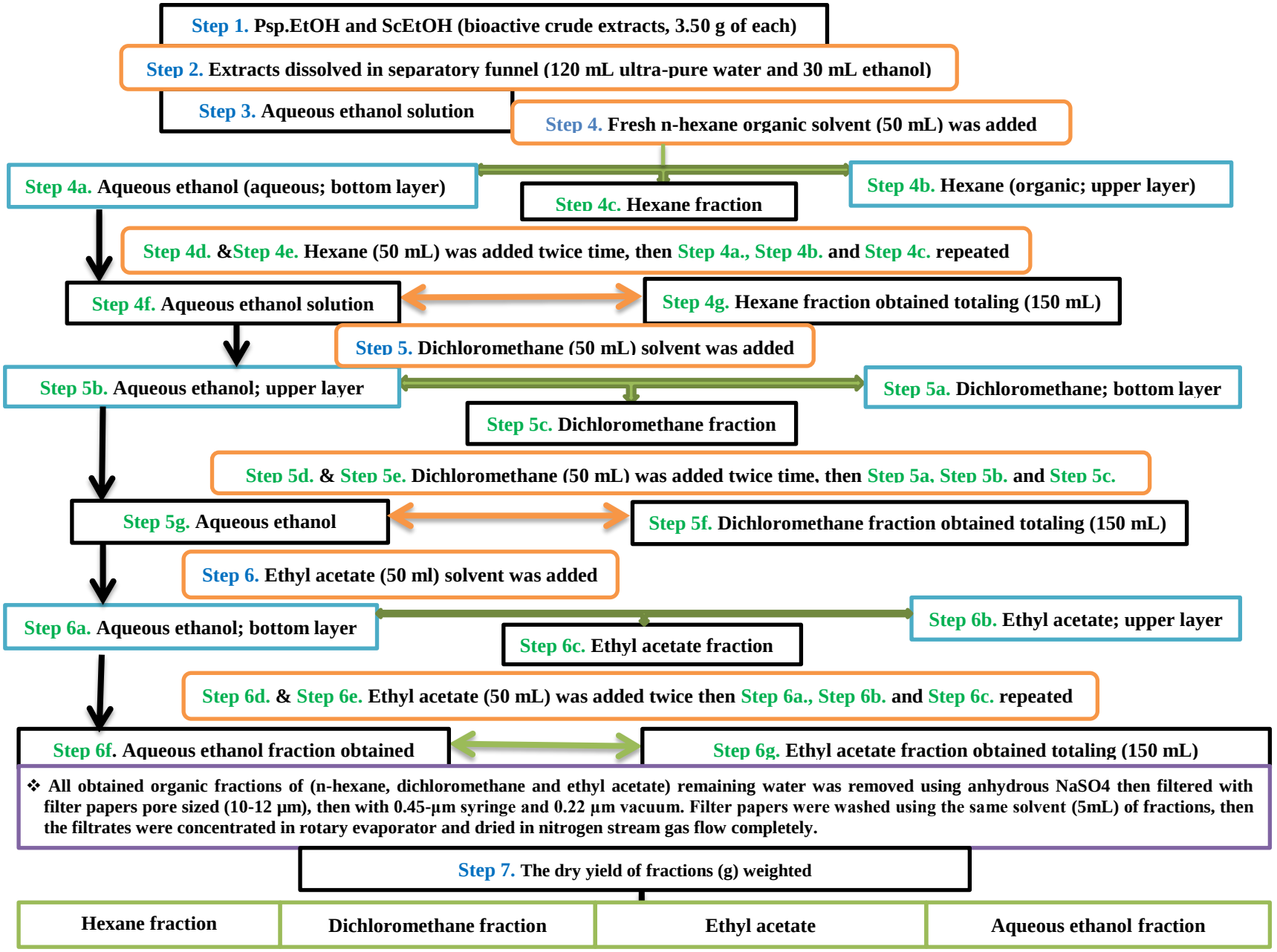


Figure 10: Flow chart for bio-guided fractionation (LLE)

3.7. Chemical characterization of the active fractions by GC-MS

3.7.1. Derivatization of samples

Gas chromatography (GC) is a widely known and used method, accepted by the Food and Agriculture Organization (FAO) for mixture analysis. It is reasonably fast, trustworthy, easy to master, with high detection sensitivity, separation efficiency, high selectivity, quick and low sample consuming. However, in GC analysis, compounds should be volatile or semi-volatile i.e. have low boiling points, good thermal stability, and low molecular polarities[102]. In order to convert compounds with low volatility that are either undetectable or detected with a low sensitivity to those with ideal detection by GC analysis, chemical derivatization techniques can be applied. Derivatization is the chemical modification of compound to produce a new one with increased volatility and separation properties that are more suitable for GC analysis[103,104].

Derivatization was done by adding methanol and acetyl chloride as a reagent. Dried fractions (5 mg) were treated with 1.5 mL of derivatization solution (methanol/acetyl chloride, 20:1, v/v), in 10 mL derivatization vessels and homogenized in the vortex (IKA, VWR, Leuven, Belgium) until complete dissolution for ~2 min. Afterwards 1 mL of hexane was added, and the mixture was placed in a water bath for 1 hour at 70°C (Figure 11). After cooling in an ice bath for 15 min, a mixture of ultra-pure water and n-hexane was added (1:4, v/v) and mixed on the vortex mixer at maximum speed in two cycles for 30 seconds at RT. In clean glass tube, the mixtures were centrifuged (2,000 rpm, 5 min, RT). The organic phase was collected, and the whole step was repeated one more time to make duplicate. Finally, remaining water was removed with an excess anhydrous sodium sulphate followed by filtration (syringe; 0.22 μM) into clean new tubes. Hexane was evaporated under a gentle nitrogen gas stream flow until complete dryness and the fraction re-suspended in 500 μL of GC-grade hexane and transferred in to 1.8 mL vials for GC-MS analysis.

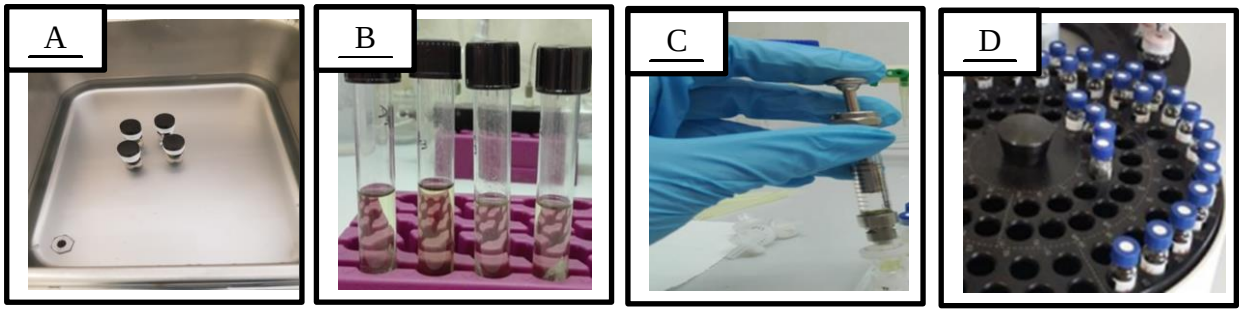


Figure 11: Some of the steps of the derivatization

Fractions during the heat treatment, in a water bath of 70ºC (A), fractions after centrifugation (B), anhydrous sodium sulphate filtration through 0.22-µm PTFE filters (C), the ready of hexane fractions in the GC-MS analyzer (D).

3.7.2. GC-MS analysis

To discover bioactive compounds and apply it on biotechnological approaches, samples were analyzed on an Agilent GC-MS (Agilent Technologies 6890 Network GC System) coupled with MS detector (5973 Inert Mass Selective Detector) in a Bruker Scion 456/GC, Scion TQ MS coupled to a ZB-5MS capillary (30m × 0.25 µm i.d., 0.25 µm film thickness from Phenomenex). The column temperature program was set as follow:

Initial oven temperature of 60°C for 1 min, increased to 120°C at the rate of 30°C/min and then increased to 250°C at 4°C/min and then ramped to 300°C at a rate of 20°C/min, and held for 4 min. A constant flow rate of 1 mL/min was used for the carrier gas (helium). Samples were injected at 300°C, operating in split less mode. Mass spectrometry (MS) was used to detect and quantify the separated compounds due to its more sensitive, faster, robust, high-resolution and accurate mass properties[102,103,104]. For the identification and quantification of bioactive compounds in fractions, the total ion mode was used.

3.7.2.1. *Expression of results*

In order to identify the compounds present in the active extract, Bruker MS workstation Software version 8.2.1 and MS Search version 2.0 was used. Peaks of eluting compounds were identified using mass spectra libraries of the National Institute Standard and Technology (NIST) applying a match quality of above 850 thresholds. Components' relative percentages were calculated based on GC peak areas with respect to total area of peaks.

3.8. Statistical analysis

All statistical analyses were performed using SPSS 26.0 statistical package for Windows (IBM SPSS Inc., Chicago, USA) software. All experiments were conducted three times. In each experiment six and eight replicates were performed in the assays of IC_{50} (color controls in duplicates) and preliminary screening respectively. Results were reported as mean ± standard error of mean (SEM). The data for all measures were tested for normality (Skewness-Kurtosis) and homogeneity (Levene's test) to see the quality of variance of homogeneity assumption; (% MCA) across groups of solvent-extracting and fractionating for the same species of algal and found to be normally distributed and homogeneous therefore, parametric statistical analysis was applied. The mean values of data were analyzed by analysis of variance (One-way ANOVA) for three groups; independent-samples t-Test for two groups was performed to identify the differences between factors. Statistical significance difference was determined at the 5% level ($p<0.05$). Once defined that differences existed among the means, post hoc pairwise multiple comparisons by Gabriel for unequal size (n) and Scheffe tests for equal n used. Prior to all statistical analyses, outliers were detected and removed using Boxplots showed the median, interquartile range, and outliers. The 50% chelating concentrations (IC_{50}) were calculated by sigmoidal fitting of the data (95% CI dose response curve).

4. RESULTS AND DISCUSSION

4.1. Copper chelating activity (Cu^{2+}CA)

In the preliminary screening, the neuroprotective capacity of crude ethanol, ethyl acetate and water extracts from *Haematococcus pluvialis* (Hp), *Isochrysis* sp., *Nannochloropsis* sp., *Phaeodactylum tricornutum* (Pt), *Porphyridium* sp., *Skeletonema costatum* (Sc), *Spirulina* sp., *Tetraselmis chui* (Tc) and *Tetraselmis* sp. CTP4 (CTP4) was assessed in terms of chelating activity of the Cu^{2+}, Fe^{2+} and Ca^{2+} at 10 mg/mL as shown in Table 9. EDTA was used as a positive control at 1 mg/mL and dimethyl sulfoxide (DMSO) was used as negative control. All extracts were dissolved in DMSO prior to the experiment. Results of the average metal chelating activity were expressed as percentage with respect to negative control. All extracts except *H. pluvialis*, *Spirulina* sp., *T. chui* and *Tetraselmis* sp. CTP4 showed higher chelating copper as can be observed in Table 9 with values ranging from 62.5% to 87.1%. The best results were found to be in *S. costatum* extracts of ethanol, ethyl acetate and water (87.1%, 76.3% and 74.2%) respectively. This could indicate that *S. costatum* extracts demonstrated higher capacity to chelate copper than others followed by ethanol extract of *Isochrysis* sp. (85.5%) and ethyl acetate extract of *Porphyridium* sp. (82.4%).

Table 9: Copper chelating activity (Cu^{2+}CA, %) of extracts

Species	extracting-solvents	Cu^{2+}CA (10 mg/mL)
H. pluvialis	ethanol	51.9 ± 0.2^{b}
	ethyl acetate	59.5 ± 0.2^{a}
	water	20.3 ± 0.4^{c}
***Isochrysis* sp.**	ethanol	85.5 ± 0.1^{a}
	ethyl acetate	67.8 ± 0.5^{c}
	water	72.6 ± 0.5^{b}
***Nannochloropsis* sp.**	ethanol	43.5 ± 0.7^{b}
	ethyl acetate	3.61 ± 0.35^{c}
	water	72.3 ± 0.4^{a}
P. tricornutum	ethanol	74.4 ± 0.6^{a}
	ethyl acetate	65.1 ± 0.9^{b}
	water	62.8 ± 1.6^{b}
***Porphyridium* sp.**	ethanol	62.5 ± 0.3^{b}
	ethyl acetate	82.4 ± 2.1^{a}
	water	52.5 ± 3.2^{c}
S. costatum	ethanol	87.1 ± 0.2^{a}
	ethyl acetate	76.3 ± 0.1^{b}
	water	74.2 ± 0.5^{c}
***Spirulina* sp.**	ethanol	60.2 ± 1.7^{a}
	ethyl acetate	52.3 ± 0.1^{b}
	water	25.5 ± 1.2^{c}
T. chui	ethanol	54.4 ± 6.5^{a}
	ethyl acetate	39.1 ± 5.6^{b}
	water	19.8 ± 4.8^{c}
***Tetraselmis* sp. CTP4**	ethanol	47.7 ± 1.3^{a}
	ethyl acetate	44.6 ± 1.3^{a}
	water	31.6 ± 0.8^{b}
***EDTA**	-	91.2 ± 0.3

Results were expressed as mean ± SEM (standard error of mean) (n = 8). For each species, values labeled with different letters are significantly different ($p < 0.05$). *EDTA = positive control, 1 mg/mL.

Next step in this study was to calculate the half-maximal chelating concentration ($IC_{50,}$ mg/mL). IC_{50} is a measure of the effectiveness of a compound to chelate 50% of copper that was found in the extract[85]. The most active extract is the one found with the lowest IC_{50} value as showed in Figure 12. We decided if the values are approximately less than 2 mg/mL to be considered as worthy enough to make further analysis.

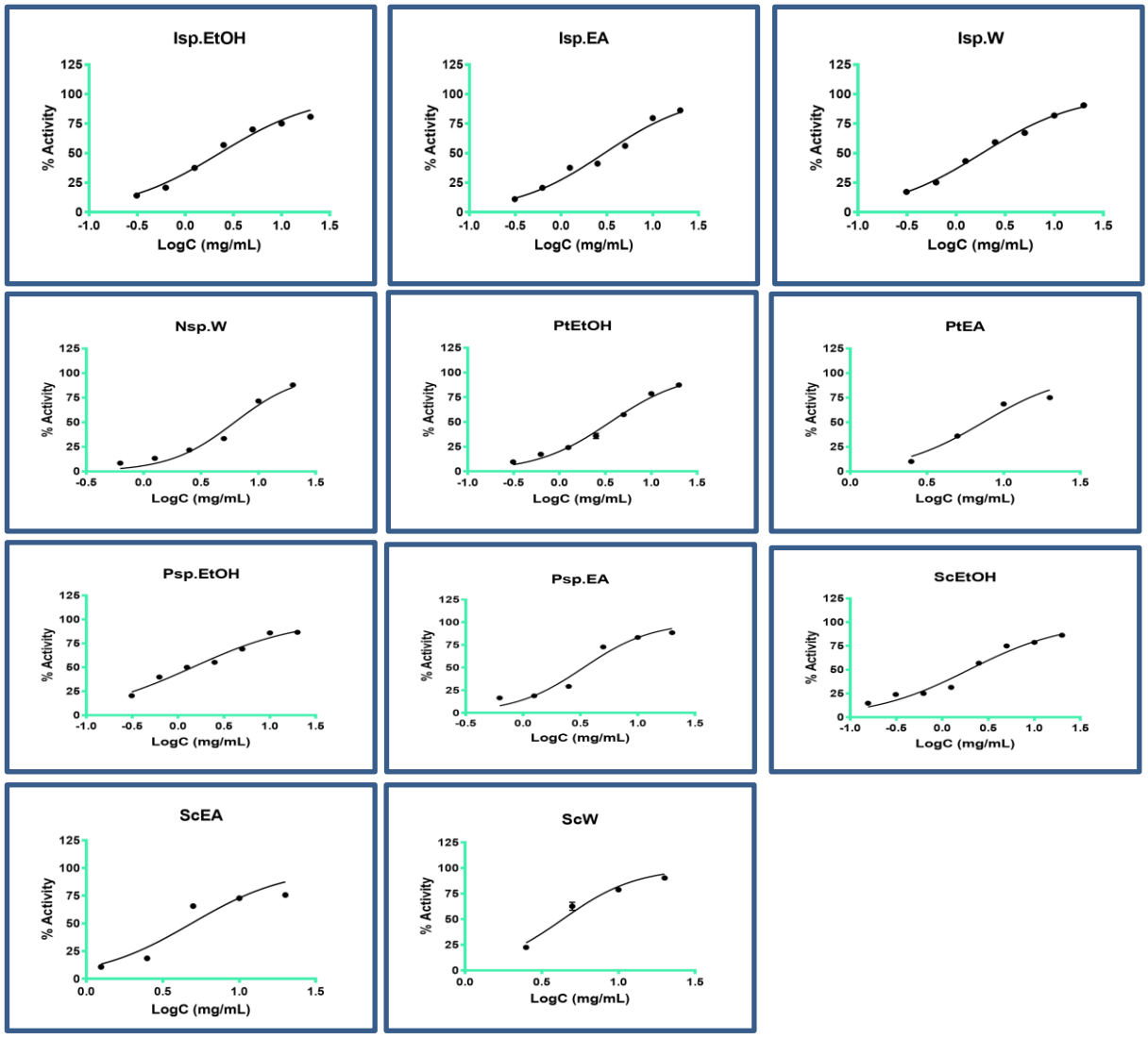


Figure 12: IC_{50} (dose response curve) of Cu^{2+}CA of extracts

Isp.EtOH = ethanol extract of *Isochrysis* sp.; Isp.EA = ethyl acetate extract of *Isochrysis* sp.; Isp.W = water extract of *Isochrysis* sp.; Nsp.W = water extract of *Nannochloropsis* sp.; PtEtOH = ethanol extract of P. *tricornutum*; PtEA = ethyl acetate extract of P. *tricornutum*; Psp.EtOH= ethanol extract of *Porphyridium* sp.; Psp.EA= ethyl acetate extract of *Porphyridium* sp.; ScEtOH = ethanol extract of S. *costatum*; ScEA = ethyl acetate extract of S. *costatum*; ScW = water extract of S. *costatum*.

Based on IC_{50} results, the most active extract with the lowest IC_{50} was identified in copper chelating activity, as can be seen in Table 10. The IC_{50} values with less than 2 mg/mL were the ones prepared from ethanol extract of *Porphyridium* sp., *S. costatum* and water extract of *Isochrysis* sp. valued with 1.44 mg/mL, 1.95 mg/mL and 1.84 mg/mL respectively.

Table 10: IC_{50} of Cu^{2+}CA of extracts with respective 95% CI

Species	**extracting-solvents**	**IC_{50}**		
		mg/mL	**CI**	**R^2**
***Isochrysis* sp.**	ethanol	2.28	2.08 – 2.49	0.982
	ethyl acetate	2.97	2.74 – 3.21	0.981
	water	1.84	1.76 – 1.92	0.991
***Nannochloropsis* sp.**	water	6.21	5.78 – 6.68	0.974
P. tricornutum	ethanol	3.58	3.41 – 3.78	0.995
	ethyl acetate	7.37	6.77 – 8.05	0.954
***Porphyridium* sp.**	ethanol	1.44	1.32 – 1.56	0.971
	ethyl acetate	3.37	3.06 – 3.72	0.952
S. costatum	ethanol	1.95	1.77 – 2.14	0.962
	ethyl acetate	4.87	4.21 – 5.68	0.881
	water	4.34	4.05 – 4.65	0.963

As chelating agent against copper, ethanol extract of the marine diatom *Porphyridium* sp. was identified as the most bioactive. The crude ethanol extract was fractionated into hexane, dichloromethane, ethyl acetate and aqueous ethanol. The polarity of the solvents used in the extraction process determines the composition and biological activity of a given extract[105]. Solvents of increasing polarity were used for the fractionation in order to separate and possibly isolate promising compounds that are responsible for neuroprotective compounds. Obtained fractions (hexane, dichloromethane, ethyl acetate and aqueous ethanol) were tested for Cu^{2+}CA, Fe^{2+}CA and Ca^{2+}CA at 10 mg/mL, 5 mg/mL and 1 mg/mL as a primary step to find an active fraction. Results shown in Table 11, hexane fractions from ethanol extracts of *Porphyridium* sp. and *S. costatum* were considerably the most active in chelating copper at 10 mg/mL, 5 mg/mL and 1 mg/mL.

Table 11: Cu^{2+}CA (%) of fractions

Crude extracts	fractionating-solvents	10 mg/mL	5 mg/mL	1 mg/mL
Psp.EtOH	crude extract	74.1 ± 0.6	67.2 ± 0.6	28.6 ± 0.1
	hexane	81.2 ± 0.1[a]	70.1 ± 0.1[a]	64.4 ± 0.1[a]
	dichloromethane	61.1 ± 1.1[b]	44.3 ± 0.7[b]	12.9 ± 0.9[b]
	ethyl acetate	46.6 ± 1.2[c]	33.8 ± 0.5[c]	13.2 ± 0.1[b]
	aqueous ethanol	25.4 ± 0.2[d]	18.1 ± 0.2[d]	5.41 ± 0.12[c]
ScEtOH	crude extract	83.4 ± 0.4	62.3 ± 1.3	22.1 ± 0.1
	hexane	92.6 ± 0.3[a]	80.4 ± 0.1[a]	64.1 ± 0.6[a]
	dichloromethane	61.4 ± 0.2[b]	42.1 ± 0.1[c]	19.4 ± 0.1[b]
	ethyl acetate	51.1 ± 0.1[c]	48.5 ± 0.1[b]	19.4 ± 0.1[b]
	aqueous ethanol	19.7 ± 0.1[d]	18.5 ± 0.1[d]	9.22 ± 0.11[c]
***EDTA**	ultra-pure water	-	-	93.2 ± 0.1

Results were expressed as mean ± SEM (n = 8). Values labeled with different letters are significantly different at $p < 0.05$. *EDTA = positive control.

IC_{50} values were obtained from IC_{50} (95% CI dose response curve) in Figure 13 to see the effectiveness and/or selectiveness of fraction at lower concentration.

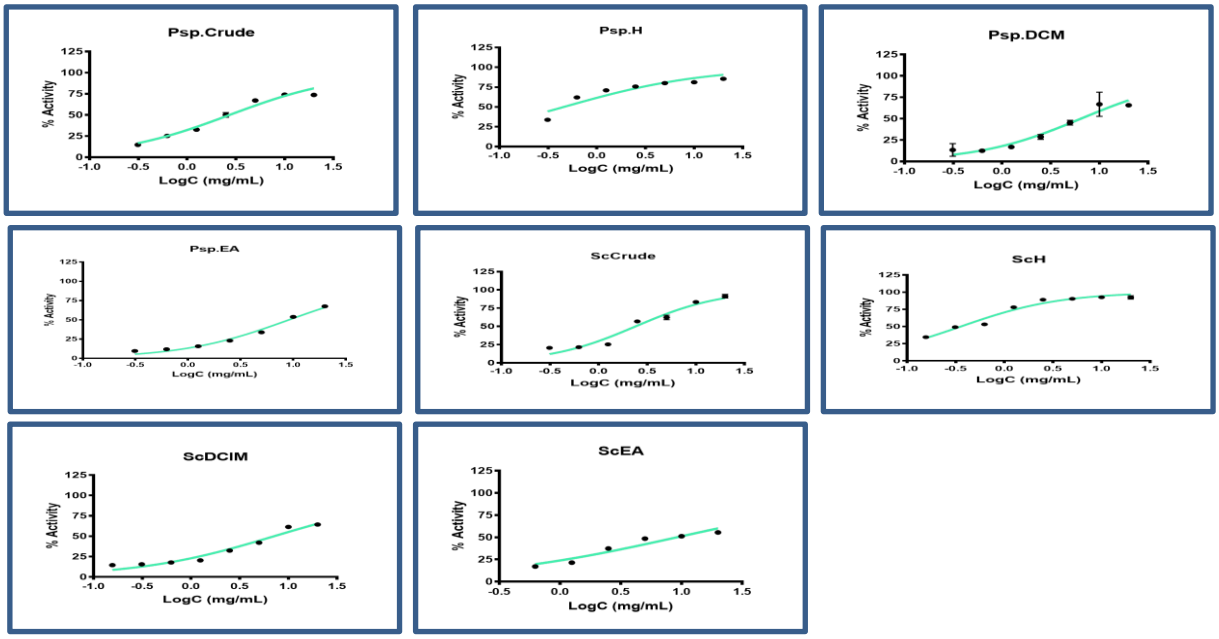


Figure 13: IC_{50} (dose response curve) of Cu^{2+}CA of fractions

Psp.Crude = crude extract of *Porphyridium* sp.; Psp.H = hexane fraction from Psp.Crude; Psp.DCM = dichloromethane fraction from Psp.Crude; Psp.EA = ethyl acetate fraction from Psp.Crude.

ScCrude = crude extract of S. *costatum*; ScH = hexane fraction from ScCrude;

ScDCM = dichloromethane extract from ScCrude; ScEA = ethyl acetate fraction from ScCrude.

In Table 12 results suggest that, the IC_{50} values of hexane fractions of ethanol extract of *Porphyridium* sp. (0.461 mg/mL) and *S. costatum* (0.361 mg/mL) were lower than dichloromethane and ethyl acetate fractions and crude. This implies that the hexane fractions of ethanol extract of *Porphyridium* sp. and *S. costatum* were more effective as copper chelating therapeutic agents. Crude extracts were re-tested for their Cu^{2+} chelation ability to make sure if the activity was maintained at 10 mg/mL, since the biomass was from different batch. As it can be seen clearly, the IC_{50} values were decreased in hexane fractions of Psp.EtOH (0.461 mg/mL) and ScEtOH (0.361 mg/mL) than in crude extract. However, in other fractions such as dichloromethane and ethyl acetate the IC_{50} values proved to be higher, meaning, chelating compounds have been concentrated in hexane fraction.

Table 12: IC_{50} of Cu^{2+}CA of fractions with respective 95% CI

Crude extracts	**fractionating-solvents**	**10 mg/mL**	**IC_{50}**		
			mg/mL	**CI**	**R^2**
Psp.EtOH	crude extract	74.1 ± 0.6	2.72	2.48 – 2.99	0.961
	hexane		0.461	0.362 – 0.561	0.864
	dichloromethane		6.48	5.55 – 7.66	0.891
	ethyl acetate		9.12	8.58 – 9.72	0.982
ScEtOH	crude extract	83.4 ± 0.4	2.41	2.16 – 2.66	0.954
	hexane		0.361	0.322 – 0.393	0.963
	dichloromethane		7.25	6.41 – 8.28	0.954
	ethyl acetate		9.17	7.95 – 9.75	0.922

Results of crude extracts at 10 mg/mL expressed as mean ± SEM.

As a result, those species can be used in the design of Alzheimer diseases chemotherapeutics, because it has been mentioned that copper is linked with neuron-related toxicity due to its redox-active nature (Cu/Cu^{2+}). However, if it is in excess then it reacts with oxygen (O_2) and generates reactive oxygen and nitrogen species (ROS and RNS). Those ROS are in the form of free radicals of cellular oxygen metabolism outcomes such as superoxide anion ($O•_2$), hydroxyl radicals (OH•), nitric oxide (NO•) and non-free radicals. Those non-free radicals have ability in producing free radicals when they are exposed for chemical reaction, namely hydrogen peroxide (H_2O_2) and peroxynitrite (ONOO). The ROS and RNS generations cause oxidative stress[106]

after neuronal cell damage and death by binding with Cu^{2+} induced reactive oxygen species. Then they deposit as aggregation of proteins such as amyloid beta peptide in senile plaque in the AD patients' brain[107]. Thus, compounds that capture Cu^{2+} resulting in preventing the generation of reactive oxygen species, and attenuate abnormal copper-protein connection that lead to increased free-radical toxicity. Moreover, it can be promising pharmacological strategy to formulate copper induced neuronal disease mainly Alzheimer's. Different studies in the literature stated that algal fractions are rich in number of compounds[108,109]. Therefore, compounds that are present in the hexane fractions of *Porphyridium* sp. and *S. costatum* were selected to be chemically characterized and tentatively identified. As it was decided in section 4.1, IC_{50} values higher than 2 mg/mL of fractions of dichloromethane, ethyl acetate fractions and crude were not promising for neuroprotective drug development and they were not subjected to characterization.

4.2. Iron chelating activity (Fe^{2+}CA)

Ethanol, ethyl acetate and water extracts of those nine different species were evaluated for their iron chelating activity (Fe^{2+}CA) at 10 mg/mL. EDTA (1 mg/mL) was used as positive control and DMSO was used as negative control. In the first phase screening, Fe^{2+} chelating activity varied greatly among extracts and/or species. Table 13 shows Fe^{2+}chelating activity found in water extracts ranging from 34.7% to 96.6% in *Porphyridium* sp. and *Tetraselmis* sp. CTP4, in ethanol extracts from 3.23% to 75.1% in *Tetraselmis* sp. CTP4 and *Isochrysis* sp. respectively. The maximum Fe^{2+} chelating capacity was observed in the water extracts of *Tetraselmis* sp. CTP4 (96.6%) and *Isochrysis* sp. (79.1%). It has been reported that results obtained with T. *chui* and *Isochrysis* sp. by Custódio et al. (2012, 2014) exhibited iron chelation capacity that contain antioxidants with potential application in the treatment of oxidative stress related diseases like AD[13,61]. *Nannochloropsis* sp. also had high chelating ability (83.2%). In the literature Goh et al. (2010) has mentioned that methanol extract of *Nannochloropsis* sp. had the most chelating ability towards iron ion[110]. Water extract of *P. tricornutum* (75.2%) and ethanol extract of *Isochrysis* sp. (75.1%) and *Porphyridium* sp. (71.7%) revealed considerable iron chelation effect. In similar finding of Kean et al. (2015) capturing ability has been identified in *P. tricornutum*[111]. It has been stated that, iron accumulation and oxidative damage are associated with neurological disorders.

Table 13: Iron chelating activity (Fe^{2+}CA, %) of extracts

Species	extracting-solvents	10 mg/mL
H. pluvialis	ethanol	6.61 ± 0.73^{a}
	ethyl acetate	4.71 ± 0.48^{b}
	water	1.92 ± 1.21^{c}
***Isochrysis* sp.**	ethanol	75.1 ± 0.1^{a}
	ethyl acetate	42.7 ± 1.1^{b}
	water	79.1 ± 1.9^{a}
***Nannochloropsis* sp.**	ethanol	19.7 ± 0.5^{b}
	ethyl acetate	4.11 ± 0.12^{c}
	water	83.2 ± 0.1^{a}
P. tricornutum	ethanol	58.8 ± 1.4^{b}
	ethyl acetate	11.8 ± 0.1^{c}
	water	75.2 ± 0.9^{a}
***Porphyridium* sp.**	ethanol	71.7 ± 0.1^{a}
	ethyl acetate	47.6 ± 0.5^{b}
	water	34.7 ± 0.2^{c}
S. costatum	ethanol	53.4 ± 0.1^{b}
	ethyl acetate	42.4 ± 0.6^{c}
	water	63.8 ± 0.9^{a}
***Spirulina* sp.**	ethanol	6.04 ± 0.62^{a}
	ethyl acetate	4.46 ± 0.27^{b}
	water	1.71 ± 0.21^{c}
T. chui	ethanol	16.6 ± 0.7^{a}
	ethyl acetate	15.8 ± 0.3^{a}
	water	4.91 ± 0.23^{b}
***Tetraselmis* sp. CTP4**	ethanol	3.23 ± 0.15^{c}
	ethyl acetate	33.2 ± 0.4^{b}
	water	96.6 ± 0.7^{a}
***EDTA**	ultra-pure water	96.1 ± 0.2

Results were expressed as mean ± SEM. Values labeled with different letters are significantly different at $p < 0.05$. *EDTA = Positive control, (1 mg/mL).

The disruption in the homeostasis of Fe^{2+} and Cu^{2+} is a main reason for increase in oxidative stress of lipid peroxidation and lead to oxidative damage of senile plaques[108]. It is a right approach to chelate Fe^{2+} and compounds that have ability to scavenge the free Fe^{2+} cations found in the brain to form a non-toxic metal complex[108].

The following step was to calculate the ($IC_{50,}$ mg/mL) of extracts that showed Fe^{2+}CA > 60%. Those were water extracts of *Tetraselmis* sp. CTP4, *Isochrysis* sp., *Nannochloropsis* sp. and *P. tricornutum* and ethanol extracts of *Isochrysis* sp., *Porphyridium* sp. and *S. costatum*. The most active extracts having higher capacity to chelate Fe^{2+} ions were the ones found with the lowest IC_{50} value as shown in Figure 14 and Table 14 were ethanol extracts of *Porphyridium* sp. (0.942 mg/mL) and *S. costatum* (0.883 mg/mL).

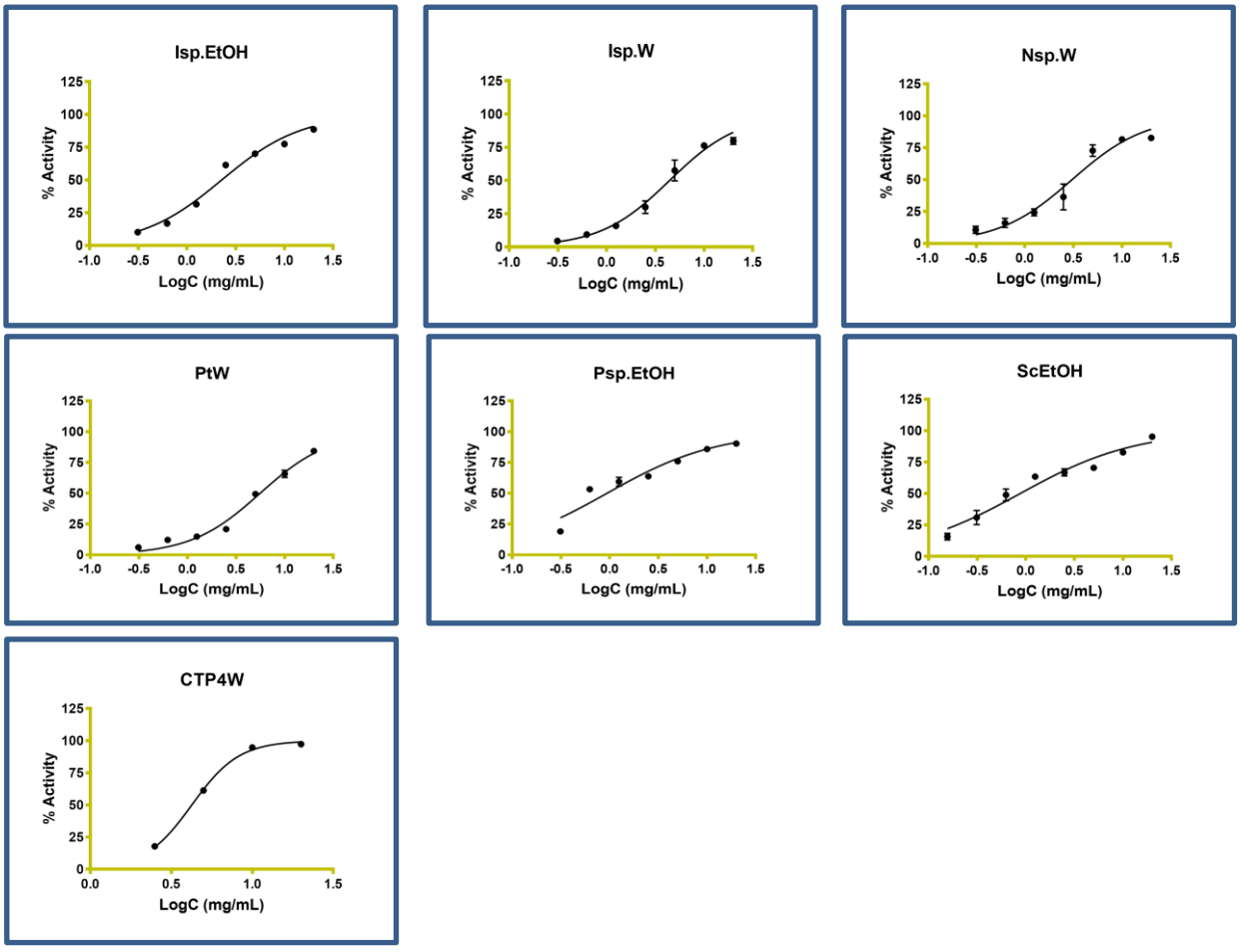


Figure 14: IC_{50} (dose response curve) of Fe^{2+}CA of extracts

PtW = water extract of *P. tricornutum*; water extract of *Tetraselmis* sp. CTP4

Error bars show the standard deviation of six replicates.

Table 14: IC_{50} of Fe^{2+}CA of extracts with respective 95% CI

Species	extracting-solvents	IC_{50}		
		mg/mL	CI	R^2
***Tetraselmis* sp. CTP4**	water	4.23	4.17 - 4.30	0.992
***Isochrysis* sp.**	ethanol	2.28	2.11 - 2.46	0.981
	water	4.43	4.11 - 4.79	0.973
***Nannochloropsis* sp.**	water	3.07	2.74 - 3.44	0.943
P. tricornutum	water	5.59	5.25 - 5.96	0.982
***Porphyridium* sp.**	ethanol	0.942	0.812 - 1.08	0.921
S. costatum	ethanol	0.883	0.771 - 0.992	0.954

Table 15 indicates that hexane fractions of ethanol extract of *Porphyridium* sp. and *S. costatum* were pointedly higher at $p<0.05$ in chelating iron at 10 mg/mL, 5 mg/mL, 1 mg/mL of 85.2%, 78.5% and 60.9%, and 97.9%, 97.3% and 88.7% respectively than fractions obtained from dichloromethane and ethyl acetate .

Table 15: Fe^{2+}CA (%) of fractions

Crude extracts	fractionating- solvents	10 mg/mL	5 mg/mL	1 mg/mL
Psp.EtOH	crude extract	77.4 ± 0.2	75.1 ± 0.7	61.6 ± 0.3
	hexane	85.2 ± 0.4^{a}	78.5 ± 0.6^{a}	60.9 ± 0.2^{a}
	dichloromethane	61.1 ± 1.1^{b}	44.3 ± 0.7^{b}	12.8 ± 0.9^{b}
	ethyl acetate	46.6 ± 1.2^{c}	33.8 ± 0.5^{c}	13.1 ± 0.1^{b}
	aqueous ethanol	6.59 ± 0.04^{d}	5.15 ± 0.06^{d}	3.41 ± 0.02^{c}
ScEtOH	crude extract	83.5 ± 1.6	62.3 ± 1.3	22.1 ± 0.1
	hexane	97.9 ± 0.3^{a}	97.3 ± 0.1^{a}	88.7 ± 0.2^{a}
	dichloromethane	61.4 ± 0.2^{b}	42.1 ± 0.1^{c}	19.4 ± 0.2^{b}
	ethyl acetate	51.2 ± 0.1^{c}	48.5 ± 0.1^{b}	19.4 ± 0.1^{b}
	aqueous ethanol	24.8 ± 0.1^{d}	17.3 ± 0.1^{d}	7.94 ± 0.12^{c}
***EDTA**	ultra-pure water	-	-	95.3 ± 0.1

*EDTA = positive control.

Results in Figure 15 and Table 16 suggest that the highest Fe^{2+}CA with low IC_{50} value were seen in hexane fractions from ethanol extracts of *Porphyridium* sp. and *S. costatum* of (0.292 mg/mL) and (0.263 mg/mL). Interestingly, our result advocates that those hexane fractions were endowed with compounds of $Fe^{2+}$and Cu^{2+}chelating activity.

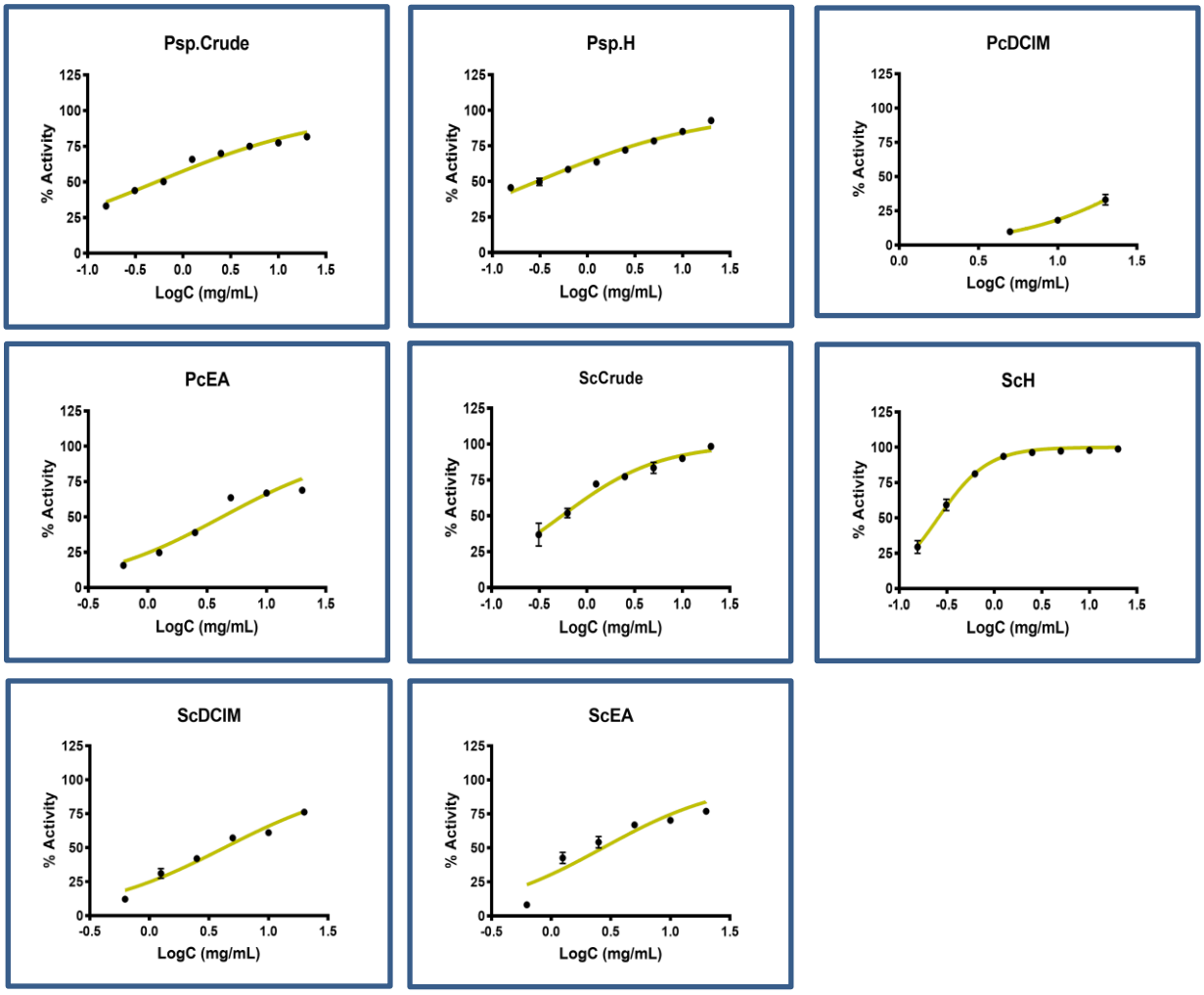


Figure 15: IC_{50} (dose response curve) of Fe^{2+}CA of fractions

Iron has a significant catalyzing effect in Fenton reaction in making reactive oxygen species by enhancing oxidative stress leading to neuron-related diseases. Even though, Fe^{2+} is an important cation for several enzymes, accumulation of Fe^{2+} has been implicated in several pathological conditions of metabolic processes like protein aggregation and oxidative stress[112]. It has been reported that, it leads to abnormal combination of protein aggregates in neuron and amyloid

plaques in the brain's mitochondria. Hydroxyl radical is very reactive, unstable, forms those modified aggregates, which are able to damage macromolecules of mitochondria and other cellular structures. Damaged mitochondria are sources of intracellular free radicals. The interactions of generated ROS and redox-active Fe^{2+} contribute to oxidative stress damage in AD patient's brain[108,112]. Thus, compounds with the capacity to chelate excess redox-active metal ions such as Fe^{2+} and Cu^{2+}, prevent or inactive uncontrolled generation of redox-metal induced reactive oxygen species complexes, inhibit oxidative stress in the Alzheimer's patient brain[112]. Those can contribute to convincingly direct design of drugs for treatment of neuron-related diseases[113].

Table 16 presents the IC_{50} values (mg/mL) of fractions of hexane and ethyl acetate from ethanolic extract of *Porphyridium* sp. and hexane, dichloromethane and ethyl acetate fractions from ethanolic extract of *S. costatum.*

Table 16: IC_{50} of Fe^{2+}CA of fraction with respective 95% CI

Crude extracts	**fractionating-solvents**	**10 (mg/mL)**	**IC_{50}**		
			mg/mL	**CI**	**R^2**
Psp.EtOH	crude extract	77.4 ± 0.2	0.522	0.471 – 0.572	0.972
	hexane		0.292	0.262 – 0.332	0.971
	ethyl acetate		4.22	3.74 – 4.78	0.933
ScEtOH	crude extract	83.5 ± 1.6	0.551	0.492 – 0.601	0.953
	hexane		0.263	0.252 – 0.274	0.994
	dichloromethane		4.25	3.88 – 4.66	0.961
	ethyl acetate		2.71	2.27 – 3.24	0.864

For that matter, *Porphyridium* sp. and *S. costatum* algal species can be potentially a source for redox metal (Cu^{2+} and Fe^{2+}) chelating compounds.

4.3. Calcium chelating activity (Ca^{2+}CA)

Apart from redox-active metal ions, in human brain, calcium is a divalent cation that regularly originates from intracellular stores in the endoplasmic reticulum to control many neuronal functions. Calcium (Ca^{2+}) has a great role for the interfacing between electrical and signal world in neuronal processes[114]. It is essential to maintain synaptic plasticity in the human brain for

Ca^{2+} dependent signaling, learning, memory and neuronal survival purposes. Calcium overload is able to cause damage to cell structures, loss of synapses and neurons cell, decline of cognitive capacity, and emotional disturbances. Alzheimer's disease is the most common Ca^{2+} induced neurodegenerative disease. Patients show loss of cognitive abilities due to short and long term neurodegeneration or cell death [114,115]. In order to sustain the neuronal process and integrity, the flow of Ca^{2+} within homo-cellular and hetero-cellular across the plasma and mitochondria membranes between intracellular organelles need to be controlled. Naturally it can be controlled by ion channels, Ca^{2+} binding proteins, ion exchangers, and voltage-operated channels (that release neurotransmitters at synaptic junctions)[116]. However, interaction of Ca^{2+} with proteins and reactive oxygen signaling species can be useful and harmful. Unregulated flow of Ca^{2+} can lead to impaired synaptic plasticity, and disassembly of synaptic connections due to proteins and neuronal damage[115]. Death of cells in the brain causes oxidative stress that leads to neurodegenerative diseases such as: Alzheimer's and brain inflammation.

Compounds that chelate calcium might be helpful. Indeed, they stabilize neuronal Ca^{2+} homeostasis, may retard cell degeneration in acute and chronic neurodegenerative conditions, and support neuronal survival. If immune-compounds were able to remove amyloid beta protein from the brain, immunization would be expected to prevent or reverse also the amyloid beta protein induced neuronal Ca^{2+} dysregulation[116]. Similarly, new chelating compounds with potential to get ride of excess flow of calcium in the brain are important ways to develop a novel therapeutic to prevent Ca^{2+}-induced neuronal diseases[114,115,116].

In present study, ethanol, ethyl acetate and water extracts of all species were tested for Ca^{2+}CA at 10 mg/mL. EGTA (1 mg/mL) and DMSO were used as positive and negative control respectively. The results show that only ethanol extracts from *Porphyridium* sp. (75.8%) and *S. costatum* (87.8%) and ethyl acetate extract of *S. costatum* (86.7%) and *Porphyridium* sp. (67.3%) had highest Ca^{2+} chelating ability presented in Table 17, however, extracts of ethanol and ethyl acetate of *S. costatum* are not significantly different at $p<0.05$ level. So that, ethanol extract of *S. costatum* is considered as the best candidate for a source of compounds with calcium chelating activity and also chelate Cu^{2+} and Fe^{2+}. Therefore, ethanol and ethyl acetate extracts of *Porphyridium* sp. and *S. costatum* were selected for IC_{50} analysis of Ca^{2+}CA.

Table 17: Calcium chelating activity (Ca^{2+}CA, %) of extracts

Species	extracting-solvents	10 mg/mL
H. pluvialis	ethanol	0.702 ± 0.122^{c}
	ethyl acetate	2.41 ± 0.76^{b}
	water	8.12 ± 0.76^{a}
***Isochrysis* sp.**	ethanol	52.8 ± 0.7^{a}
	ethyl acetate	38.1 ± 0.2^{b}
	water	6.67 ± 0.46^{c}
***Nannochloropsis* sp.**	ethanol	15.5 ± 0.6^{a}
	ethyl acetate	7.98 ± 0.15^{b}
	water	13.2 ± 1.5^{a}
P. tricornutum	ethanol	35.2 ± 1.1^{b}
	ethyl acetate	48.1 ± 0.5^{a}
	water	47.4 ± 0.5^{a}
***Porphyridium* sp.**	ethanol	75.8 ± 0.1^{a}
	ethyl acetate	67.3 ± 1.3^{b}
	water	28.6 ± 0.1^{c}
S. costatum	ethanol	87.8 ± 1.7^{a}
	ethyl acetate	86.7 ± 1.6^{a}
	water	24.3 ± 0.8^{b}
***Spirulina* sp.**	ethanol	48.8 ± 0.4^{a}
	ethyl acetate	19.4 ± 0.3^{b}
	water	7.12 ± 0.31^{c}
T. chui	ethanol	48.9 ± 0.5^{b}
	ethyl acetate	57.2 ± 2.1^{a}
	water	23.1 ± 2.2^{c}
***Tetraselmis* sp.CTP4**	ethanol	54.7 ± 1.1^{a}
	ethyl acetate	12.3 ± 0.3^{b}
	water	58.6 ± 1.5^{a}
***EGTA**	-	85.9 ± 0.5

Results were expressed as mean ± SEM (n = 8). Values labeled with different letters are significantly different ($p < 0.05$). *EGTA = Positive control, 1 mg/mL.

Interestingly, results shown in Figure 16, ethanol extracts of *Porphyridium* sp. and *S. costatum* displayed the most calcium chelating activity. Bioactive compounds of *Porphyridium* sp. and *S. costatum* could chelate 50% of free calcium ions that are found in both species at low concentration of 0.832 mg/mL and 1.02 mg/mL respectively (Table 18). Therefore, both extracts proceeded to fractionation stage to concentrate compounds that are responsible in chelating of Ca^{2+} and redox-active cations.

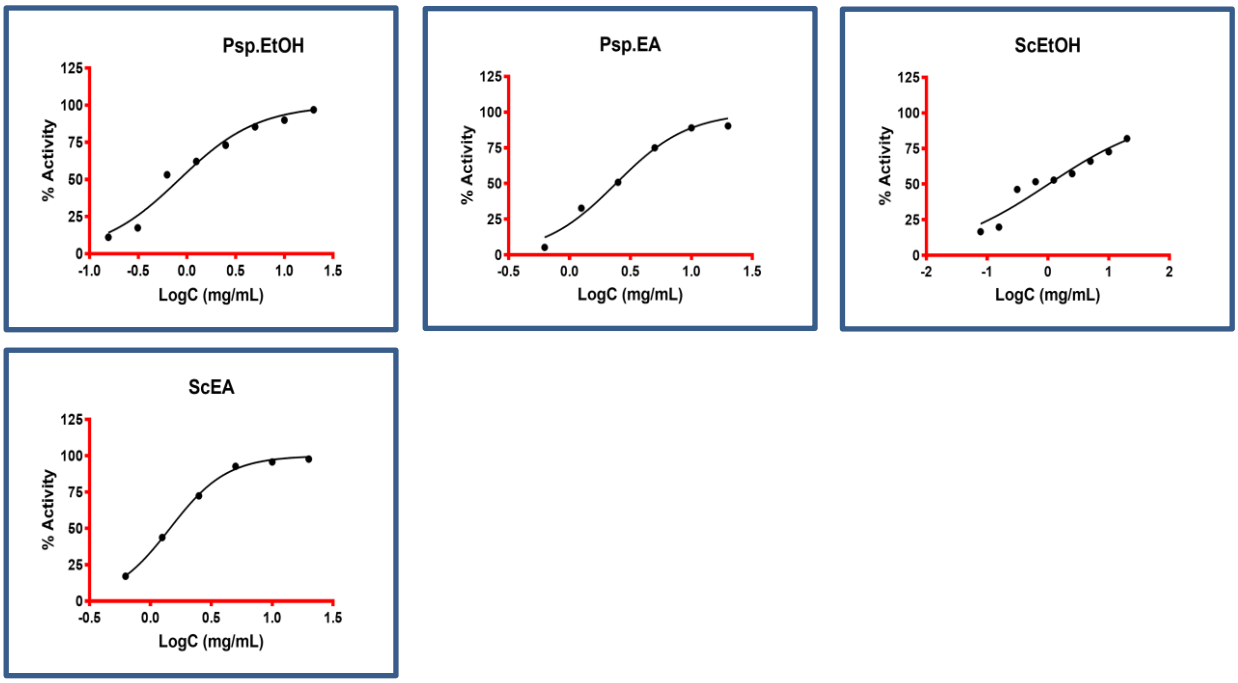


Figure 16: IC_{50} (dose response curve) of Ca^{2+}CA of extracts

Table 18: IC_{50} of Ca^{2+}CA of extracts with respective 95% CI

Species	**extracting-solvents**	**IC_{50}**		
		mg/mL	**CI**	**R^2**
***Porphyridium* sp.**	ethanol	0.832	0.762 - 0.911	0.972
	ethyl acetate	2.41	2.26 - 2.55	0.981
S. costatum	ethanol	1.02	0.883 - 1.19	0.923
	ethyl acetate	1.45	1.45 - 1.47	0.992

Ethanol extracts of *Porphyridium* sp. and *S. costatum* were subjected to fractionation using organic solvent of hexane, dichloromethane and ethyl acetate. We obtained fractions re-evaluated for calcium chelating activity at 10 mg/mL, 5mg/mL and 1 mg/mL as initial analysis to test their activity. According to results that are visualized in Table 19 the hexane fraction of ethanol extract of *Porphyridium* sp. (95.4%, 93.8% and 71.5%) and *S. costatum* (97.1%, 96.2% and 88.5%) showed maximum activity among others at 10 mg/mL, 5mg/mL and 1 mg/mL respectively. The next step was to test the hexane fractions 50% chelating concentrations (IC_{50}).

Table 19: Ca^{2+}CA (%) of fractions

Crude extracts	**fractionating -solvents**	**10 mg/mL**	**5 mg/mL**	**1 mg/mL**
Psp.EtOH	crude extract	80.1 ± 0.3	70.2 ± 0.3	52.2 ± 0.2
	hexane	95.4 ± 0.1[a]	93.8 ± 0.3[a]	71.5 ± 0.2[a]
	dichloromethane	52.5 ± 0.1[d]	47.6 ± 0.6[c]	37.8 ± 0.1[b]
	ethyl acetate	62.5 ± 0.1[b]	62.9 ± 0.9[b]	38.4 ± 0.1[b]
	aqueous ethanol	56.2 ± 0.5[c]	47.7 ± 0.1[c]	29.9 ± 0.1[c]
ScEtOH	crude extract	88.6 ± 0.7	76.5 ± 1.3	36.2 ± 1.1
	hexane	97.1 ± 0.1[a]	96.2 ± 0.1[a]	88.5 ± 0.1[a]
	dichloromethane	80.1 ± 0.6[b]	72.2 ± 0.6[b]	34.5 ± 0.3[c]
	ethyl acetate	81.5 ± 0.9[b]	71.4 ± 0.6[b]	42.6 ± 0.2[b]
	aqueous ethanol	73.4 ± 0.2[c]	67.5 ± 0.1[c]	26.1 ± 0.1[c]
***EGTA**	ultra-pure water	-	-	91.1 ± 0.1

Psp.EtOH = ethanol extract from *Porphyridium* sp.; ScEtOH = ethanol extract from *Skeletonema costatum*. *EGTA = positive control.

As clearly shown in the Figure 17 that confirming hexane fractions of *Porphyridium* sp. and *S. costatum* exhibited 50% chelation at lower concentration when compare to the others with the IC_{50} values of 0.451 mg/mL and 0.181 mg/mL (Table 20) respectively.

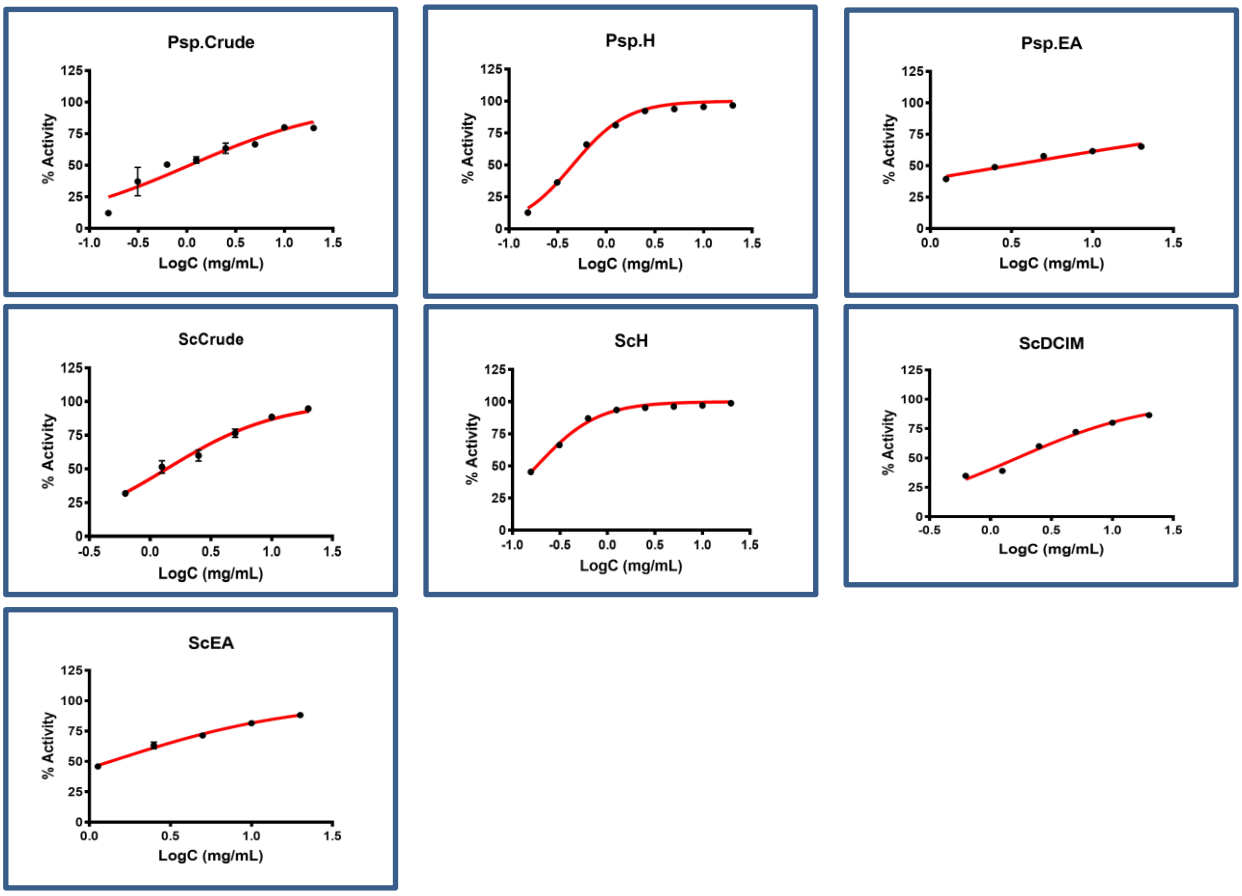


Figure 17: IC_{50} (dose response curve) of Ca^{2+}CA of fractions

Table 20: IC_{50} of Ca^{2+}CA of fractions with respective 95% CI

Crude extracts	**fractionating-solvents**	**10 (mg/mL)**	**IC_{50}**		
			mg/mL	**CI**	**R^2**
Psp.EtOH	crude extract	80.1 ± 0.3	1.06	0.88 – 1.27	0.891
	hexane		0.451	0.432 – 0.471	0.992
	dichloromethane		4.43	3.85 – 5.12	0.901
	ethyl acetate		3.03	2.75 – 3.31	0.963
ScEtOH	crude extract	88.6 ± 0.7	1.36	1.26 – 1.47	0.972
	hexane		0.181	0.173 – 0.192	0.994
	dichloromethane		1.66	1.54 – 1.77	0.981
	ethyl acetate		1.35	1.24 – 1.45	0.984

Clearly as can be noticed in Table 11, Table 15 and Table 19, hexane fraction of ethanol extract from *Porphyridium* sp. and *S. costatum* presented high chelating activity at 10 mg/mL, 5 mg/mL and 1 mg/mL for copper, iron and calcium. The values for Cu^{2+}CA are 81.2%, 70.5% and 64.4%, and with an IC_{50} value of 0.461 mg/mL for *Porphyridium* sp., and 92.6%, 80.4%, and 64.1%, and with an IC_{50} value of 0.361 mg/mL for *S. costatum* respectively. Similarly, for Fe^{2+}CA, 85.2%, 78.5% and 60.9% with an IC_{50} of 0.292 mg/mL, and 97.9%, 97.3% and 88.7% and with an IC_{50} value of 0.263 mg/mL for *Porphyridium* sp. and *S. costatum* respectively.

In the case of calcium chelating activity of hexane fraction from ethanol extract of *Porphyridium* sp. and *S. costatum* also contributed in slight proximity to each other at 10 mg/mL, 5 mg/mL and 1 mg/mL with values of 95.4%, 93.8% and 71.5% with an IC_{50} = 0.451, and 97.1%, 96.2% and 88.5% with IC_{50} value of 0.181 mg/mL respectively. This explains that compounds that are responsible for the activity are from the same polarity family, since it is known that the activity of target compounds and the solvent depends on polarity as it dictates reciprocal solubility, which means non-polar compounds are better-extracted using non-polar solvents. Hexane (a non-polar solvent) has been used widely all over the world as a solvent for extracting saturated, mono and poly unsaturated fatty acid or lipids[91]. Since, microalgae are rich in fatty acids, carotenoids, sterols, vitamins, and lipids, different methods have been applied to extract those compounds[91]. Therefore, both extracts are expected to have metabolites of fatty acid, sterols, vitamins, and lipids groups.

In general, at 10 mg/mL, extracts with greater than 50% MCA are considered as active in chelating metals. Taking this in consideration, ethanol, ethyl acetate and water extract of nine species were significantly varied among each other. As general comparison, extracts showed capacity to chelate Cu^{2+}, than Fe^{2+} and Ca^{2+}. The Fe^{2+} and Ca^{2+} chelating activity of extracts were moderate; the ethanol and ethyl acetate extract had higher MCA than water except Fe^{2+}CA water extract of *Tetraselmis* sp. CTP4 (96.6%), *Isochrysis* sp. (79.1%), *Nannochloropsis* sp. (83.2%), *P. tricornutum* (75.2), *S. costatum* (63.8%); Cu^{2+}CA of water extract of *Isochrysis* sp. (72.6%), *Nannochloropsis* sp. (72.3%), *P. tricornutum* (62.8%), *Porphyridium* sp. (52.5%) and *S. costatum* (74.2%) and Ca^{2+}CA of water extract of *Tetraselmis* sp. CTP4 (58.6%) were significant.

The differences of activity within the same species can be due to the difference in polarity of extracting-solvents type, extract preparation methods, state of chemicals and concentrations, environment and algal content of bio-active constituents. Those factors can potentially affect the

extracted metal chelating compounds[105,117,118]. To the best of our knowledge, Cu^{2+}CA, Fe^{2+}CA and Ca^{2+}CA of hexane fractions from ethanolic extract of *Porphyridium* sp. and *S. costatum* were reported for the first time. In addition, the chelation activity of *Porphyridium* sp. and *S. costatum* were not showed as metal chelating selecting, meaning that both presented copper, iron and calcium chelation activity. Which was the claim of synthetic chelator such as EDTA and EGTA, that chelate all metals found in the brain, that cause deficiency of useful bio-metals in the brain[44]. Algal extracts from different batches were proved that the percentage of metal chelating activity at the same concentration were approximately steady. It has been reported that microalgae are naturally capable of adapting to different seasons and conditions (temperature, light and pH)[119]. Despite the fact that, the percentage of metal chelating activity of the same species has shown differences amongst the batches, which are common because the cultivating conditions, methods in extraction and fractionation procedures can influence compounds concentration and biochemical composition of the microalgae[105].

Organic solvents such as hexane, dichloromethane, ethyl acetate were used to accumulate (concentrate) the compounds in their polarity difference. Solvents with polarity difference have a great factor to extract drug lead compounds from algal extract. Those fractionating-organic solvents were chosen with their polarity difference in order to make them suitable for research purposes including chemical characterization. Hexane is allowed by the Food and Drugs Administration (FDA) to be used in food and analytical laboratories for scientific investigation[67]. Both species extracts of non-polar (hexane) fractions possessed an excellent source of chelating compounds able to bind Fe^{2+}, Cu^{2+} and Ca^{2+}. That are pointing to a possible beneficial role as cation chelators having class of fatty acids, vitamins, sterols and others[61]. Thus, marine microalgae have proved that, as a potential source of compounds with neuroprotective capacity.

4.4. Chemical Characterization

In the past decades, marine microalgae gained popularity due to previously discovered novel and unique bio-active compounds[120]. In the result and discussion section, the hexane fractions from ethanol extract of algal species of *Porphyridium* sp. and *S. costatum* were concluded as the most promising in terms of copper, iron and calcium chelating activity.

In this section, those fractions were analyzed by using GC-MS in order to potentially identify their main neuron-protective compounds. GC-MS is a technique usually employed for separation, identification and quantification of volatile and semi-volatile compounds[118].

Algal species are composed of non-volatile compounds such as fatty acids and their various substituted forms. Derivatization was done to convert into derivatives suitable for GC. Treating the sample with methanol and acetyl chloride using derivatization (whose details were described in Section 3.7.1) were the main tasks. Then, GC-MS separated compounds of hexane fraction from ethanol extract of *Porphyridium* sp. and *S. costatum*, and tentatively identified compounds using GC-MS presented in (Table 21) and (Table 22) respectively. The peaks were identified by comparing linear retention times (minute), total area of the compounds, and their relative contribution to the total composition (percentage of total area). Those results matched with spectrum fragmentation pattern of the mass spectra in NIST library[121].

While interpreting the GC-MS result, the functional group has been modified from methylated ester ($RCOO\text{-}CH_3$) or methoxy ($RO\text{-}CH_3$) to carboxylic acid (RCOO-H) or alcoholic functional group (RO-H) respectively. The mechanism performed by substituting hydrogen in the place of methyl from their methyl attached portion. This process has advantages in the creation of volatile compounds, however, it might lead to confusion in distinguishing between the presence of methylated esters and methoxy in a natural form in the sample and from derivatization function[118].

The GC-MS analysis identified a total of 44 and 42 peaks from *Porphyridium* sp. and *S. costatum* of which 19 and 22 were successfully identified (Table 21) and (Table 22) respectively.

Table 21: Identified compounds present in hexane fraction of Psp.EtOH using GC-MS

Retention time (min)	**Compounds**	**chemical classes**	**total area (%)**
23.4	Hexadecanoic acid	SFA	27.8
30.9	5,8,11,14-Eicosatetraenoic acid	PUFA	13.4
31.1	5,8,11,14,17- Eicosapentaenoic acid	PUFA	7.49
27.2	9,12-Octadecadienoic acid	PUFA	7.22
22.6	7-Hexadecenoic acid	MUFA	3.48
27.4	11-Octadecenoic acid	MUFA	2.05
31.5	11,14-Eicosadienoic acid	PUFA	1.75
27.2	9-Octadecenoic acid	MUFA	0.881
25.7	Tetrahydropyranyl ether of citronellol	ether	0.681
31.3	cis-5,8,11,14,17- Eicosapentaenoic acid	PUFA	0.401
22.2	9,12,15-Octadecatrienoic acid	PUFA	0.359
26.6	γ-linolenic acid	PUFA	0.294
31.4	4,7,10,13,16,19-Docosahexaenoic acid	PUFA	0.205
31.6	9-Eicosenoic acid	MUFA	0.201
34.7	6,9,12,15-Docosatetraenoic acid.	PUFA	0.159
23.6	Pregna-5,9(11)-dien-20-ol-3-one ethylene ketal	sterol	0.155
34.4	4,7,10,13,16-Docosapentaenoic acid	PUFA	0.123
14.4	Octadecanoic acid	SFA	0.095
13.7	13-Octadecenoic acid	MUFA	0.052
-	Unidentified compounds	-	28.9
Total			**95.7**

According to GC-MS results three main chemical functional groups from *Porphyridium* sp. (Table 21) were identified such as:

- Fatty acids
  - Two saturated fatty acids (SFA)
  - Five monounsaturated fatty acids (MUFA)
  - Ten polyunsaturated fatty acids (PUFA)
- One sterol

- One ether

✓ Unidentified compounds

Table 22: Identified compounds present in hexane fraction of ScEtOH by (GC-MS)

Retention time (min)	**compounds**	**chemical classes**	**total area (%)**
18.6	Tetradecanoic acid	SFA	31.7
23.2	9-Hexadecenoic acid	MUFA	21.9
23.7	Hexadecanoic acid	SFA	12.1
14.9	1,1-Dodecanediol	alcohol	3.48
22.3	9,12,15-Octadecatrienoic acid	PUFA	1.83
41.7	Cholesterol	sterol	1.51
30.8	5,8,11,14,17-Eicosapentaenoic acid	PUFA	1.35
27.4	trans 13-Octadecenoic acid	MUFA	1.21
20.1	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	alcohol	0.991
24.7	9,12-Octadienoic acid	PUFA	0.937
27.3	9-Octadecenoic acid	MUFA	0.924
38.1	Tetracosanoic acid	SFA	0.827
27.9	Octadecanoic acid	SFA	0.686
24.2	3,3,6-Trimethyl-1,5-heptadien-4-ol	alcohol	0.683
30.9	9,10-Dihydroxy octadecanoic acid	PUFA	0.348
26.8	8,11,14,17-Eicosatetraenoic acid	PUFA	0.346
27.5	Phytol	alcohol	0.329
23.3	7-Hexadecenoic acid	MUFA	0.223
24.1	7,10-Hexadecadienoic acid	PUFA	0.159
37.9	15-Tetracosenoic acid	MUFA	0.142
36.1	Docosanoic acid	SFA	0.118
37.8	Gamma-sitosterol	sterol	0.102
-	Unidentified compounds	-	10.2
Total			**92.1**

Analyzed three chemical functions from *S. costatum* were as follow:

- ❖ Fatty acids
  - ✓ Five saturated fatty acids (SFA)
  - ✓ Five monounsaturated fatty acids (MUFA)
  - ✓ Six polyunsaturated fatty acids (PUFA)
- ❖ Four long-chain alcohols
- ❖ Two sterols
- ✓ Unidentified compounds

Among tentatively identified compounds present in hexane fractions of Psp.EtOH and ScEtOH visibly SFA, PUFA, alcoholic long-chain alcohols and MUFA were dominant in number. Those most dominant compounds were reported as bioactive compounds with biomedical activity in the pharmacological fields to treat diseases like cancer, oxidative stress neuron-related[122], viral, fungal, bacterial, diabetic, hypercholesterolemia and inflammation[123]. Similar chemical groups were previously identified from hexane extract of algal species in which polyunsaturated fatty acids[124] and steroids were the most abundant compounds[13], as a promising cation chelating agent and antioxidant. Therefore, compounds found in *Porphyridium* sp. and *S. costatum* can have neuron-protective ability. Compounds such as hexadecanoic acid (27.8%); 5,8,11,14-eicosatetraenoic acid (13.4%); 5,8,11,14,17-eicosapentaenoic acid (7.49%) and 9,12-octadecadienoic acid (7.22%) were the most abundant found in *Porphyridium* sp., and tetradecanoic acid (31.7%); followed by 9-hexadecenoic acid (21.9%), hexadecanoic acid (12.1%) and 1,1-dodecanediol were the leading in *S. costatum*.

It has been reported that hexadecanoic acid was identified from non-algal natural extracts of *Rutacea chalepensis* leaves as Fe^{2+} chelating agent[88] and from *Spongia officinalis* and *Cystoserio compressa* as antimicrobial agent[125]. Previously, hexadecanoic acid has been mentioned as most abundant in *Phaeodactylum* species[126]. As can be observed in Table 21, the most abundant peak identified by GC-MS was hexadecanoic acid with 27.8% of total peak area in which it has been described as antioxidant from algal *Sun clorella*[106] and also from the Red Algae *Laurencia brandenii*. Hexadecanoic acid was recorded as cytotoxic agents[123,127]. Thus recognizes that *Porphyridium* sp. could be significant source of bioactive compounds with neuroprotective capacity as well.

Apart from a high content of fatty acids, sterols were commonly found in algal species such as *Nannochloropsis* sp., *Tetraselmis* sp., *Chlorella vulgaris* and *Cyanophora paradoxa*. Naturally

occurring cholesterol-like triterpenes in plants and animals contribute to the regulation of membrane fluidity and permeability and form part of the structural components of cell membrane in different organisms. Due to their biological activities, may act as ROS scavengers[13], with antioxidant, anti-inflammatory and anti-hypercholesterolemic effects. Sterols improve neurological functions by preventing central nervous system from neuronal inflammation and by protecting from free radicals, β-amyloid-induced neurotoxicity leading to improved cognition in AD patients[13,128]. In this study, we have identified two sterols from *Porphyridium* sp. *(*Pregna-5,9 (11)-dien-20-ol-3-one ethylene ketal) with 0.155% at retention time of 23.6 min (Table 21) and from *S. costatum* in the form of cholesterol with 1.51% at retention time of 41.7 min (Table 22). In this consideration, both sterols could provide a valuable source for neuroprotective capacity.

It is obviously known that pharmaceutical industries have been desperately searching for a novel compounds that have not been previously identified. In this study out of 44 and 42 detected peaks, 19 and 22 were successfully identified using NIST library, with 95.7% and 92.1% of total peak area from hexane fraction of ethanol extract of *Porphyridium* sp. and *S. costatum* respectively. However, among unrevealed compounds, the largest peaks presented by *Porphyridium* sp. were as unidentified compounds (28.9%) of total peak area. Unidentified compounds were also presented in *S. costatum* at 10.2% of total area. Therefore, the majority of volatile or semi-volatile compounds present in the hexane fractions were successfully identified.

At this stage of study, it is difficult to pinpoint one specific compound that is responsible for cation chelator as a neuroprotective compound. However, the results shows that the most abundant compounds of saturated fatty acid of hexadecanoic acid, polyunsaturated fatty acid of 5,8,11,14-eicosatetraenoic acid and 5,8,11,14,17-eicosapentaenoic acid from *Porphyridium* sp. and mono unsaturated fatty acid of 7-hexadecenoic acid from, and saturated fatty acid of tetradecanoic acid, mono unsaturated fatty acid of 9-hexadecenoic acid and alcohol of 1,1-dodecanediol form *S. costatum* might act as cation chelating compounds, possessing neuroprotective activity.

Among identified compounds, long-chain fatty acids are the most abundant, polyunsaturated fatty acids were clearly seen as the most dominant compounds in the hexane fraction from ethanolic extract of *Porphyridium* sp. and *S. costatum.* It has been well explained that hexane extracts of microalgae are a significant source of polyunsaturated fatty acids such as 5,8,11,14,17-eicosapentaenoic acid which was identified as a dominant compound from hexane

extract of *T. chui*[13]. In this study, results show that *Porphyridium* sp. and *S. costatum* were reach in polyunsaturated fatty acids of 5,8,11,14-eicosatetraenoic acid and 5,8,11,14,17-eicosapentaenoic acid, and 5,8,11,14,17-eicosapentaenoic acid respectively. *Porphyridium* sp. and *S. costatum* exhibited a higher ability to chelate copper, iron and calcium in both polar and non-polar fractions. Genuinely it implies that compounds who are responsible to chelate the cations, may have different chemical character. However, Cu^{2+}, Fe^{2+} and Ca^{2+} chelating compounds found in the slightly polar fractions of dichloromethane (PI = 3.10) and ethyl acetate (PI = 4.40) performed less than in non-polar solvent of hexane (PI = 0.10) since the lowest IC_{50} values were obtained with the hexane fractions. This implied that Cu^{2+}, Fe^{2+} and Ca^{2+} chelating activity seemed to be mainly expressed in less polar and/or non-polar compounds[61].

The structure, conjugate bond, electron donor and/or acceptor sites of these compounds (or functional groups) are the main participants in the chelation mechanism[13].

The term chelator is derived from Greek word "*chele*" which means "*crab's claw*" or holding with a strong grip. The chelation complex is formed by a ligand or molecule containing at least two donor groups (or coordination numbers) connected to their substrates or ions to bind metal ions and form stable complexes[52]. Chelating agents are organic or inorganic compounds capable of binding metal ions. Chelating atoms can form either two covalent linkages the term bidentate refers to two donor groups or two coordinate linkages and so, bidentate chelators, tridentate chelators are linked to three coordinates or covalent linkages[37] as chelating rings and so on. Other types of chelating ligands are possible, like EDTA, which is a hexadentate ligand. Some chelators are able to form multi dentate complexes. They can permeate cell membranes upon binding metal ions[37,52].

Chelating agents form covalent linkages or coordinate and multi dentate ligands with atoms attached to the metal. Mainly atoms like S, N and O function as ligand atoms in the form of chemical groups like –SH, –S-S, $–NH_2$, =NH, –OH, $–OPO_3H$, or >C=O[37]. The following figure shows the formation of metal ligand complexes using mono, bi and polydentate ligands (chelators) Figure 18[37].

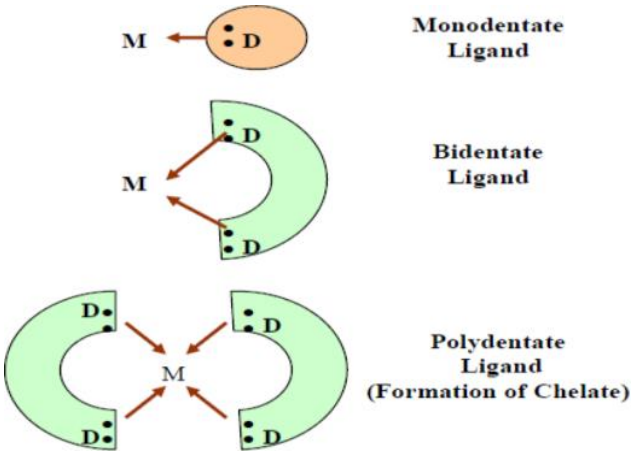
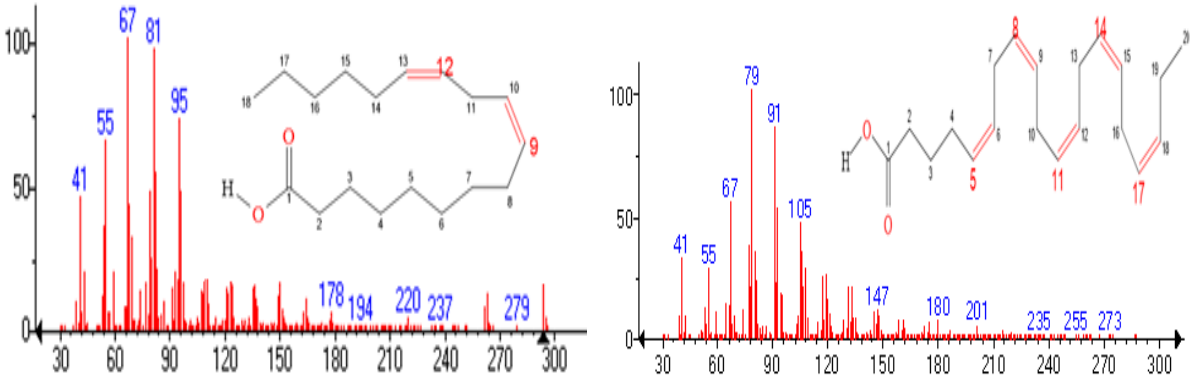


Figure 18: Formation of metal ligand complexes due to chelators

M implies metals to be chelate by means of dentate (D) or chelating site.

Compounds that were identified in this study, unsaturated fatty acids especially polyunsaturated fatty acids might be responsible for the chelation character. Since, they have double bonds[91], the delocalization of π-electrons enhances the lipophilicity (ability to dissolve in oils, fats, lipids, and non-polar solvents) of the complexes, ending up to block the metal binding sites. Chelate formation through ring size, number of aromatics and heterocyclic rings (rings, which contain sulfur, nitrogen, and/or oxygen) are responsible for the metal chelating activity and their metal complexes formation[129]. Polyunsaturated fatty acids contain C=O at the active chelating site, since it contains π-electrons and also two pair donating electrons in the oxygen atoms that makes the compound able to chelate metal ions[37].

Compounds that have been identified in this study such as 9,12-octadecadienoic acid (Z,Z) (left) at retention time of 27.2 min and 5,8,11,14,17-eicosapentaenoic acid (right) at retention time of 31.1 min are good examples having poly dentate groups due to C=O and π-electrons as a metal chelating site (Figure 19).



9,12-Octadecadienoic acid (Z,Z) 5,8,11,14,17-Eicosapentaenoic acid

Figure 19: Examples of chelating compounds having poly-dentate site

Compound 9,12-otadecadienoic acid (Z,Z) was from hexane fraction of Psp.EtOH and 5,8,11,14,17-eicosapentaenoic acid was from hexane fraction of *Sc*EtOH.

In the compound 9,12-octadecadienoic acid (Z,Z) the 4 chelating sites are the following: two oxygen atoms that are bonded at the first carbon atom (each oxygen atom contains a pair of electrons) and π-electrons that are found in the carbon atoms 9 and 12 to form ligand-metal (Cu^{2+}, Fe^{2+} and Ca^{2+} that are found in the brain) bonds. Equally, 5,8,11,14,17- eicosapentaenoic acid, has 7 chelating sites: two oxygen atoms and π-electrons in carbon atom of 5,8,11,14 and 17 that could chelate the excess Cu^{2+}, Fe^{2+} and Ca^{2+} ions of overload in the Alzheimer patients brain.

Natural chelators characterized by same distribution as the metal with greater affinity having low toxicity able to penetrate via cell membranes, rapidly soluble in water and ultimately eliminate toxic metal by forming stable and non-toxic complexes. They regularly rescue the cells by shielding biological targets from further damage, loss and death[37].

However, most of the compounds that were identified in this study could be metal chelators; are non-polar and insoluble in water. By using metal chelating agents, it have been possible to reduce the cation concentrations in the brains of patients with AD, leading to a small, but significant improvement in cognitive function[130].

In order to investigate the exact compound, further studies are very important and needed. We recommend that hexane fraction of both species *Porphyridium* sp. and *S. costatum* should be re-fractionated again, separate fatty acid mixture into one fraction rich in saturated fatty acids and the other rich in unsaturated acids. These obtained sub-fractions should be re-tested for their

cation chelating activity of (Cu^{2+}, Fe^{2+} and Ca^{2+}). Through this the number of compounds can be reduced and it becomes easy to understand the mechanism to target specific compounds with neuroprotective capacity.

In this study only GC-MS separation and characterization technique was used to investigate volatile compounds in the fractions, however, some new, non-volatile compounds might be found in the fractions as neuron protectors. So that, in order to characterize non-volatile compounds, other non-volatile detecting analytical method would be recommended such as Liquid Chromatography coupled with Mass Spectrometry (LC-MS).

To the best of our knowledge and based on reports of previous publications that address metal chelating activity of microalgae, there are no reports on the new source of neuroprotective compounds in terms of chelating cations such as Cu^{2+}, Fe^{2+} and Ca^{2+} of *Porphyridium* sp. and *S. costatum.* The results of nine species with calcium chelating activity were documented for the first time. However, the identified compounds were already reported and characterized by NIST. Thus, *Porphyridium* sp. and *S. costatum* couldn't be a promising source of new-compounds for the application of pharmaceutical drug formulation. Since, pharmaceutical industries dreadfully investigate for a new drug candidate that have not been previously identified and characterized by NIST library. Still, countless unexplored bioactive compounds from marine microalgae have been gaining great attention due to its potential as novel bioactive compounds. As a result, scientists have been investigating new bio-active compounds. Other species have been considered to have neuroprotective capacity apart from that already referred (of *Porphyridium* sp. and *S. costatum*).

5. CONCLUSION

Overall results indicate that microalgae extracts are potentially valuable sources of neuroprotective compounds in terms of copper, iron and calcium chelating agents, which provide a new insight into their use as a source of therapeutic compounds for metal-induced neurodegenerative disease such as AD. According to results, *Porphyridium* sp. and *S. costatum* are promising strains for natural products of new neuroprotective agents for designing as a good alternative ingredients in pharmaceuticals industries and including in the nutrition industries, in order to prevent and treat cation-induced neurodegenerative diseases specially Alzheimer's.

A successful GC-MS analysis has done for hexane fractions about 19 and 22 volatile compounds with 95.7% of *Porphyridium* sp. and 92.1% of *S. costatum* confirmed using NIST library respectively. In this study, many biologically beneficial compound groups were identified such as long chain saturated and unsaturated (mono and poly) fatty acids, ether, alcohols and sterols. Among those, hexadecanoic acid (27.8%); 5,8,11,14-eicosatetraenoic acid (13.4%); 5,8,11,14,17-eicosapentaenoic acid (7.29%) and 9,12-octadecadienoic acid (7.22); and tetradecanoic acid (31.7%), 9-hexadecenoic acid (21.9%), hexadecanoic acid (12.9%) were found to be the most promising.

The plotted chromatograms, spectra and the structure of most abundant compounds that were tentatively identified from hexane fraction from ethanol extract of *Porphyridium* sp. and *S. costatum* were referred in the section of Annex 1 and Annex 2.

6. PERSPECTIVES FOR FUTURE STUDIES

Due to a matter of time, Cu^{2+}CA of water extract from *Isochrysis* sp. and Ca^{2+}CA of ethyl acetate extract from *S. costatum* were not been further investigated. Results suggested that water extract from *Isochrysis* sp. seems selective towards Cu^{2+}CA with an IC_{50} value of 1.84 mg/mL and it is not active in chelating Ca^{2+} whereas less active towards Fe^{2+} with an IC_{50} = 4.43 mg/mL. Ethyl acetate extract of *S. costatum* was significantly active in chelating Ca^{2+} with an IC_{50} = 1.45 mg/mL than Cu^{2+}CA with an IC_{50} = 4.87 mg/mL and reduced efficacy to chelate Fe^{2+}. Therefore, both extracts of algae could be a source to design selective-capturing biomolecules for the pharmaceutical industries and the production of food supplements. Water extract of *Isochrysis* sp. showed as selective copper chelator and ethyl acetate extract of *S. costatum* showed as selective calcium chelator.

It has been stated that, cation chelator should possess high enough selectivity to remove the targeted metal ion excluding other interference in specific extracts, basically by those essential metal ions which are present in significant concentrations[44]. For this reason, both needed to be fractionated and re-tested to evaluate their cation-selective chelating ability. If their fractions maintain their selective ability, it would be recommended to separate, characterize it in order to investigate, to point out in charged specific compounds and to design cation-selective-induced neurodegenerative disease like Alzheimer's.

Porphyridium sp. and *S. costatum* were not selective towards each chelating activity, in other hand both are active in chelating copper, iron and calcium with approximately low range of variation of concentration, which was one of the drawbacks of synthetic metal chelators (EDTA and EGTA) meant as universal cations chelators which most probably lead to have deficiency of useful cations[131].

Moreover, hexane fractions of *Porphyridium* sp. and *S. costatum* should be re-fractionated, in order to obtain concentrated and limited active compounds. Therefore, further studies still needed to isolate specific bioactive compounds and then reconfirming of the sub-fractions to their neuroprotective properties in terms of copper, iron and calcium chelation.

In this study, only volatile compounds were identified using GC-MS however non-volatile compounds should be identified using another method such as LC-MS in order to search for new source of possible active non-volatile compounds with neuroprotective capacity.

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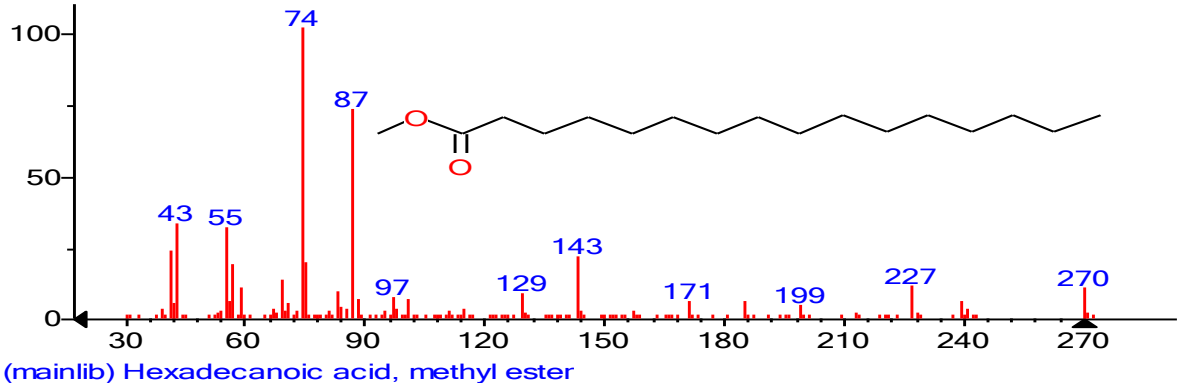
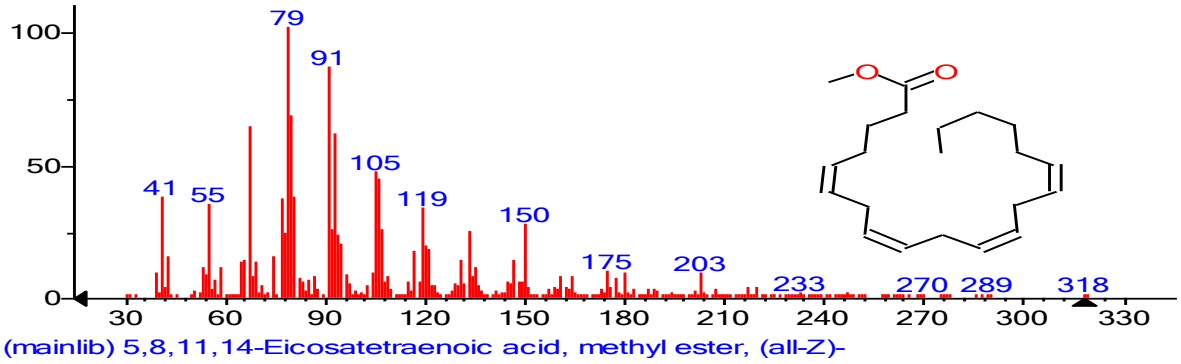
ANNEX

Annex 1: Tentatively identified the most abundant compounds detected in hexane fraction of ethanol extract from *Phaeodactylum* sp. by GC-MS

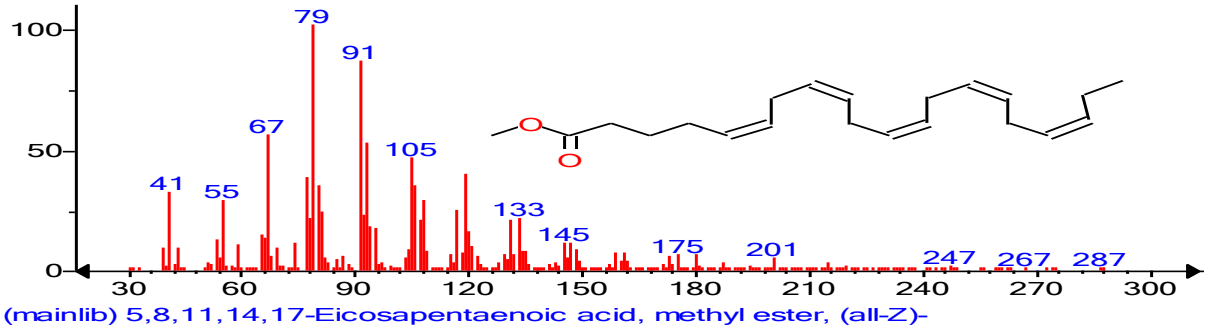
Note: All presented compounds in the Annex are identified derivatives compounds using GC-MS, without replacement of hydrogen in place of methyl group.



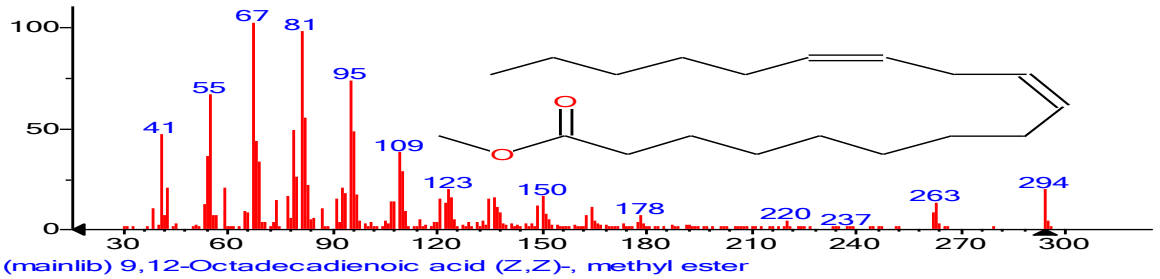
Hexadecanoic acid, methyl ester ($C_{17}H_{34}O_2$)



5,8,11,14-Eicosatetraenoic acid, methyl ester, (all-Z)- ($C_{21}H_{34}O_2$)

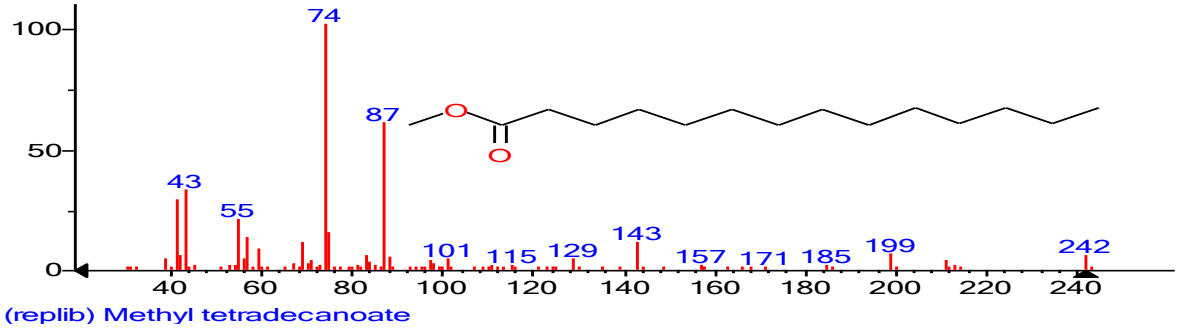


5,8,11,14,17-Eicosapentaenoic acid, methyl ester, (all-Z)- ($C_{21}H_{32}O_2$)

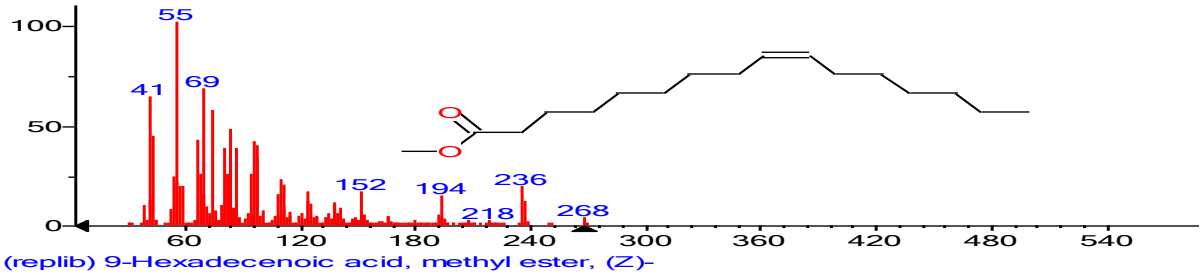


9,12-Octadecadienoic acid (Z,Z)-, methyl ester ($C_{19}H_{34}O_2$)

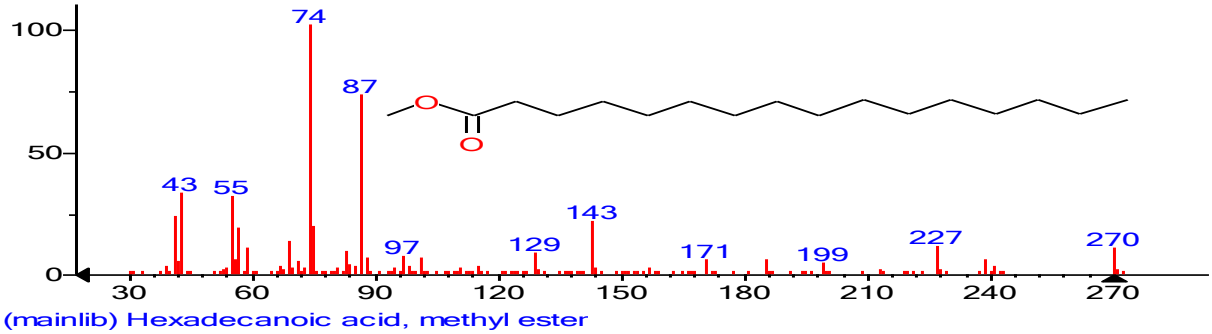
Annex 2: Identified most abundant compounds present in hexane fraction of Ethanol extract from *Skeletonema costatum* using GC-MS



Methyl tetradecanoate ($C_{15}H_{30}O_2$)



9-Hexadecenoic acid, methyl ester, (Z) ($C_{17}H_{32}O_2$)



Hexadecanoic acid, methyl ester ($C_{17}H_{28}O_2$)