SEGISMUNDO GARCÍA VALVERDE

ISOLATION AND IDENTIFICATION OF BIOACTIVE COMPOUNDS FROM MICROALGAE

UAlg
UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

2023

SEGISMUNDO GARCÍA VALVERDE

ISOLATION AND IDENTIFICATION OF BIOACTIVE COMPOUNDS FROM MICROALGAE

Mestrado em Inovação Química e Regulamentação /

Erasmus Mundus MSc in Chemical Innovation and Regulation

Trabalho efetuado sob a orientação de:

Professora Luísa Barreira

Faculdade de Ciências e Tecnologia

2023

Declaration of Authorship

I declare that I am the author of this work, which is original. The work cites other authors and works, which are adequately referred in the text and are listed in the bibliography.

_____

Segismundo García Valverde

Acknowledgements

I would like to express my gratitude to my supervisor, Prof. Luísa Barreira, for welcoming me to Faro and to Universidade do Algarve, for introducing me to the exciting world of microalgae and for guiding me through the process of writing this thesis. Her expertise and constructive feedback were crucial for shaping the direction of this work.

I also want to thank all the students, researchers, and staff of CCMAR and MarBioTech who were my colleagues during the development of this thesis. All of them taught me meaningful lessons during my stay there, and offered support and advice anytime I needed it. I want to specially thank PhD student Mélanie Silva, who worked with me on daily basis, and with whom I shared joy, frustrations, and numerous enriching conversations. Thanks to her patience and commitment this academic journey was a fulfilling experience.

I also would like to thank all the good Portuguese people that welcomed me in Faro and showed me an unknown angle of my neighbour country, where a part of me belongs from now on. Thanks to them, during my stay in Faro I did not only progress academically, but I also enjoyed a wonderful culture and gastronomy as well as astonishing landscapes and a pleasant lifestyle.

I also want to thank the Erasmus Mundus programme, specifically the Erasmus Mundus MSc in Chemical Innovation and Regulation (ChIR) from which I enjoy a studentship (grant agreement 619824-EMJMD). Thanks to the programme management team, for bringing me the opportunity to live in different countries while I study what I am passionate about.

Finally, and above all, thanks to my friends and family. Despite living far away from you all, the thought of seeing you again gives me strength to keep moving forward every morning.

"The ocean is a desert with its life underground
And a perfect disguise above"
-A Horse With No Name (America)

"Ars longa vita brevis"
-Hippocrates

Resumo

O termo "microalga" descreve uma ampla variedade de espécies de algas microscópicas, tanto eucariotas (como as diatomáceas ou as algas verdes) quanto procariotas, como as cianobactérias. As microalgas compõem um grupo pouco explorado de organismos, com menos de 10 % das espécies descritas em relação ao total estimado. Geralmente, são organismos fotoautotróficos que capturam CO_2 como fonte de carbono e utilizam luz solar como fonte de energia. São até 50 vezes mais eficientes que as plantas superiores na fotossíntese, o que lhes confere maior rendimento de biomassa e maior taxa de crescimento. Além disso, são um grupo altamente biodiverso, e podem ser encontradas espécies adaptadas para sobreviver em uma ampla gama de condições ambientais.

O metabolismo das microalgas é altamente influenciado pelas condições ambientais do cultivo, tornando possível modificá-las de forma controlada para controlar seus ritmos de crescimento e otimizar a composição bioquímica das microalgas de acordo com os interesses comerciais de seu cultivo. Entre os compostos naturais encontrados nas microalgas estão ácidos gordos, proteínas, vitaminas, polifenóis, pigmentos carotenoides, entre muitos outros. Graças às suas propriedades, as microalgas são úteis para a descoberta e produção de produtos naturais com aplicações terapêuticas, nutricionais, na produção de biocombustíveis e até mesmo na biorremediação e tratamento de águas. Além disso, devido às suas características naturais, os processos produtivos que utilizam microalgas estão alinhados com as políticas de bioeconomia circular promovidas pela União Europeia para acelerar o progresso em direção a uma economia com baixas emissões de carbono.

Apesar de todas as vantagens das microalgas, os produtos delas derivados ainda não conseguiram entrar no mercado para substituir os concorrentes atuais (por exemplo, combustíveis fósseis ou medicamentos sintetizados por processos altamente poluentes). Isso deve-se aos altos custos do cultivo e processamento das microalgas, bem como a problemas de escalabilidade. Uma das possíveis soluções para tornar os produtos de microalgas economicamente viáveis é a inovação. Ao descobrir novas espécies e conhecer melhor as características e composição das microalgas, será possível otimizar as suas condições de cultivo e processamento, permitindo que entrem no mercado como uma alternativa verde e competitiva.

Historicamente, a pesquisa tem se concentrado na extração de biocombustíveis das microalgas, mas na última década, a extração de compostos de valor acrescentado e a produção de nutracêuticos têm ganhado maior relevância, uma vez que é necessário extrair o máximo possível de produtos das microalgas para capturar completamente o valor de seu cultivo.

O objetivo deste trabalho foi identificar compostos bioativos da microalga *Tetraselmis striata* CTP4. Esta espécie foi isolada na Ria Formosa, Portugal, e tem mostrado resultados promissores na produção de biocombustíveis e nutracêuticos, além de ter sido cultivada com sucesso à escala industrial. No entanto, sua composição e muitas de suas propriedades ainda não foram completamente descritas.

Para cumprir os objetivos deste trabalho, a biomassa foi processada com a ajuda de um moinho de bolas de aço inoxidável e foram preparados extratos de hexano, etanol e acetato de etilo usando um sistema automatizado de extração (EDGE®). O uso de solventes com diferentes polaridades permitiu alargar o espectro de compostos extraídos. Além disso, o etanol e o acetato de etilo são compatíveis com a produção de preparações alimentares. Os extratos foram submetidos a testes de atividade antioxidante através dos ensaios ABTS, DPPH e FRAP. Também foi testada sua atividade na regulação da hipertensão arterial através de ensaios de inibição da enzima conversora da angiotensina (ACE). Todos os ensaios foram realizados em diferentes concentrações para calcular o parâmetro IC_{50} de cada extrato em cada teste. Foi constatado que o extrato de acetato de etilo era o mais bioativo, com resultados de IC_{50} de 4,1 ± 0,7 mg/mL, 6,6 ± 0,9 mg/mL e 4,0 ± 2,0 mg/mL para os testes ABTS, DPPH e FRAP, respetivamente, além de um valor de 0,40 ± 0,07 mg/mL para o ensaio de inibição da ACE.

O extrato de acetato de etilo foi submetido a fracionamento por HPLC originando 41 frações. Algumas das frações com menor massa foram combinadas para obter a massa necessária para a análise da bioatividade, enquanto que as frações que tinham massa suficiente foram mantidas como foram coletadas inicialmente. Dessa forma, foram obtidas 16 frações finais (A-P) que foram submetidas aos mesmos testes de bioatividade que o extrato original de acetato de etilo. As frações C, G, N e O foram identificadas como as frações com os maiores níveis de bioatividade em relação ao extrato original de acetato de etilo. Isso significa que os compostos bioativos estavam concentrados nestas frações.

Para a identificação tentativa de alguns dos compostos bioativos presentes nas frações, recorreu-se à cromatografia líquida acoplada à espectrometria de massa. Os espectros obtidos para cada uma das frações foram comparados com espetros encontrados nas bases de dados (NIST, Wiley) que contêm centenas de milhares de espectros. Foram identificados tentativamente 16 compostos, muitos dos quais possuem funcionalidades antioxidantes ou têm similaridades estruturais com inibidores conhecidos da ACE. Além disso, a estrutura principal vanillin-semicarbazone do composto número 14, 4-(hexyloxy)vanillin-semicarbazone, é uma molécula descrita na literatura como um potencial agente antitumoral e com comprovadas propriedades antioxidantes e antibacterianas, entre outras.

Este estudo abrange os primeiros passos necessários para a descoberta de moléculas interessantes em microalgas. Pesquisas futuras devem repetir o fracionamento do extrato, isolar os compostos de interesse e confirmar a sua estrutura através de ressonância magnética nuclear. Posteriormente, os compostos devem ser purificados e submetidos a diferentes testes e ensaios clínicos para confirmar as suas propriedades biológicas e estudar o seu modo de ação. Posteriormente, a sua possível produção em larga escala, e comercialização como medicamentos, nutracêuticos ou alimentos funcionais poderá ser equacionada.

Em resumo, este estudo confirmou as propriedades antioxidantes dos extratos de *Tetraselmis striata* CTP4, além de revelar as suas propriedades anti-hipertensivas. Também abriu caminho para a identificação de alguns dos compostos bioativos responsáveis pelas propriedades observadas. Dessa forma, expandiu-se o conhecimento sobre esta espécie de microalga e consolidou-se o seu futuro promissor como fonte sustentável de compostos bioativos.

Palavras-chave: Microalgas, antioxidante, anti-hipertensivo, compostos bioativos, compostos de alto valor.

Abstract

Microalgae are an untapped resource with numerous potential commercial applications in biofuels production, drug discovery and production, food and feed, cosmetics, and environmental remediation, among other fields. They display unique properties such as tuneable growth rates and biochemical composition, higher photosynthetic efficiency than higher plants, and low water intensity cultivation. Also, there is a wide variety of microalgae species that can be cultivated in diverse environmental conditions, and it is estimated that most of the species have not been described yet. In short, the cultivation of microalgae offers a green alternative to many goods, products, and processes. However, projects involving products derived from microalgae cultivation struggle to be economically feasible because of high costs, inefficient processes, and problems of scalability from laboratory to industrial scale. Expanding our knowledge on microalgae is key to fully unlock their potential. For instance, the study of new promising strains could yield the discovery of natural compounds with interesting therapeutical or nutritional applications. This work is focused on the study of the bioactivity of *Tetraselmis striata* CTP4, a promising strain of microalgae isolated in Faro, Portugal. Biomass was processed and extracts of different solvents were elaborated and tested for their antioxidant (ABTS, DPPH, and FRAP assays) and antihypertensive (angiotensin converting enzyme - ACE - inhibition assay) activity, finding the ethyl acetate extracts as the most bioactive overall. This extract was further divided in fractions using preparative HPLC and the fractions tested again to narrow on the bioactive compounds. Finally, the four most interesting fractions were analysed through LC-MS to tentatively identify the compounds that possibly caused the observed bioactivity. Sixteen compounds were tentatively identified, most of them with antioxidant moieties and some similarities to traditional ACE inhibitors. Interestingly, compound 14, 4-(hexyloxy)-vanillin-semicarbazone, is a derivative of the vainillin-semicarbazone molecule, proven to have anticancer, antioxidant, antibacterial, and anti-inflammatory activity and is here described for the first time in *T. striata* CTP4, and, to the best of our knowledge, in microalgae in general. In summary, This work opened the door for the identification of bioactive compounds in *T. striata* CTP4.

Keywords: Microalgae, antioxidant, antihypertensive, bioactive compounds, high-value compounds.

Subject Index

Figure index

Table index

Abbreviations and acronyms

PUFAs – Polyunsaturated fatty acids

EPA – Eicopentaneoic acid

DHA – Docosahexanoic acid

ROS – Reactive oxygen species

DNA – Deoxyribonucleic acid

ACE – Angiotensin-converting enzyme

EU – European Union

EFSA – European Food Security Agency

EC – European Commission

GM – Genetically modified

DMSO – Dimethylsulfoxide

HPLC – High performance liquid chromatography

TCA – Trichloroacetic acid

ABTS - 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid

DPPH - 2,2-diphenyl-1-picrylhydrazyl

LC-MS – Liquid chromatography – Mass Spectrometry

FRAP - Ferric iron reducing power

EDGE® - Energized dispersive guided extraction system

SET - Single-electron transfer

ACN – Acetonitrile

ELSD - Evaporative light scattering detector

PAD - photo diode array detector

1. Introduction

1.1 Microalgae: Characteristics and market potential

The term microalgae describes a wide range of microscopic algae species. It comprises both eukaryotic (e.g., diatoms, green algae) and prokaryotic (e.g., cyanobacteria) mainly photoautotrophic organisms. They make up the most biodiverse group of living organisms, and they are present in most ecological niches in both marine and terrestrial waters. More than 50,000 species of microalgae have been described, but the total number is estimated to be around 800,000[1].

Microalgae are generally autotrophic organisms that use CO_2 as their carbon source together with sunlight to obtain energy and perform photosynthesis. Microalgae are the predominant component of phytoplankton and they are accountable for more than 40 % of the global photosynthetic primary production (oxygen supply)[2]. In fact, microalgae are 10-50 times more efficient than higher plants in transforming solar energy, consequently, they display much higher biomass production yield and faster growth rates[3].

Due to the high biodiversity of the group, it is possible to find microalgae species adapted to survive in all sorts of environments, including a wide range of pH, temperature, salinity, and light exposure conditions. Furthermore, microalgal metabolism is highly conditioned by growth conditions, meaning that growth rate and biochemical composition can be tuned and optimised for any given species and purpose[4].

There is a vast array of valuable biomolecules that can be obtained from microalgal biomass, including but not limited to antioxidants, pigments, anti-inflammatory compounds, essential fatty acids, polyunsaturated fatty acids, proteins, vitamins, polyphenols, and polysaccharides[5]. Actually, although land plants are highly exploited for their production of natural products, the diversity of compounds produced by algal species is estimated to be over 10 times greater than those produced by land plants[6].

In addition, thanks to their adaptability, microalgae cultivation can be carried out using waste inputs such as wastewater. In this way, high value biomass can be created while remediating polluted influxes and capturing CO_2. This goes in line with the principles of circular bioeconomy, which the European Union embraces to accelerate progress towards a circular, low-carbon economy[7].

All these characteristics made microalgae a very appealing subject of study in the recent years, as commercial applications can be found in diverse areas: biofuels, high-value chemicals, bioactive compounds with application in the cosmetic and pharmaceutical industries, nutrition, and environmental remediation, among others[8].

However, commercialization of microalgae-derived products still faces some important challenges, most of them related with the scalability of the cultivation and the economic feasibility of the projects. It is for this reason that innovation is much needed to finally unlock the full potential of microalgae as a source of food, energy and high value compounds, as outlined in Figure 1[9].

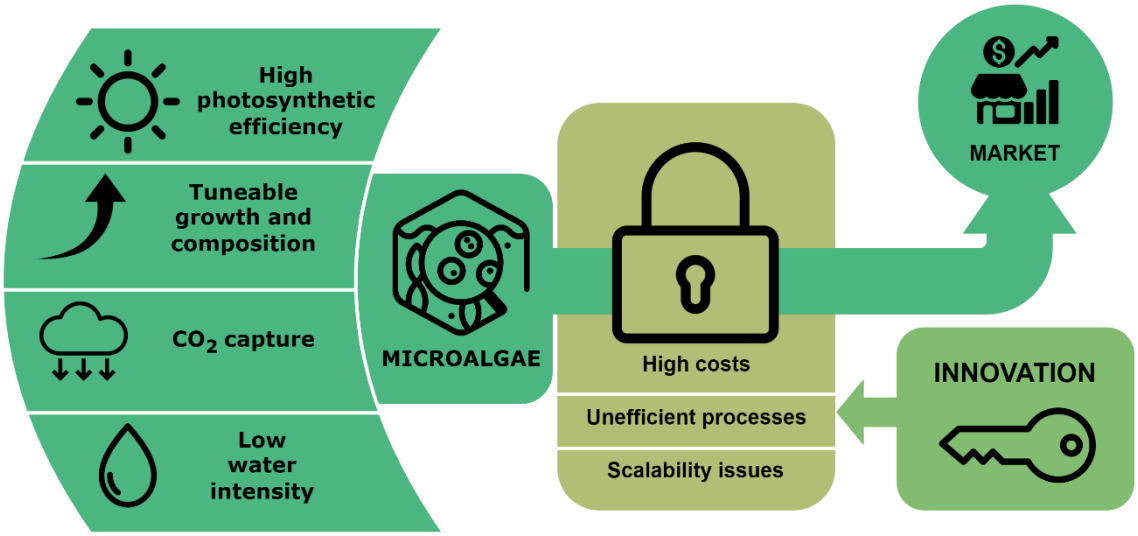


Figure 1. Innovation is the key to unlock the path of microalgal technologies to the market.

1.2 Historical overview

Microalgae have been used as a food source for thousands of years. The first evidence traces back to China 2000 years ago, when *Nostoc* was consumed to survive through a famine. Several other cultures around the world are known to regularly consume microalgae, specially being a high-valued food source for communities in arid regions where other food sources are limited[8]. For instance, the population of Chad harvest *Arthtrospira* ('dihé') from Lake Kossorom daily to use it as a food. The species *N. commune*, *N. flageliforme* and *N. punctiforme* are traditionally consumed in China, Mongolia, and South America. In Japan, the cyanobacterium *Aphanotheca sacrum* is considered a special delicacy. Indigenous Mexican communities collected blue-green

algae from Lake Texcoco to prepare a dry cake known as 'tecuitlatl', according to early Spanish chroniclers[10].

Despite this rich background, laboratory cultivation of microalgae only started in late 19th century and commercial large-scale production in the second half of the 20th century. In the 1950's concerns raised about insufficient protein supply for a fast-growing world population, and algal biomass was presented as a good candidate to solve the issue. The first Algal Mass-Culture Symposium took place in 1952 at Stanford University, where new technologies were suggested for mass exploitation of microalgae apart from being harvested from the environment. *Arthrospira* was declared a 'food for the future' in 1974's United Nations World Food Conference and kept gaining popularity in later World Health Organization and Food and Agriculture Organization events, being presented as an alternative to achieve food of high quality with low environmental impact, rich in minerals and protein and safe to be administered to children[8,10].

In this context, interest in applied algal culture continued and the systematic examination of algae for biologically active substances, particularly antibiotics, began. During the second half of the century different events increased interest in certain applications. The space race fuelled research on microalgae as CO_2 capture systems. Later, with the raise of environmental technology, microalgae were used to improve the quality of wastewater and in bioremediation processes[11].

Due to the 1970's oil crisis, large investments were made in research of microalgae as a renewable energy resource under the 'Aquatic Species Programme' funded by the US Department of Energy. The most important conclusion of the programme was that 'the conditions that promote high productivity and rapid growth (nutrient abundance) and the conditions that induce lipid accumulation (nutrient limitation) are mutually exclusive. Further research will be needed to overcome this barrier probably involving genetic manipulation of algal strains to increase photosynthetic efficiency or to increase constitutive levels of lipid synthesis in algal strains.' Another research-intensive campaign worth mentioning is the Japanese Biological CO_2 Fixation Programme during the 1990's, although its outcomes are not as clear[12].

Research in microalgae has been driven both by basic research, for instance when screening for high value molecules or isolating undescribed strains, and by applied research, when bursts of interest came from the political priority to solve certain problems

(e.g., oil crisis). This context quickly generated a vast innovation pathway that brought advances in phycology, biochemistry, nutrition, pharmacology, engineering, pollution remediation and genetics, among others[12].

Efforts are now focused on mid and downstream processing and genetic manipulation to improve the efficiency and thus the economic viability of the derived products. In this sense, plants called 'biorefineries' are now being assessed as an option to further increase the market potential of microalgae by combining the extraction of different derivatives in one single installation (e.g., biofuels, high value chemicals and wastewater remediation)[13].

Figure 2 shows the evolution of research interest in different topics concerning microalgae over time. It must be considered that because of the commercial sensitivity of applied research, most of the studies on microalgae pre-2005 were not published in the form of academic papers. Interest in microalgae for food and nutrition, biofuels, biorefineries and high-value compounds grew exponentially in the 2005-2015 period, while a moderate linear growth is observed in wastewater treatment with microalgae.

The excitement over biofuels slowed down around 2015 when food and nutrition and high-value chemicals became the two most important microalgae research topics. This is because the scientific community reached a consensus around the economical non-viability of biofuels, that can only be achieved if combined with other processes such as the extraction of high-value chemicals, the production of nutrients or the treatment of wastewater in systems known as biorefineries. In fact, biorefineries also show an exponential increase and are expected to surpass biofuels as a research trend in the following years.

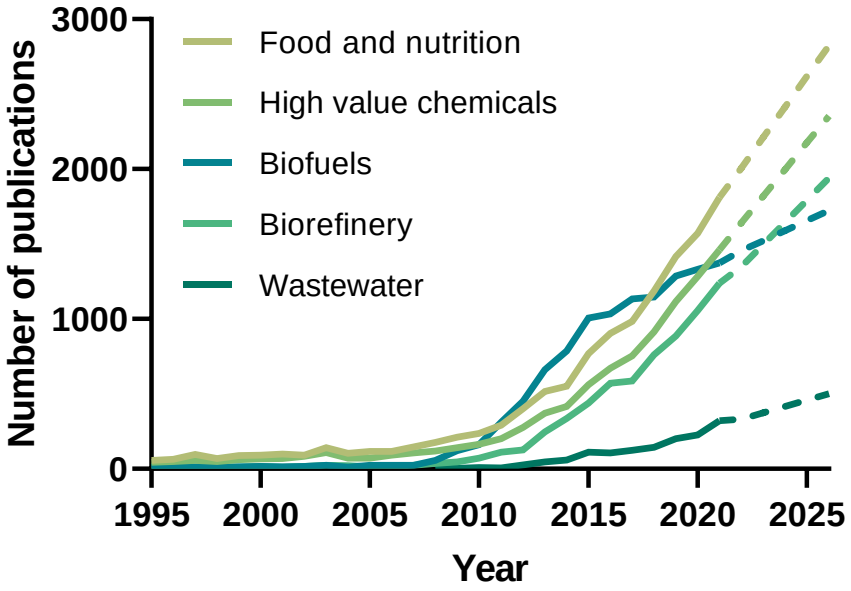


Figure 2. Number of publications by category of microalgae applications over time. Results obtained on January 30th 2023, from Web of Science search engine over a period until 2021 with the following keywords: TS = (microalgae AND (protein OR food OR nutrition OR feed OR novel food OR functional food)) for food and nutrition, TS = (microalgae AND (molecules OR bioactive OR compound)) for high-value molecules, TS = (microalgae AND (wastewater OR polluted water OR water remediation)) for wastewater treatment, TS = (microalgae AND (fuel OR biofuel OR oil OR biooil)) for biofuels and TS = (microalgae AND biorefinery) for biorefineries. Dotted lines are a linear extrapolation into the future from period 2015-2020 data. Updated and adapted from [2].

1.3 Commercial applications of bioactive compounds from microalgae

The transition from laboratory culture to viable commercial plants is not easy and certainly not equal for different microalgae derivatives. Challenges include[13]:

- High installation and operation costs, specifically in downstream processing as algae must be separated from culture medium in which they are very diluted, usually a few grams of biomass per litre.
- Difficulties to control culture conditions, especially in open ponds, where environmental abiotic conditions (e.g., temperature, pH, light intensity) play an important role.
- Microbial contamination with strains different from the one of interest (leading to the production of an undesired species) or with algal predators (leading to culture collapse).

Because of these bottlenecks, commercially viable products are mainly high value compounds which balance the production costs with a high sell price and bulk products that need little-to-no extraction like whole microalgae formulations for food. Some of the most relevant commercial applications are reviewed as follows, highlighting the extraction of high-value chemicals for being the subject matter of this work.

The importance of the commercialization of bioactive compounds from microalgae goes beyond the search for profit, but it unlocks a whole new range of natural products that can be used as drugs, cosmetics, food, or nutraceuticals, and provides additional channels of revenue for microalgae use in critical industries such as biofuels production or wastewater management.

1.3.1 Microalgae for human and animal nutrition

As explained before, microalgae original purpose was to serve as a food, from the traditional harvesting techniques in different lakes around the world to the large scale cultivation ponds. The main factors to be assessed when trying to approve a microalgae-derived product for human and/or animal consumption are chemical composition, presence of biogenic and xenobiotic toxicants, protein quality and microbial contamination[14].

Chemical composition of microalgae varies largely from strain to strain and even in batches of the same strain, as these organisms change their biochemical composition depending on the growing conditions. In general terms, microalgae are rich in protein. High-protein content was the original reason to consider these organisms as food. They show an average content of 40 % of protein that can grow up to 70 % for certain strains and growth conditions. The second major constituent is lipids and then carbohydrates, although they present higher variability. These values are similar to the ones of meat and eggs[14].

The most common microalgal product for nutrition purposes is dry biomass. This simple microalgal product contains natural antioxidants such as carotenoids and phenolic compounds, as well as vitamins, essential amino acids, minerals, poly unsaturated fatty acids (PUFAs), and other bioactive compounds that grant anti-inflammatory and anticoagulant properties. All of these in addition to the high-quality protein and carbohydrate content[15].

Dry microalgal biomass is usually found in the form of capsules or powder and labelled as food supplement. For now, only a few formulations have been approved in the EU or USA. The limiting factors for the development of this novel food are the strong colour and flavour, that restrict public demand and acceptance. It can also be incorporated in other food preparations such as yogurts or mayonnaise to enhance their nutritional properties, but usually causing changes in colour, flavour or the food matrix that potentially hinder market acceptance, although some products are quickly improving in this sense[16].

An alternative to dry microalgae biomass are extracts of microalgae. These extracts are obtained after a simple treatment of the whole dry biomass. Because downstream processing is being added, the costs are higher, but there are some interesting advantages. For example, beneficial bioactive compounds are much more bioavailable when the cell walls are disrupted. Extracts are a good option when trying to increase specific nutritional values in food, for instance, adding 0,5 % w/v of *Pavlova luteri* lipid extract to yogurt can improve by 300 % the total n-3 PUFAs content[14].

Dry microalgae can also be used as an alternative source of protein for animal feeding. Non-ruminants (poultry, pigs, rabbits) cannot digest algae's wall cellulosic material, so only processed algal biomass or thin-wall microalgae could compete with current feed. The few studies published in this sense agree on microalgae being a good partial substitute for conventional protein, although further research is needed to fully understand the effects of the substitution in animal health and in the organoleptic properties of the products derived from the animals. However, microalgae for feeding of ruminants has also failed to attract the interest of researchers and industry, because of the large amount of algal biomass required and the technical difficulties present in the process. Results on microalgae animal feeding are however still to be settled and further research is needed in this area[14].

1.3.2 Microalgae for high-value bioactive compounds

Microalgal biomass contains a high number of metabolites of commercial interest in different fields. Large scale production of some bioactive compounds such as vitamins, proteins, PUFAs, pigments has proven to be profitable. Many of these compounds (carotenes, oleic acid, vitamin E, vitamin B12, lutein, phycocyanin) present antifungal, antiviral, antioxidant, anti-inflammatory or antibiotic actions, among others, making them

attractive for the pharmaceutical industry, but also for cosmetics, nutraceuticals and food supplements or additives[2].

Table 1 shows some examples of bioactive compounds proven to be extractable from microalgae and marketable. Carotenoids and fatty acids are probably the most studied and optimized groups of compounds but it is also possible to extract polysaccharides with uses in the pharmaceutical and food industry[17]; polyphenols and several other types of antioxidants; and pigments like phycobiliproteins, phycocyanin or chlorophyl, along with diacylglycerols, sterols, and linear alkadienes[18].

Table 1. Selected examples of bioactive compounds extracted from production. Adapted and completed from [6].

Substances	**Microalgal species**	**Dry weight (%)**	**Properties**
Carotenoids			
Lutein	*Dunaliella salina*	0.4-0.8 %	Antioxidant, eye health
β-Carotene	*D. salina*	10 %	Antioxidant, vitamin A precursor
Fucoxanthin	*Chaetoceros* sp., *Cylindrotheca* sp., *Odontella* sp., *Phaeodactylum* sp., *Isochrysis* sp.	1.5%-2.0 %	Antioxidant, antidiabetic, anticancer, neuroprotective
Astaxanthin	*Haematococcus* sp., especially *H. pluvialis*	1%-8 %	Antioxidant
Essential fatty acids			
Eicopentaneoic acid (EPA)	*Phaeodactylum tricornutum*, *Monnodus subterraneus*, *Porphyridium cruentum*, *Amphora* sp.	0,7 %-6.1 % of the total lipids	Anti-inflammatory, cardiovascular health, mental health
Docosahexanoic acid (DHA)	*Arthrospira platensis*, *Rhizosolenia setigera*, *Thalassiosira stellaris*, *Crypthecodinium cohnii*, *Isocrysis.*	17.5 %-30.2 % of total lipids	Anti-inflammatory, brain health, eye health, cardiovascular health

In contrast with the irregularity and uncertainty of the microalgal biofuels, there is a good market trend guaranteed for bioactive compounds from microalgae by the fact that consumer preferences are changing towards environmentally friendly products. For instance, PUFAs demand is likely to become unsustainable in the future because most of

it comes from fish oil. Chemical synthesis is not likely to be profitable due to the complexity of natural compounds, so extraction from microalgae could be an attractive solution[6].

On the other hand, it must be considered that bioactive compounds are usually secondary metabolites in microalgae. This means that cellular concentrations in wild strains are rather low. For the harvesting of these compounds from microalgae to be profitable and interesting, advances in genetic engineering must happen to improve the growth and biochemical profile of microalgae. By using mutagenesis, adaptative evolution, and other genetic modification techniques, wild strains could be optimized to produce certain products thus creating microalgae-based 'bio-factories'[5].

This research work is focused on screening a strain of microalgae looking for antioxidant and antihypertensive activities, and trying to identify the compounds that cause them. These compounds are further described in the following sections.

1.3.2.1 Antioxidant compounds

The agreed definition for an antioxidant compound is "a substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate"[19]. Antioxidant molecules in microalgae (and in most of the living organisms) are produced to protect the cell from reactive oxygen species (ROS), i.e., by reducing oxidative stress. Generally, ROS refers to derivatives of the O_2 molecule that are more reactive than oxygen itself; sometimes as free radicals with at least one unpaired electron ($O_2^{-}\cdot$, $OH\cdot$), and sometimes as nonradical molecules (H_2O_2, $^1O_2$)[20].

Oxidative stress is a complex chemical and physiological phenomenon that accompanies most of the biotic and abiotic stresses of an organism and it is caused by the overproduction and accumulation of ROS. ROS are not malign themselves as they serve key biological function (defence against pathogens and cell signalling, among others). It is only their chemistry that causes harm. ROS have very diverse toxicities and mechanisms of action. For instance, hydrogen peroxide is not particularly toxic, but it can diffuse throughout cell membranes and oxidize sulfhydryl groups of amino acids thus causing key enzymes to denature. It also reacts with DNA and with transition metals present in the cell generating highly reactive hydroxyl radicals, that promote lipid peroxidation and protein and nucleic acid denaturation. There are no enzymes able to

deactivate these latter radicals, so they can ultimately lead to cell death if the concentration of antioxidants is not enough no quench them[21].

In human health, oxidative stress is related to a wide variety of diseases. Cancer can be driven or promoted by chromosomal modification and oncogene activation caused by oxidative stress. Oxidative stress is both a primary and a secondary cause for atherosclerosis and thus of cardiovascular diseases. It also plays a pivotal role in the development of neurological diseases like Alzheimer's, as well as in respiratory and kidney diseases and in rheumatoid arthritis[22].

ROS accumulation can be triggered by diet rich in fats, sugars, and processed food; a sedentary lifestyle, alcoholism and smoking, or environmental factors such as exposure to pollution or radiation. Oxidative stress can be prevented by avoiding the conditions mentioned and including antioxidant-rich food and supplements in the diet[23].

Antioxidants work by scavenging the free radicals. They stabilize them either accepting or donating electrons. The human body has some endogenous antioxidants like gluthathione or L-arginine, but exogenous antioxidants like vitamin E and C, flavonoids, polyphenols, and tocopherols are also fundamental to keep the oxidative balance[22]. As Figure 3 shows, a lot of different antioxidants from mentioned categories can be found in microalgae.

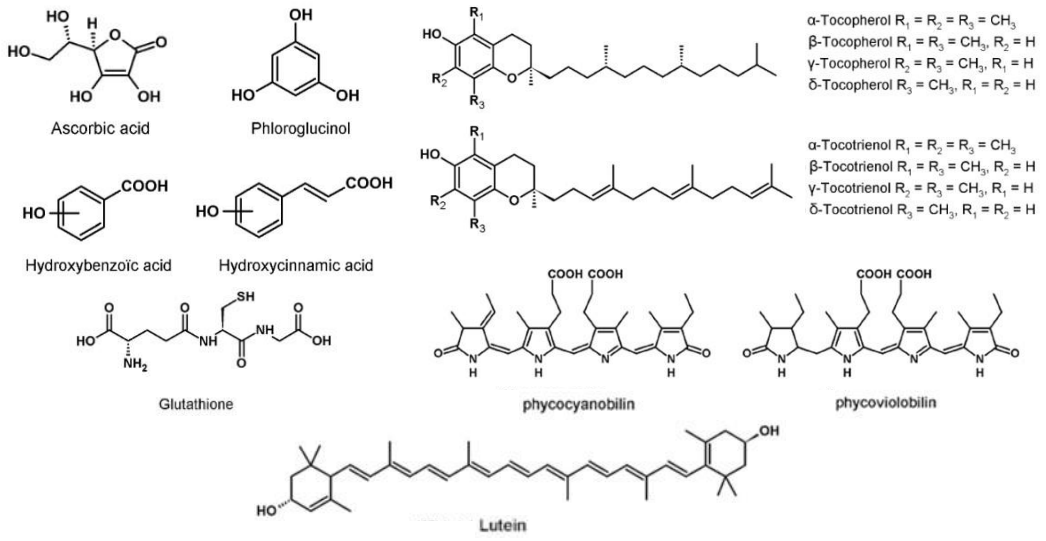


Figure 3. Examples of antioxidant compounds found in microalgae. Adapted and summarized from[21].

1.3.2.2 Antihypertensive compounds

Hypertension is a chronic disease in humans. It is a very common multifactorial disorder, and it is considered one of the major causes of premature death. It is defined by permanently presenting a blood pressure over a certain threshold. Hypertension increases with age, and it is deeply related to cardiovascular diseases. The causes of it are yet to be understood but some very relevant factors are: high body mass index, insulin resistance, alcohol, tobacco and salt intake, low potassium and calcium intake, stress, sedentary lifestyle[24].

There are around 1.5 billion people in the world affected by hypertension, and 40 % of them are "uncontrolled" and need to take at least two hypotensive drugs. The most common therapeutic target is the angiotensin-converting enzyme (ACE). Briefly, ACE converts angiotensin into angiotensin II which increases blood pressure through vasoconstriction. This process is framed in the renin-angiotensin-aldosterone system (Figure 4)[25].

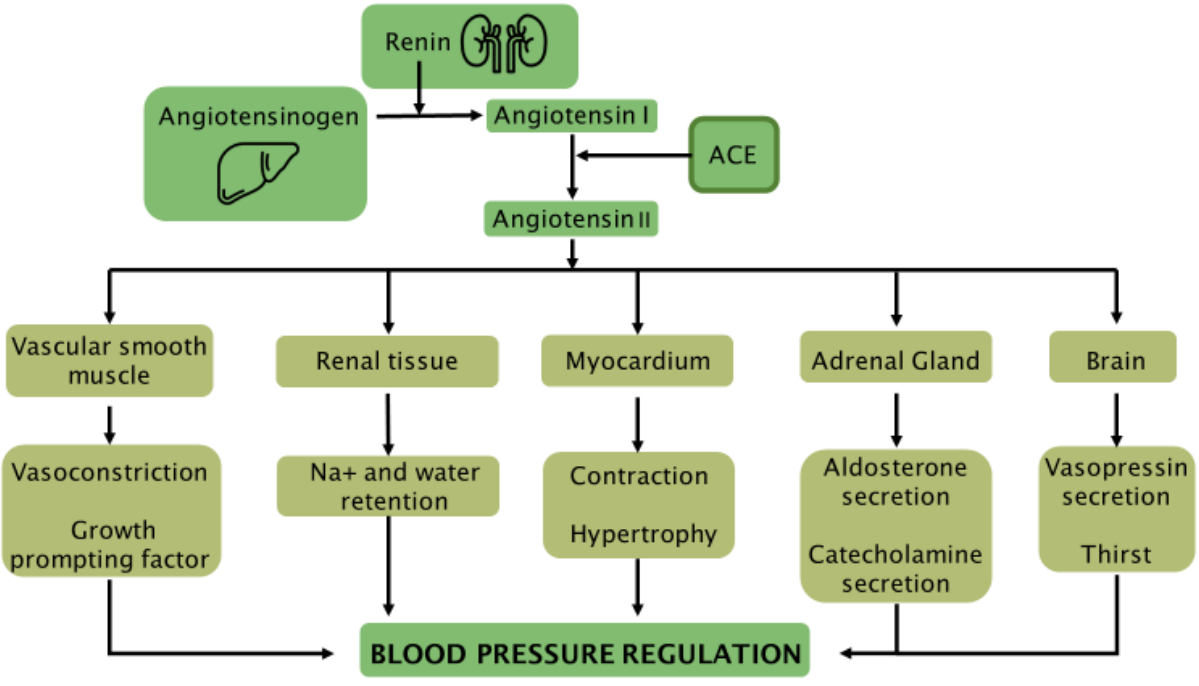


Figure 4. Renin-angiotensin system and its effect on different tissues. Adapted from[26].

Most of the discovered and marketed drugs to treat hypertension, such as captopril, enalapril, lisinopril and benzapril, work by inhibiting ACE and thus reducing angiotensin II blood concentration. However, synthetic drugs cause severe side effects such as erectile disfunction, angioedema and congenital malformations[25].

It is in this context that natural biopeptides arise as a highly attractive solution. Some biopeptides have ACE inhibiting activity while little-to-no side effects. These substances are often found in foods like fish, eggs or mil; but also in microalgal extracts. In fact, the ACE inhibitory activity of microalgal extracts has been a subject of interest for the last 30 years since it was first studied in *Chorella vulgaris*[26].

The biopeptides found in microalgae are usually short chain biopeptides consisting in 2-12 amino acids (Figure 5), they are stable in the mammalian gastrointestinal tract, and most of them are non-competitive inhibitors of ACE. There is a wide range of different hypotensive biopeptides obtainable from microalgae. Different species yield biopeptides variable in length, functional groups, and inhibitory activity. It is for this reason that it is fundamental to screen new species and strains for ACE inhibitory activity, as they may reveal improvements in hypertension treatment[25].

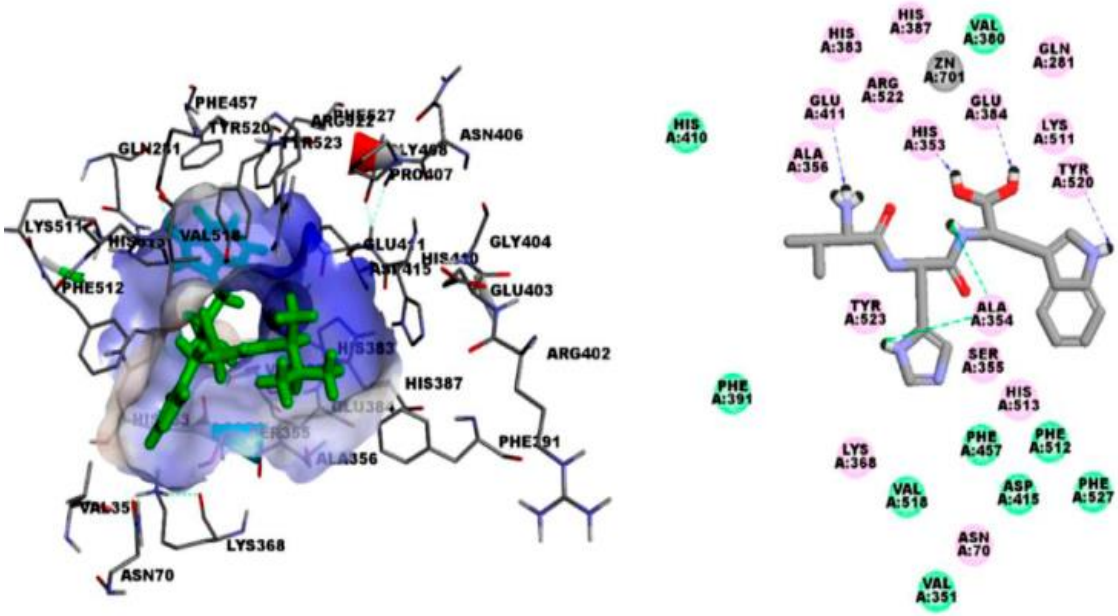


Figure 5. Molecular docking of ACE and the biopeptide VHW (Val-His-Trp) found in *Chlorella vulgaris* [27]. Adapted from [25].

1.4 Safety and regulatory concerns

Microalgae, as a new source of chemicals and food, can pose various risks to human health and the environment. These risks include the presence of allergens, toxins, pathogens, heavy metals, and pesticides. Also, as discussed before, the innovation pathway of microalgae aims towards genetic modification, thus creating new risks that must be thoroughly evaluated before placing related products in the market. It is the job of the different regulation agencies to decide whether the consumption of microalgae products or derivatives is safe[28].

Food allergy is an exacerbated immune reaction of the human body to harmless compounds present in food, specifically, to some antigens in the form of proteins or polysaccharides. The introduction of novel foods with little to no presence in nowadays markets can unveil unknown allergic conditions. The allergenicity of microalgae is yet to be fully described. Most of the published studies are focused on airborne microalgae and they report allergic reactions for several strains, but most of the reactions happened in people who were already sensitive to moulds or food. For now, consensus is that allergy to microalgal products can pose a serious risk for already sensitive patients, but not for the broader public. Actually, some of the strains that have been reported as allergens, have also been described as a source of anti-allergenic compounds[29,30].

Some marine algae are known for their production of toxins that gives rise to serious poisoning diseases in human health, especially when they are concentrated in fish and shellfish. Microalgae of the Chlorellaceae family, to which *Chlorella* belongs, have never been reported to synthesize toxins, however, some microalgae like *Aphanizomenon flos-aquae* have been reported to produce significant levels of toxins. Considering that large-scale cultures are never axenic, i.e., they do not contain only the strain of interest, especially in open-pond cultures, biological contamination with toxic species is a risk to consider. However, the presence of biohazardous toxins in microalgae culture is not common enough to be a matter of serious concern. Still, constant monitoring of undesired species and toxins present in the culture is fundamental to avoid accidents in this sense[31,32].

Another risk associated to their consumption as food is the amount of nucleic acids that microalgae contain. Total nucleic acid content is about 4 %-6 %, which is higher than most plant-based food. Nucleic acid metabolism in humans leads to the accumulation of

uric acid ultimately causing gout and other related diseases. This is one of the most important factors to determine the recommended highest daily intake[30].

There are some other risks not associated with microalgae intrinsically but with the cultivation process. Every material, surface and chemical used in the cultivation process can potentially transfer contaminants into the final product. Furthermore, open systems like raceway ponds (which are the most usual when it comes to microalgae for food cultivation) are susceptible to be contaminated by airborne materials[31].

A critical aspect of the cultivation process is the quality of the water. Some production schemes include low quality water (not drinkable) to couple microalgae production to wastewater treatment. This increases the risk of heavy metal and pesticides bioaccumulation in microalgae. The EU regulations allow for production of microalgae derived products with not drinkable water if the final product complies with the food safety regulations. For now, all examined products in the market found heavy metals levels below safety limits. Also, pesticide contamination and accumulation is a matter of concern[30]. The ability of the biomass to accumulate heavy metals or pesticides from water depends on the characteristics of each strain (e.g., their cell wall biochemistry) and the water conditions (i.e., pH, salinity, temperature, etc). On a final note, closed cultivation systems such as photobioreactors or greenhouses grant a better control on water quality than open systems like raceway ponds or marine water systems[31].

In Europe, the main regulation for food is the Regulation 178/2002 on food law established by the European Food Safety Agency (EFSA). However, the consumption of algae is a rather new phenomenon for the Europeans, and there are specific regulations for what is considered 'novel food'. According to the novel food regulation (Regulation 2015/2283 of the European Parliament and of the Council), novel foods means 'any food that was not used for human consumption in a significant degree before 15 May 1997 (when the first regulation on novel foods came into force), irrespective of the dates of accession of member states to the Union'[30].

The microalgae accepted as non-novel foods (significantly consumed before May 1997) are *Aphanizomenon flosaquae, Arthrospira platensis, Chlorella luteoviridis, Chlorella pyrenoidosa, Chlorella vulgaris* and an undefined *Spirulina* sp. Every other ingredient or preparation from microalgae must be authorised following the procedure which includes direct authorization from the EC and, almost always, a safety assessment by EFSA. There

is a simplified process for novel foods that have a proven record of consumption in third countries, from which microalgae could take advantage from. During the last decades, a lot of microalgae products have been accepted as novel foods, such as the diatom *Odontella aurita*, *Tetraselmis chui*, or DHA-rich oil from *Ulkenia* sp.[32].

All the safety assessment studies performed by EFSA must be based on experiments, research, and data previously published or collected and funded by the applicant. In exchange, the applicant gets a five-year preference and exclusivity on the intellectual property of the data provided. In practice, this means market exclusivity, because another applicant should have to collect and fund all the data again to get an authorization during those five years[32]. This is an excellent mechanism to ensure a high level of protection of human health and environment whilst fostering innovation but may be a burden for small innovators.

Finally, it is important to mention the safety and regulatory concerns over genetically modified (GM) microalgae. As discussed earlier, genetic modification is needed to bioengineer new microalgal strains with enhanced properties and biochemical characteristics in order to make their cultivation economically feasible[32,35]. European regulations for genetically modified microalgae are different from the one mentioned above, and they pose restrictions on their use and interaction with the environment to ensure human and environmental health, as GM organisms can heavily disrupt ecosystems. In this sense, applicable law are directives 2009/41/EC and 2001/18/EC, that regulate research and production of genetically modified organisms and Regulation 1829/2003/EC, that covers GM food and feed[36].

1.5 Microalgae used in this work

This work is focused on the isolation and identification of bioactive compounds from *Tetraselmis striata* CTP4. Isolation of *T. striata* CTP4 was first reported in 2016[33]. It was discovered during the screening of samples collected in a marsh area (Ria Formosa) in Algarve, Portugal, near a wastewater treatment plant. This microalga species was isolated while screening for robust, lipid-rich species for biofuel production. Its characterization unveiled a fast-growing and euryhaline strain with interesting properties and biochemical profile, which meant high potential for several commercial applications.

T. striata CTP4 has a relatively large cell size (9-22 mm) and lacks a flagellum (Figure 6), which makes it sediment easily, thus reducing biomass concentration and dewatering

costs. The first tests showed a good ability of lipid induction under nutrient depletion (3-fold increase in lipid content). Overall, it showed potential to produce a biofuel compliant with several international regulations which require high levels of lipids with as little unsaturation as possible [33].

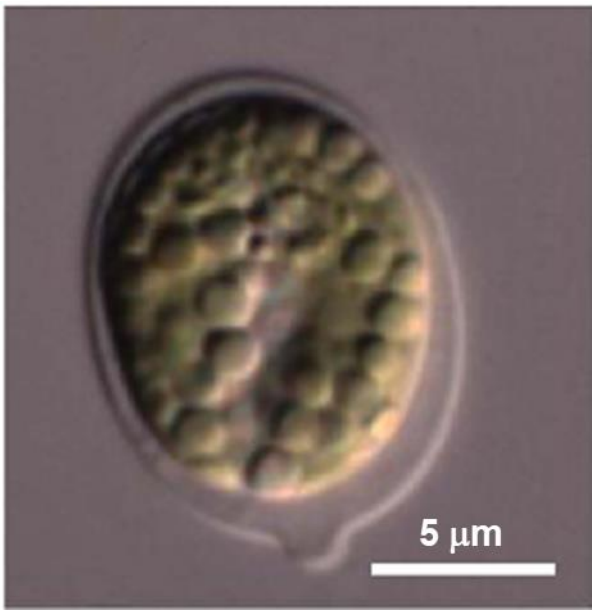


Figure 6. *Tetraselmis striata* CTP4 under the microscope. Adapted from [33].

Three years after its isolation, *T. striata* CTP4 was reported to have been cultivated in an industrial facility, specifically in a 100 m^3 photobioreactor built by Allmicroalgae, S.A.[34]. It is important to study the properties of the strain both at laboratory and industrial scale, as some of them are likely to change. The industrially produced strain showed a highly interesting biochemical profile with high protein (31.2 %) and dietary fibres (24.6 %) content, but low lipid content, similar to that of the soybean. Also, a rich carotenoid and vitamins outline was described, along with moderate antioxidant activity. These properties reveal *T. striata* CTP4 as a good candidate for several commercial applications apart from biofuel production.

Since its isolation and large scale production, *T. striata* CTP4 has been studied for several applications such as wastewater treatment[35], CO_2 sequestration[36], and production of functional foods[37] and feed[38]. It also has been genetically improved to produce higher amounts of pigment and fatty acids[39], and cultivated under different thermal and light stimuli to improve the production of high value compounds[40].

2. Objectives

The main goal of this work is to isolate and identify antioxidant and antihypertensive compounds from the microalgae *T. striata* CTP4. To achieve this goal, specific objectives were set:

- Evaluate the antioxidant and antihypertensive activity of *T. striata* CTP4 extracts produced with mainly food grade not toxic solvents (e.g., ethyl acetate, ethanol, and hexane).
- Produce fractions enriched in bioactive compounds.
- Analyse the most bioactive fractions and identify compounds possibly responsible for the observed activity.

The present work aims to further expand the knowledge on the properties of *T. striata* CTP4 and its bioactive compounds, hoping to consolidate its position as a promising microalga with high potential for different commercial applications.

3. Materials and methodology

3.1 Chemicals

Solvents with different purity grades were used: methanol, ethyl acetate and acetonitrile were HPLC grade, DMSO and ethanol (99.8 %) were analytical reagent grade and hexane was commercial grade. Milli-Q water was produced inhouse through a Milli-Q Advantage A10 Ultrapure Water Purification System (Merck, Germany). Trichloroacetic acid (TCA), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium chloride, potassium persulfate, gallic acid, sodium phosphate dibasic and sodium phosphate monobasic were purchased from Sigma-Aldrich (Germany). Ferricyanide was purchased from Acros Organics (Belgium). Ferrous chloride and ferric chloride were purchased from VWR (Portugal).

Angiotensin Converting Enzyme from rabbit lung (≥2.0 units/mg protein, modified Warburg-Christian), and captopril were purchased from Sigma-Aldrich (Germany). Peptide Abz-Gly-Phe(NO_2)-Pro was purchased from Bachem (Switzerland).

3.2 Microalgal biomass

T. striata CTP4 biomass was grown in-house (MarBioTech, CCMAR, UAlg) during summer 2022 using a closed system of polyethylene sleeves, starting from the Petri dishes that had the original isolate. The biomass was freeze-dried and preserved in dried and sealed conditions at room temperature until used.

3.3 Experimental design

The experimental design is based on the classical chemistry research approach of separation and test cycles. The approach was envisioned as a 'funnel' that starts at the top end with the microalgal biomass. This is shown in Figure 7 and described as follows.

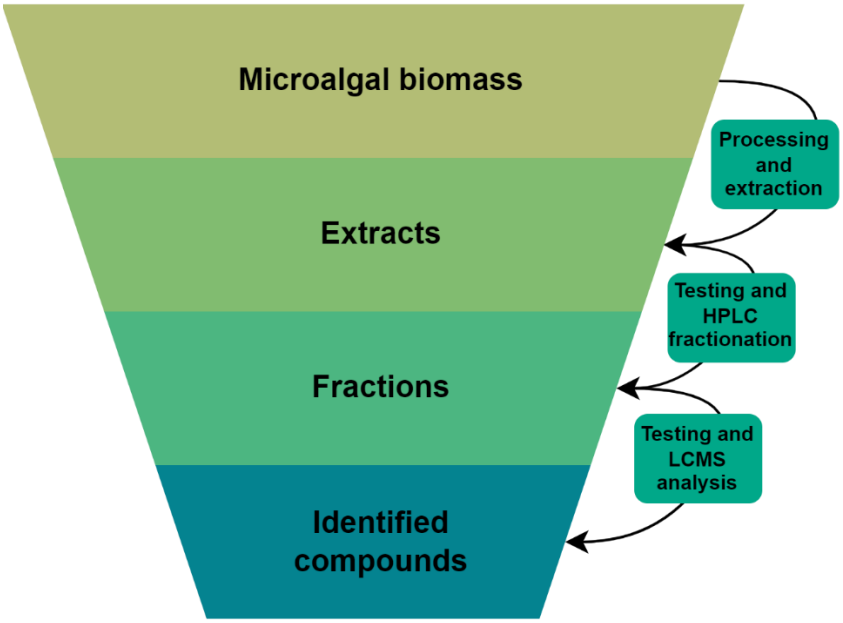


Figure 7. Funnel experimental approach.

The biomass was processed into a fine powder and then extracted using non-toxic food grade solvents with different polarities (e.g., ethyl acetate and ethanol), and hexane because of its low polarity, thus covering a wide range of extracted compounds. Each extract was then tested for antioxidant and antihypertensive activities at different concentrations. Results were analysed and the most active extract advances to the next phase, which is separation in fractions through high performance liquid chromatography (HPLC). The resulting fractions were then tested again for antioxidant and antihypertensive activities. Finally, the most interesting fractions were sent to liquid chromatography coupled with mass spectrometry (LC-MS) analysis to tentatively identify the compounds possibly responsible for the analysed bioactivity.

3.4 Biomass processing and extraction

Microalgal biomass was dried in an Alpha 1-2 LDPlus freeze-dryer (Martin Christ) at 0.041 mbar and -63 ºC during 48 h. It was then pulverised using a planetary ball mill (Retsch PM 100). The biomass was introduced in the sealed jar along with the milling stainless-steel balls. The mill performs radial oscillations in horizontal position. It was programmed at 450 rpm for 5 minutes, with 10 second stops for cooling every 30 seconds. This avoids possible degradation of labile compounds. Finally, the chunky biomass was transformed into a green fine powder. This process was done just before the extraction process to avoid losses by evaporation. The combination of freeze-drying and ball milling ensures the breaking of functional cell structures facilitating the later extraction with minimum loss of bioactive compounds.

The extraction was carried out with an energized dispersive guided extraction system (EDGE®). The EDGE is an automated solvent extraction instrument commercialized by CEM. It combines pressurized liquid extraction and dispersive solid phase extraction, and it claims to be quicker and simpler than Soxhlet and other extraction methods[41]. Figure 8 shows the EDGE instrument and its extraction cell (Q-cup).

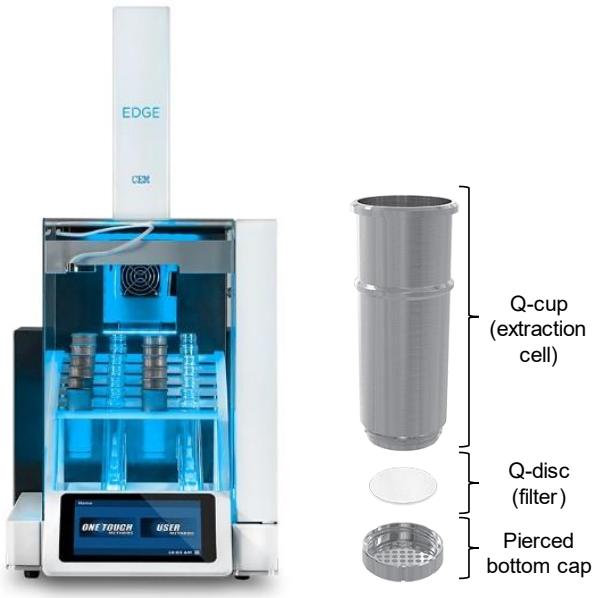


Figure 8. EDGE extractor (left) and scheme of an EDGE extraction cell or Q-cup (right). Adapted from[41].

To perform an extraction with EDGE, the sample and some sand are added to the Q-cup, that is also equipped with a filter at its bottom. The sand helps to promote contact between

the biomass and the solvents, facilitating the extraction. EDGE's robotic arm transfers the Q-cup into a sealed chamber where programmed amounts of solvents are added from the bottom to the gap between the cell and chamber walls and from the top. The walls of the sample are heated up until a programmed temperature, causing the solvent to expand and disperse into the sample through the holes in the bottom of the Q-cup. Due to the system being sealed, pressure builds up allowing for the solvent to perform over its boiling point at room temperature and it does not evaporate out of the Q-cup. After a set time of extraction, the extract is collected in a glass collection vial and the Q-cup is transferred to its original position[42]. A schematic visualization of this process is shown in Figure 9.

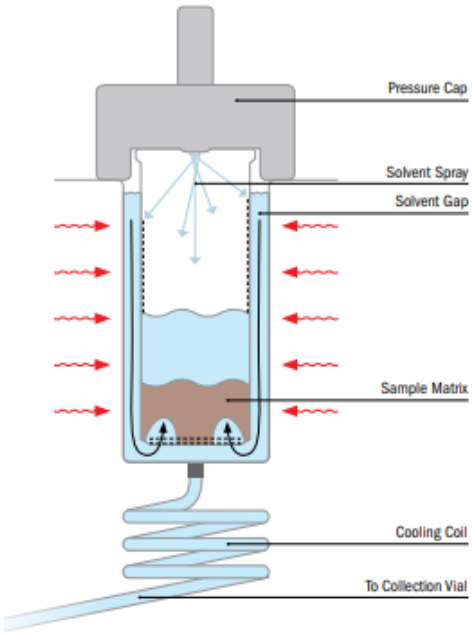


Figure 9. Schematic representation of the Q-cup during an EDGE extraction. Retrieved from[41].

The extraction of the microalgal biomass followed the usual protocol developed in-house. Firstly, the Q-cup was assembled including a S1 sandwich filter of two cellulose layers with a glass fibre layer in between. Then, 1 g of microalgal biomass was added to the Q-cup along with 1 g of fine sand. The Q-cup was placed in the EDGE rack waiting for the program to start. The programme included, for each Q-cup, 3 cycles of extraction of 10 minutes (total of 30 minutes) using 20 mL of solvent in each extraction. Temperature was set at 30 ºC. It also included washing cycles at the beginning of the run and every time solvent was changed. This process was carried out 5 times for ethanol and ethyl acetate extractions and 6 times for hexane extraction to collect enough extract for the activity tests and future fractionation. The collection vials were dried with a gentle nitrogen flow

and the remaining solid extract was weighed and redissolved in DMSO or left in the solid state, depending on the later assay.

The yield of the extraction was calculated dividing the weight of the extract after drying by the weight of the biomass introduced in the Q-cup, following equation 1.

$$Yield\ (\%) = \frac{w_{dry\ extract}}{w_{dry\ initial\ biomass}} \cdot 100 \qquad \text{(eq. 1)}$$

Regardless of the extracts being dissolved in DMSO or in the solid state, samples were always stored in the cold to avoid degradation of labile compounds. A long-term stock was stored at -80 ºC to minimize the degradation and a short-term stock was stored at -20 ºC. Stock concentration was always 20 mg/mL.

3.5 Bioactivity assays

Bioactivity assays were performed in 96-well microplates. This technique falls under the umbrella term of high throughput assays, that allow to test a high number of samples and concentrations for different activities in a simple manner and short time span using just microplates, pipettes, and a microplate reader. Microplates absorbance was read using a Synergy HT Multidetection Microplate Reader (BioTec Instruments, USA).

3.5.1 Antioxidant activity assays

This set of assays tests the ability of the sample to act as antioxidant through different mechanisms. On the extracts, these assays were performed for 5, 2.5, 1.25, 0.625, 0.3125 and 0.15625 mg extract/mL in DMSO in sextuplicate for three consecutive days (*N*=3). They always included two colour controls for each sample. Every plate included negative and positive controls in sextuplicate along with their colour controls. Gallic acid was the reference antioxidant chosen as positive control. For the fractions, antioxidant assays were performed for 1 mg/mL in triplicate for three different days, and controls were also prepared in triplicate.

According to the literature, Ferric Iron Reducing Power (FRAP) reaction is based on a single-electron transfer mechanism, while ABTS and DPPH assays' reactions are more complex and include a combination of single-electron transfer (SET) and hydrogen atom transfer[43]. This must be kept in mind when analysing the results.

3.5.1.1 ABTS Assay

The ABTS assay is a very extended antioxidant activity assay for a wide range of matrix samples, including algae and plants. Briefly, during the reaction, the antioxidant reduces the $ABTS^{\cdot+}$ radical into $ABTS^{2-}$. The radical is blue coloured (λ_{max}=734 nm) while the anion is colourless. The anion is firstly turn into the radical by reaction with potassium persulfate, as shown in Figure 10.

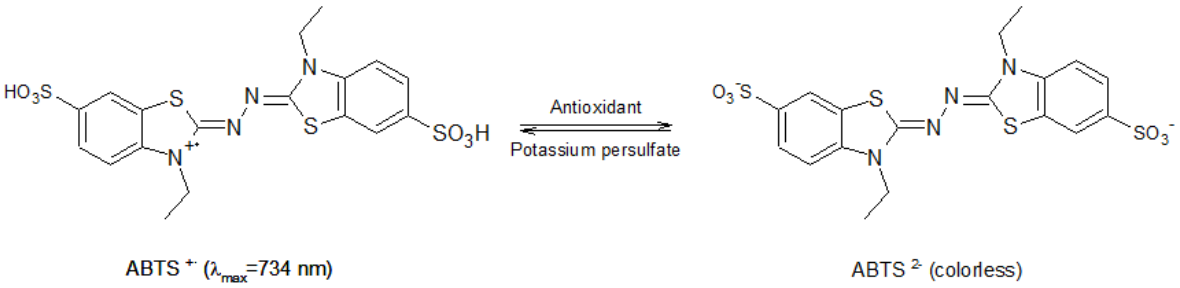


Figure 10. ABTS assay mechanism.

The protocol used was based on that of Re et al.[44] with some modifications. A stock solution of ABTS was prepared dissolving 0.003514 g of potassium persulfate with 0.0203 g of ABTS in 5 mL of distilled water. The solution was stored at 4 ºC in the dark. Before using the stock solution, absorbance at 734 nm was measured and the concentration of the solution corrected with ethanol if needed until an absorbance around 0.7. The rate of dilution needed was then applied to elaborate a working solution of ABTS, that was freshly made every day.

The 96-well plate included:

- Sample wells with 10 µL sample and 190 µL of ABTS.
- Negative control wells with 10 µL of solvent (DMSO) and 190 µL of ABTS.
- Positive control with 10 µL of a 1 mg/mL solution of gallic acid and 190 µL of ABTS. Although this control is not used in the calculations, it acts as a guarantee of the good functioning of the assay.
- Colour controls with 10 µL of sample and 190 µL of distilled water.

After the reagents are added, the microplate was incubated for 6 min in the dark and the absorbance read at 734 nm. The % of radical scavenging was measured based on the decolouration of the sample (equation 2).

$$Activity\ (\%) = \frac{A_{C^-} - A_{sample}}{A_{C^-}} \cdot 100 \qquad \text{(eq. 2)}$$

Where:

- A_{C^-} is the absorbance of the negative control.
- A_{sample} is the absorbance of the sample.

Also, the IC_{50} parameter was estimated for the extract samples. It is defined as the concentration of extract needed to scavenge 50 % of radical.

3.5.1.2 DPPH Assay

DPPH assay is also a very popular antioxidant test. It is based on the reaction of the stable radical DPPH·, strongly purple coloured, with an antioxidant that reduces the radical to its DPPH non-radical form, losing the purple colour (Figure 11).

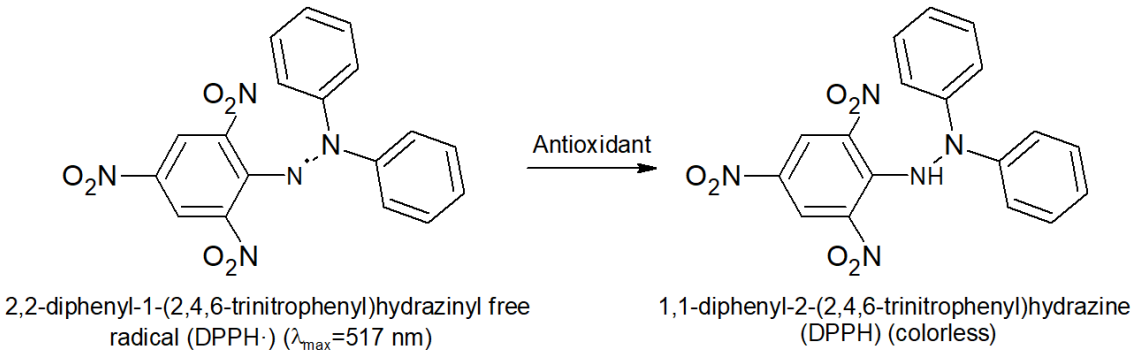


Figure 11. DPPH assay mechanism.

The protocol was based on that of Blois[45] with some modifications. A 120 μM solution of DPPH was prepared with 0,0024 g of DPPH and 50 mL of methanol. This solution was stored at 4 ºC in the dark and only used in a one-week period. The 96-well plate included:

- Samples wells with 22 μL sample and 200 μL of DPPH.
- Negative control wells with 22 μL of solvent (DMSO) and 200 μL of DPPH.
- Positive control with 22 μL of a 1 mg/mL solution of gallic acid and 200 μL of DPPH. Although this control is not used in the calculations, it acts as a guarantee of the good functioning of the assay.
- Colour controls with 22 μL of sample and 200 μL of methanol.

After the reagents were added, the microplate was incubated 30 min in the dark and then absorbance was read at 517 nm. The percentage of radical scavenging was estimated using equation 2. Also, the IC_{50} parameter was estimated for the extract samples.

3.5.1.3 FRAP Assay

This method is based on the ability of the samples to reduce ferric iron (Fe^{3+}). Antioxidant substances in the sample will reduce potassium ferricyanide ($Fe(CN)_3$) to form potassium ferrocyanide ($Fe(CN)_2$). This latter compound reacts with ferric chloride ($FeCl_3$) to form a ferric-ferrous complex that displays and absorption maximum at 700 nm, showing a blue-green colour (Figure 12). The reaction happens at an acidic pH upon addition of trichloroacetic acid.

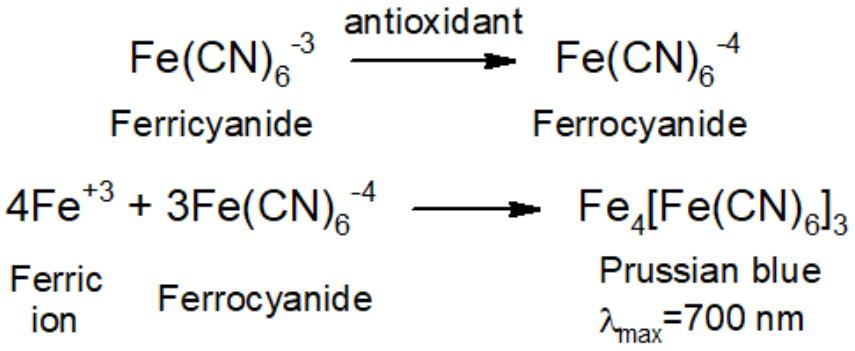


Figure 12. FRAP assay mechanism.

The protocol is based on that of Megías et al.[46] with some modifications. The following solutions were prepared:

- Ferricyanide solution (1 % w/v in distilled water)
- Trichloroacetic acid (10 % w/v in distilled water)
- Ferric chloride solution (0.1 % w/v in distilled water)

The 96-well plate included:

- Samples wells with 50 µL sample, 50 µL of distilled water and 50 µL of ferricyanide.
- Negative control wells with 50 µL of solvent (DMSO), 50 µL of distilled water, and 50 µL of ferricyanide. Although this control is not used in the calculations, it acts as a guarantee of good functioning of the assay.

- Positive control with 50 μL of a 1 mg/mL solution of gallic, 50 μL of distilled water, and 50 μL of ferricyanide.
- Colour controls with 50 μL of sample and 100 μL of distilled water.

The microplate was then incubated for 20 min at 50 ºC. Then, 50 μL of trichloroacetic acid and 10 μL of ferric chloride were added to every well but the colour controls, that were completed with 60 μL of distilled water. The microplate was then incubated for 10 min at 50 ºC and, finally, absorbance was read at 700 nm.

Because this assay is based on the production of colour, the percentage of reducing activity was estimated following equation 3. Also, the IC_{50} parameter was estimated for the extract samples.

$$Activity\ (\%) = \frac{A_{sample}}{A_{C+}} \cdot 100 \qquad \text{(eq. 3)}$$

Where:

- A_{C+} is the absorbance of the positive control (gallic acid)
- A_{sample} is the absorbance of the sample.

3.5.2 Antihypertensive activity assay

This assay is based on the ability of ACE to hydrolyse the peptide Abz-Gly-Phe(NO_2)-Pro or 'substrate', to generate o-aminobenzoglycine (Abz-Gly) and a coproduct. Abz-Gly is a fluorescent substance with $\lambda_{excitation}$ = 365 nm and $\lambda_{emmission}$ = 405 nm. If the microalgal sample can inhibit the enzyme, less fluorescence will be detected. For this assay, the 96-well microplate used included a black coating between the wells to avoid interferences in the measurement of fluorescence. The bottom of the wells remained transparent. Captopril was selected as the positive control. It is a widely known and used antihypertensive drug that inhibits ACE activity.

Extracts were tested in different concentrations, specifically 1, 0.5, 0.25, 0.125, 0.0612, 0.0306 mg/mL. Each of the measurements was done in triplicate during three different days (*N*=3). There was no need of including a colour control in this assay because of it being based on fluorescence measurement. Fractions were tested at 1 mg/mL in triplicate during three different days.

The protocol followed was the one designed by Toldrá et al.[47]. The following stock solutions were prepared:

- Buffer A: 18.171 g of Tris base and 0.136 mg of $ZnCl_2$ were added to 500 mL of distilled water. pH was corrected with HCl 1 M until measuring 8.3. The solution was kept in 4 ºC until use.
- Buffer B: 1.8171 g of Tris base were added to 100 mL of distilled water. pH was corrected using HCl 1 M until measuring 8.3. The solution was kept at 4 ºC until use.
- Buffer C: 1.8171 g of Tris base and 6.5745 g of NaCl were added to 100 mL of distilled water. The solution was kept at 4 ºC until use.
- ACE stock solution (500 mU/mL): 1 mL of buffer A and 1 mL of glycerol were added to ACE 1 U. 10 aliquots of 200 μL were elaborated and stored at -20 ºC until use.
- Substrate solution: 10.35 mL of buffer C were added to 50 mg of substrate and 1 mL aliquots were elaborated. The aliquots were sonicated until complete dissolution and stored in the dark at -20 ºC.
- Captopril solution: 2.2 mg of captopril were added to 50 mL of buffer B. The solution was kept at 4 ºC until use.

Every assay day, the following working solutions were freshly prepared:

- ACE working solution (18 mU/mL): 180 μL of ACE stock solution dissolved in 4820 μL of buffer B.
- Substrate working solution (0.45 mM): 1 mL of substrate stock solution dissolved in 21.2 mL of buffer C.
- Captopril working solution (1 mM): 25 μL of captopril stock solution to 4975 μL of buffer B.

The 96-well plate included:

- Sample cells with 50 μL of sample and 50 μL of ACE working solution.
- Positive control with 50 μL of captopril working solution and 50 μL of ACE working solution.

- Negative control with μL of buffer B and DMSO. This mix is adjusted in each case to match the amount of DMSO in each controlled sample after diluting the extract in Buffer B to reach the desired concentration. In this way, the interference caused by the DMSO disrupting the ACE enzyme is eliminated. The control also includes 50 μL of ACE working solution. Apart from that, DMSO concentration was always kept below 5 % to minimize this effect.

The microplate was incubated at 37 ºC while shaking during 10 min inside the microplate reader. The substrate was incubated at 37 ºC during 10 min in an oven. After that time, 200 μL were quickly added to each well and the kinetic reading was started with $\lambda_{excitation}$ = 365 nm and $\lambda_{emmission}$ = 405 nm during 40 min with readings every 5 min.

For each well, the slope of kinetic curve (fluorescence over time) was calculated and used in equation 4 to calculate the inhibitory activity of the sample.

$$ACE\ inhibitory\ activity\ (\%) = 100 - \left(\frac{slope_{sample}}{slope_{C-}} \cdot 100\right) \quad \text{(eq.}$$

Where:

- $slope_{sample}$ is the slope of the kinetic curve of the sample.
- $slope_{C-}$ is the slope of the kinetic curve of the negative control

3.6 HPLC Fractionation

The most promising extracts were selected for further study. Fractionation was conducted in a LC-20AP (Shimadzu), equipped with an SPD-M40 photo diode array detector (PAD) and an LTII low temperature evaporative light scattering detector (ELSD). Several analytical runs were carried out with a low volume analytical column and a small sample injection (20 μL of extract 50 mg/mL in acetonitrile) to design the fractionation scheme and to optimize the solvent composition scheme. All the samples and eluents were filtered through 0,22 μm pore filters.

The ELSD and PAD chromatograms obtained during the analytical runs where analysed and compared to design a convenient fractionation scheme. The final scheme included a 70 min run with the collection of 41 fractions, approximately one every 1 minute and 40 seconds. The solvent scheme started with 75 % acetonitrile (can; solvent A) and Milli-Q water (solvent B) increasing linearly until 100 % ACN after 30 min. After 55 minutes,

the percentage of A started decreasing until it reached 75 % again in minute 60 and stayed still until finish. The semi-preparative column used was a Luna 5u C18(2) 100A, 250 x 10.0 mm (Phenomenex). The injection volume was 200 µL of 100 mg/mL extract. Fractions were collected by an automatic collector in 10 mL test tubes. This process was repeated 15 times in order to collect enough mass of each fraction for the activity assays. Data was acquired and processed with the LabSolutions Shimadzu software. The 41 fractions were concentrated under a gentle N_2 flow, transferred to amber vials, dried, and weighed.

3.7 LC-MS analysis

The fractions were dissolved in methanol until 0.1 mg/mL, filtered in 0.22 µm filters and sent to the analytical services of CCMAR, where they were analysed. The chromatographic separation was achieved using a Thermo Scientific ultimate 3000 UHPLC (Bremen, Germany). The column was a Thermo Scientific Accucore RP-18 (2.1 × 100 mm, 2.6 µm). The mobile phase composition was prepared with water (A) and acetonitrile (B), both containing 0.1 % of formic acid. The gradient (in v/v %) started with 100 % of A for 2 min, increased linearly to 30 % of B in 13 min, to 100 % of B in 16 min, was maintained at 100 of % B for 4 min, returned to 100 % of A in 1 min and then was maintained at 100 % of A for 4 min before the next run. The flow rate was 0.3 mL/min. The injection volume was 5 µL.

Mass analysis was performed on an Orbitrap Elite (Bremen, Germany) mass spectrometer with a Heated ElectroSpray Ionization source. Acquisition was performed under positive and negative polarities. HR-MS data were acquired using the following ionization parameters: spray voltages, 3.7 kV (positive polarity) and 4.0 kV (negative polarity); sheath gas, 40 arbitrary units; auxiliary gas, 10 arbitrary units; heater temperature, 300 ºC; capillary temperature, 350 ◦C; S-Lenses RF level, 64.9 %. Scan range was 100–1000 m/z. Fragmentation spectra were obtained by Collision Induced Dissociation by running the system in data dependent mode with dynamic exclusion.

LC-MS profiles were analysed using XCalibur FreeStyle software. The compounds were tentatively identified based on comparison of the mass spectra with those in NIST 17.0 library, Wiley MS library, and other public databases (e.g., MAINLIB, REPLIB, W10N14M1.001, and W10N14M2 libraries). Identification was focused on the peaks present in the chromatograms and compounds were considered tentatively identified if

they appeared at least two times in the peak and showed probability values higher than 70 %.

3.8 Data analysis

Statistical analysis of the data was performed using Microsoft Excel and Graphpad Prism 8.0. Outliers were processed under Dixon's Q rules with 95% of confidence. One- and two-way ANOVA tests were used for sample comparison. Differences were considered significant if $p<0.05$. IC_{50} was calculated through a non-linear fit of the set of data to a curve following equation 5, where Y is response and X is the logarithm of the sample concentration. *Top* and *Bottom* are fit parameters for the top and bottom asymptotes of the fitting curve.

$$Y = Bottom + \frac{Top-Bottom}{1+10^{\log IC50-X}} \quad \text{(eq. 5)}$$

4. Results and discussion

4.1 Extraction

The yield of extraction was the highest for ethanol (15 %), while it remained around 5 % for the hexane and ethyl acetate extracts. This is in agreement with the internal reference values for extractions of *T. striata* CTP4 with the same equipment and in the same conditions.

Table 2. Extraction yields.

Solvent	Dry biomass (g)	Yield (g)	Yield (%)	Internal reference value (%)
Ethanol	5.08	0.761	15.0	14.1
Ethyl acetate	5.02	0.232	4.62	5.20
Hexane	5.98	0.357	5.97	3.80

4.2 Bioactivity assays on extracts

The microalgal biomass was extracted with three different solvents of decreasing polarity: ethanol, ethyl acetate and hexane. The hexane extracts usually contain non-polar compounds such as lipids, fatty acids, hydrocarbons, and pigments. The ethanol extract captures polar and moderately polar compounds such as phenolic compounds, flavonoids, alkaloids, and water-soluble pigments like chlorophylls. Also, sugars, organic acids, and vitamins. Ethyl acetate, as a moderately polar solvent has a wide range of extracted

compounds, some of them overlapping with the ethanol and hexane extracts. Terpenoids, sterols, and glycosides are expected to be found in this extract[15].

4.2.1 Antioxidant activity

Antioxidant activity was assessed through three assays: ABTS, DPPH and FRAP. The results are visually summarized in Figures 13, 14 and 15. Ethyl acetate can be easily identified as the most active extract in each of the tests. ABTS and DPPH show very similar profile as both tests work under a complex combination of single-electron transfer (SET) and hydrogen atom transfer mechanisms, so both tests are expected to cover a similar range of antioxidant compounds.

On the other hand, FRAP assay works under a mostly SET mechanisms, which in this case generates slightly different results because it may not capture the activity of hydrogen-transfer antioxidant compounds. The hexane extract activity is lower than in the other two tests. Also, because this test takes the positive control as the reference value, it is common to see activities over 100 %.

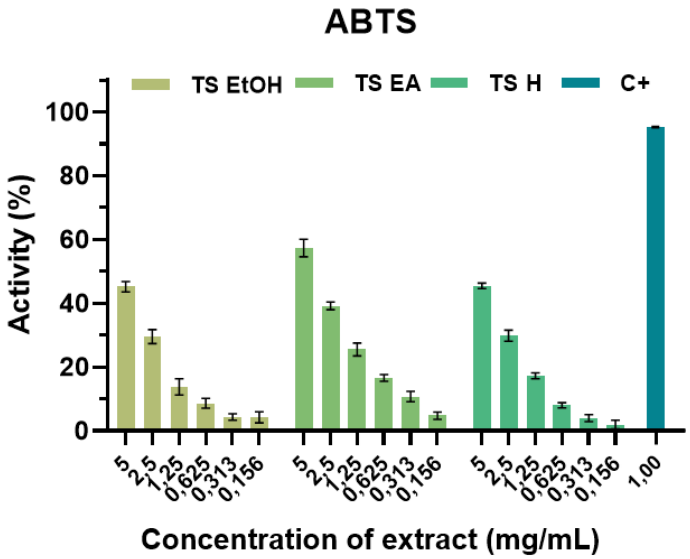


Figure 13. ABTS assay results for ethanol, ethyl acetate and hexane extracts. For each tested concentration, assays were performed in sextuplicate three days in a row (*n*=18). TS EtOH: Ethanol extract, TS EA: Ethyl acetate extract. TS H: Hexane extract, C+: positive control (gallic acid). Error bars indicate a 95% interval of confidence.

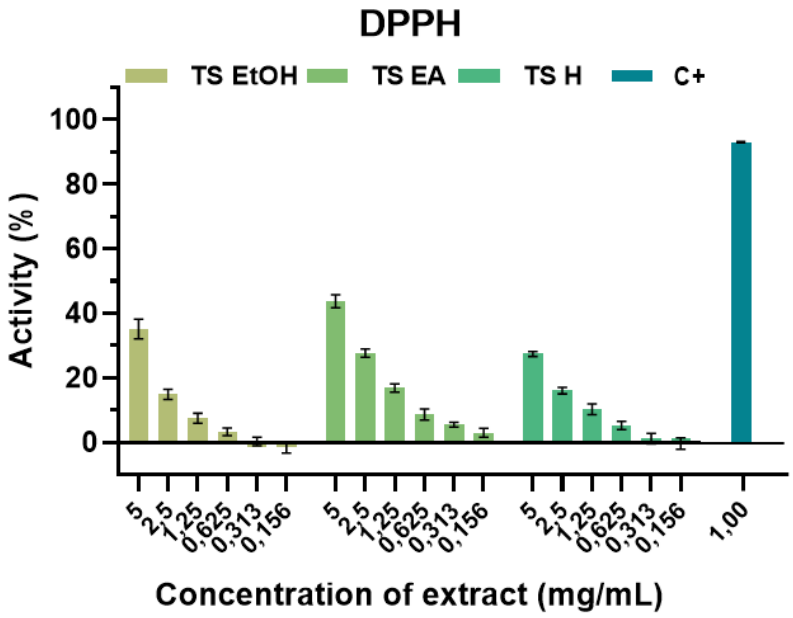


Figure 14. DPPH assay results for ethanol, ethyl acetate and hexane extracts**.** For each tested concentration, assays were performed in sextuplicate three days in a row (*n*=18). TS EtOH: Ethanol extract, TS EA: Ethyl acetate extract. TS H: Hexane extract. C+: positive control (gallic acid). Error bars indicate a 95% interval of confidence.

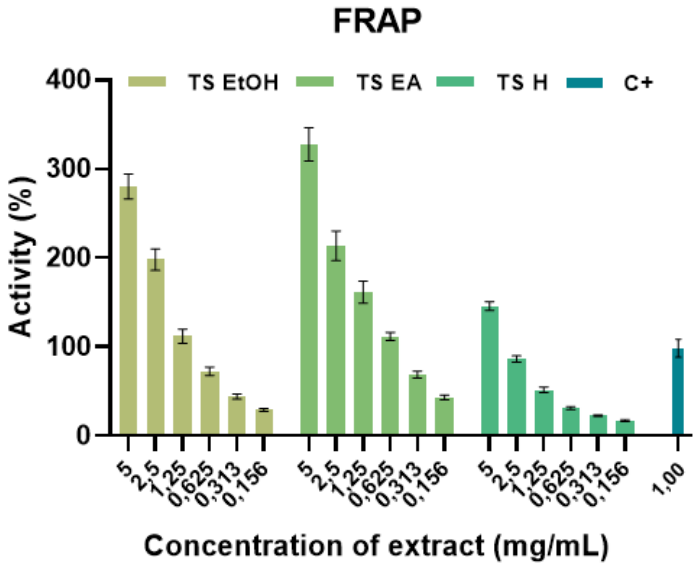


Figure 15. FRAP assay results for ethanol, ethyl acetate, and hexane extracts. For each tested concentration, assays were performed in sextuplicate three days in a row (n=18). TS EtOH: Ethanol extract, TS EA: Ethyl acetate extract. TS H: Hexane extract. C+: positive control (gallic acid). Error bars indicate a 95% interval of confidence.

To compare the results with the ones in literature, IC_{50} was calculated for each extract in each test as the concentration required to reach 50 % of the maximum activity. Results are shown in Table 3.

Table 3. Estimated IC_{50} values for different extracts in antioxidant tests with 95 % interval of confidence (mg/mL).

Solvent	ABTS	DPPH	FRAP
Hexane	5.41 ± 0.90^{a}	$5.52\pm3.03^{a,b}$	19.9 ± 11.2^{b}
Ethyl acetate	4.15 ± 0.70^{b}	6.64 ± 0.93^{a}	4.26 ± 1.81^{a}
Ethanol	6.27 ± 0.62^{a}	9.27 ± 1.13^{b}	5.18 ± 1.45^{a}

Note: Different superscripts (^{a,b}) indicate statistically significant differences between results for the same test.

Low values of IC_{50} mean higher antioxidant potential. Results show low values of this indicator for the hexane and ethyl acetate extracts in ABTS and DPPH tests. Ethanol extract values are comparatively higher in these two tests. However, the FRAP test, that, as discussed before, works under a different mechanism, shows ethyl acetate and ethanol with low values of IC_{50}. Hexane is the least active extract for this test.

Positive controls were 99.8 ± 0.8 % for the ABTS test and 93.0 ± 0.6 % for the DPPH test. These results were not used for the calculations but to confirm that the assays were working properly. In the case of the FRAP assay, the absorbance of the positive control I used itself to establish the 100 % value, so the negative control (11.1 ± 0.9 %) was used to assess the functioning of the assay.

Carotenoids and polyphenols are the two main groups of antioxidant compounds present in microalgae. Polyphenols can act as antioxidants both by the SET and hydrogen transfer mechanisms, while carotenoids work mainly by hydrogen transfer[48]. The hexane extract shows very low activity on FRAP and moderate activity in ABTS and DPPH. Knowing that the FRAP assay only detects antioxidant activity from SET mechanism, it is likely that the hexane extract contains mainly carotenoids, while the ethyl acetate and the ethanol extract probably contain a mixture of carotenoids and polyphenols, more concentrated in the ethyl acetate extract.

These results do not match the ones published by Silva[15], that reports the hexane extract to have the highest antioxidant activity in the three performed tests, with IC_{50} values for the hexane extract of 2.18 mg/mL in the ABTS test, 2.55 mg/mL in the DPPH test and 1.82 mg/mL in the FRAP test. However, the biomass used in the referred study was produced, extracted, and processed by different means that the one used in this work. The biomass was cultivated by an external company, and the details of the process are not

reported. Also, it was spray-dried and stored over two years before the tests. Spray-drying is an alternative for freeze-drying that subjects the biomass to high temperatures during a short period of time, which may cause degradation of bioactive compounds; that, along with the long period of conservation, may have degraded some of the compounds present in it. Furthermore, the disruption and extraction processes were performed using a bead beater, that may yield extracts with different extract compositions from those processed and extracted by ball milling and EDGE. These differences might explain the high variation between the results.

The study from Pereira et al.[34] also reports different IC_{50} values from the ones estimated in this work. The publication reports >10 mg/mL for the hexane and ethanol extracts both in DPPH and ABTS, and values under 1.5 mg/mL for every extract in the FRAP test. However, DPPH and ABTS values for the ethyl acetate extract are similar to the ones estimated (2.6 and 6.9 mg/mL, respectively).

The fact that the work of Pereira also reports ethyl acetate extracts having the highest antioxidant potential of the three studied extracts may indicate the presence of a common ground in both studies. The biomass studied by Pereira was produced in industrial conditions, specifically in 100 m^3 photobioreactors, rather than in a laboratory environment. However, it was analysed shortly after production, as it was done in this study and contrary the study from Silva, in which samples were stores long-term before analysis. This fact highlights the susceptibility of the ethyl acetate extracts to prolonged storage. Ethyl acetate is a medium polarity extract able to extract microalgal carotenoid pigments which usually display high antioxidant activity. Carotenoid pigments, however, are extremely prone to chemical and light oxidation and easily degrade during storage of biomass even in short periods of a few months[49]. Differences in processing and extraction from this study to the one from Pereira, namely using a disperser and non-automatic extraction, contribute to explain the remaining differences found in the results for the two other extracts.

The obtained results are relatively low when compared with the ones reported for other microalgae of the *Tetraselmis* genus. For instance, 1 mg/mL of methanolic extract of *T. chuii* showed 71.4 % inhibition of DPPH[50], while none of the three extracts studied in this work go beyond 50 % at that concentration. Moreover, the hexane extract of *Tetraselmis* sp. has a reported IC_{50} value of 2.41 ± 0.40 mg/mL[51], almost half of what was found in

this study. However, the IC_{50} values found for *Tetraselmis* microalgae are way lower than the ones found for other green microalgae, for instance for *Chloroccocum infusionum, or Desmodesmus intermedius*[52]. Actually, the antioxidant activity of microalgae from the *Tetraselmis* genus is considered as interesting for industrial applications[50].

Considering the results obtained for the 3 extracts, and since the ethyl acetate extract showed consistent antioxidant potential in the three performed tests, this extract was selected for further studies.

4.2.2 Anti-hypertensive activity

The three extracts showed concentration-dependent ACE inhibition activity (Figure 16). At a concentration of 1 mg/mL, the three extracts showed >50 % ACE inhibition, with IC_{50} values of 0.5 ± 0.2 mg/mL for the ethanolic extract, 0.40 ± 0.07 mg/mL for the ethyl acetate extract, and 1.1 ± 0.9 mg/mL for the hexane extract. There are not significant differences between the IC_{50} values because of the high uncertainty reported for the ethanol and hexane extracts. The IC_{50} calculated for the ethyl acetate extract is more precise and reliable. Although the IC_{50} measures do not show clearly which extract has the highest activity, comparison between individual activity values for each concentration in Figure 16 confirms that in fact the ethyl acetate extract is the one with the highest activity, specially at high concentrations.

The only reported value of ACE inhibition for *T. striata* CTP4 was found in Silva[15], with the ethanolic extract yielding just 0.79 % of inhibition at 1 mg/mL. This result differs vastly from the one found in the current work (56.6 %). This may be caused by the differences in production, conservation and processing discussed above. Also, the assay performed in the study from Silva study showed very low inhibition values (10 % to 0 %) for a well-known ACE inhibitor, enalapril, that was used as a positive control. This also differs from the values we found for another well-known inhibitor used as positive control, captopril, and might answer to protocol differences that should be further studied. The inhibition value for our positive control, captopril 1 mM, was over 90 % on average, confirming the reliability of the assay used in this work.

There is evidence of similar microalgae, also from the *Tetraselmis* genus, displaying antihypertensive activity reported in Nova et al.[53]. This work showed that the ACE inhibition activity of horse mackerel fillets sprayed with hydrolats of the microalga was increased up to four-fold when compared to the non-sprayed fillets. Outside the

Tetraselmis genus, there are numerous microalgae with ACE inhibition activity, from which active biopeptides have been isolated, for instance, the biopeptide Tyr-Met-Gly-Leu-Asp-Leu-Lys from *Isochrysis galbana* with IC_{50} of 0.03 mg/mL, or the biopeptide Gly-Met-Asn-Asn-Leu-Thr-Pro with IC_{50} of 0.09 mg/mL isolated from *Nanochloropsis oculata*. There are similar peptides isolated from different species of microalgae such as *Chlorella vulgaris* or *Spirulina platensis*, all showing similar IC_{50} values[54,55,56,57]. The IC_{50} values found for these biopeptides are about 10 times lower than the ones we found for the extracts (0.4-1.1 mg/mL). However, bioactive compounds are expected to be diluted in the initial extracts. Therefore, IC_{50} values are expected to decrease after fractionation of the extracts, possibly making these extracts promising antihypertensive agents.

To the best of our knowledge, there is no other publication exploring the ACE inhibition activity of *T. striata* CTP4 or other microalgae from the *Tetraselmis* genus produced and processed in similar conditions. Thus, further research is needed in other to correctly assess the ACE inhibition activity of *Tetraselmis* sp. and the potential presence of bioactive peptides. In this work, the ethyl acetate extract was identified as the one with the highest ACE inhibition activity.

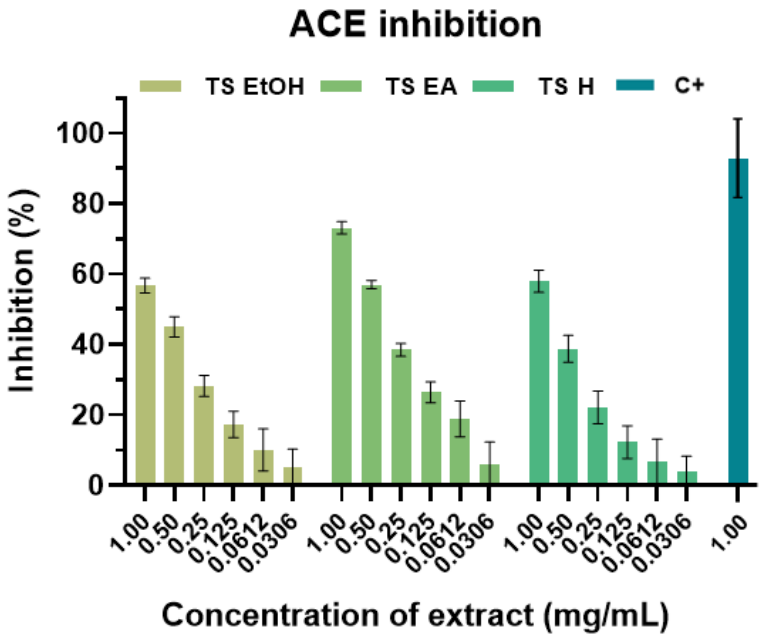


Figure 16. ACE inhibition assay results for ethanol, ethyl acetate, and hexane extracts. For each tested concentration, assays were performed in triplicate three days in a row (*n*=9). TS EtOH: Ethanol extract, TS EA: Ethyl acetate extract. TS H: Hexane extract. C+: positive control (captopril). Error bars indicate a 95% interval of confidence.

4.3 HPLC Fractionation

The results revealed that the ethyl acetate extract of *T. striata* CTP4 had higher antioxidant and antihypertensive activity, so it was selected for HPLC fractionation and LC-MS analysis to tentatively identify individual compounds contributing to these effects.

The analytical run yielded a chromatogram with numerous peaks of variable intensity (Figure 17) and highly spread throughout the whole chromatogram. When compared, ELSD and PAD chromatograms allow to identify which of the compounds causing the peaks absorb UV-Vis light. Peaks around 33, 37, 42 and 46 min showed intense ELSD signals but no PAD signals. This indicates the presence of non-absorbing compounds in the extract. This information is used as guidance for later analysis and discussion.

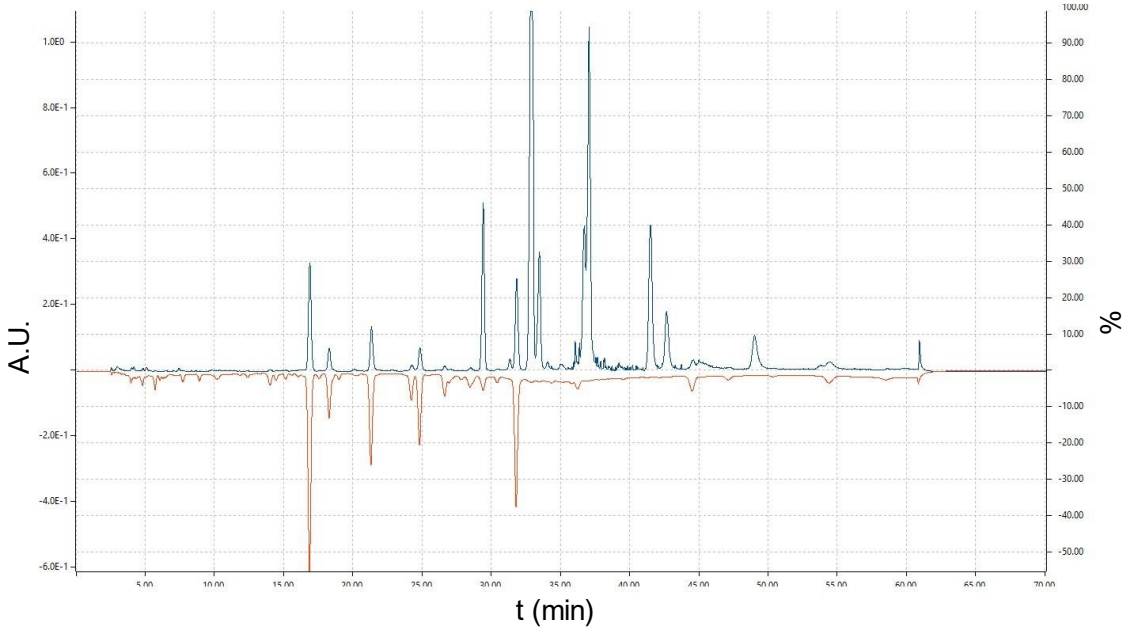


Figure 17. ELSD (blue, scattering percentage in the right axis) and PAD (orange, absorbance units in the left axis) chromatogram of the analytical run of the HPLC fractionation of the ethyl acetate extract.

Some of the 41 fractions were pooled together into a vial and dried again, elaborating new fractions (Figure 18). This was done mainly in the fractions with little-to-no peaks in the chromatogram and low mass yield, so they could be properly tested in the next step. Fractions covering a single and high peak, like fractions G or J, yielded enough mass to be tested by themselves (e.g., fractions 4-8 were pooled together to elaborate fraction B). In this way, 16 new fractions (A-P) were elaborated from the 41 initial fractions.

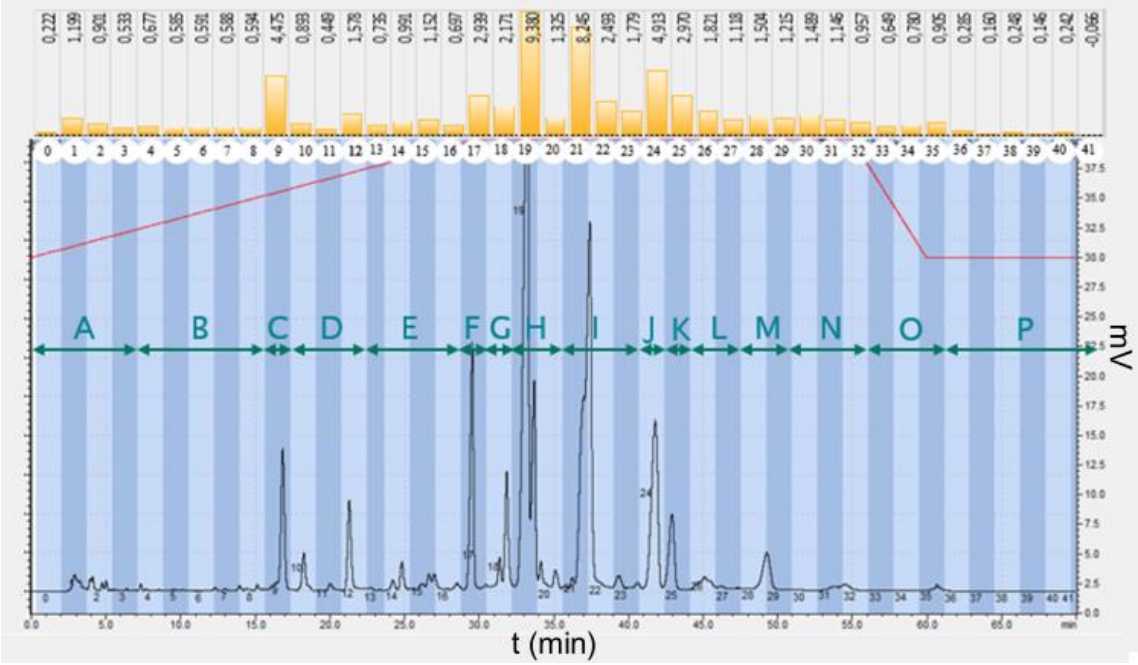


Figure 18. Fractionation scheme superposed with the weigh obtained in each of the fraction (mg) and the ELSD chromatogram of the analytical run.

The total collected weigh was 65.74 mg, starting from 310 injected mg, which translates into an absolute yield of 21 % This yield is similar the reported for similar microalgal fractionation processes[58]. However, there might be room for improvement in the fractionation step to better recover the injected mass. This could be optimized by designing a fractionation scheme with less fractions and less runs to reduce the high amount of manual work subject to mistakes and losses in this step.

Figure 19 shows some of the fractions collected. The difference in colour in between the samples and between the fractions and the dark green crude extract confirm that there was indeed a successful separation of at least different groups of compounds. The colour profile of each fraction was used in the later discussion of the results.

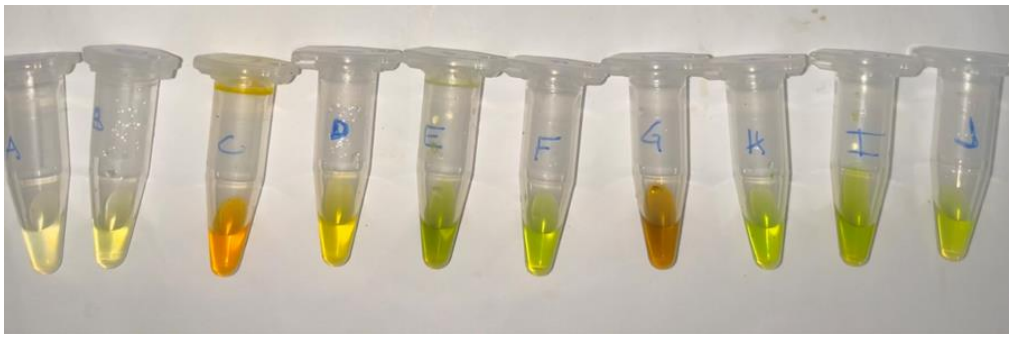


Figure 19. Some of the final fractions after HPLC fractionation.

4.4 Bioactivity assays on fractions

The 16 fractions were tested along with the crude extract. Results are expressed in % of activity of the crude extract, thus 100 % of activity represents the same bioactivity as the crude extract. Less than 100 % activity means that the compounds present in the fraction are less active, than the crude extract, so the compounds causing the bioactivity of the extract are "diluted" by other non-active compounds or are not present at all. On the other hand, more than 100 % activity means that the most bioactive compounds are concentrated in the fraction, and it shows even greater activity than the crude extract.

4.4.1 Antioxidant activity

ABTS, DPPH and FRAP test were performed again in each of the fractions. Results are shown in figures 20-22.

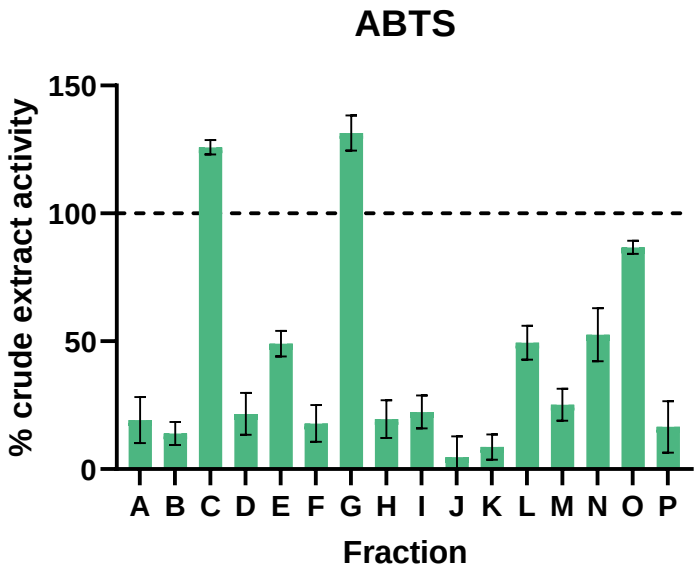


Figure 20. ABTS assay results for the fractions of the ethyl acetate extract. Fractions were tested at 1 mg/mL on triplicate during three days in a row (*n*=9). Error bars indicate 95% interval of confidence. Dotted line represents 100% of crude ethyl acetate extract activity, thus values over the dotted line correspond to fractions more concentrated in bioactive compounds than the ethyl acetate crude extract.

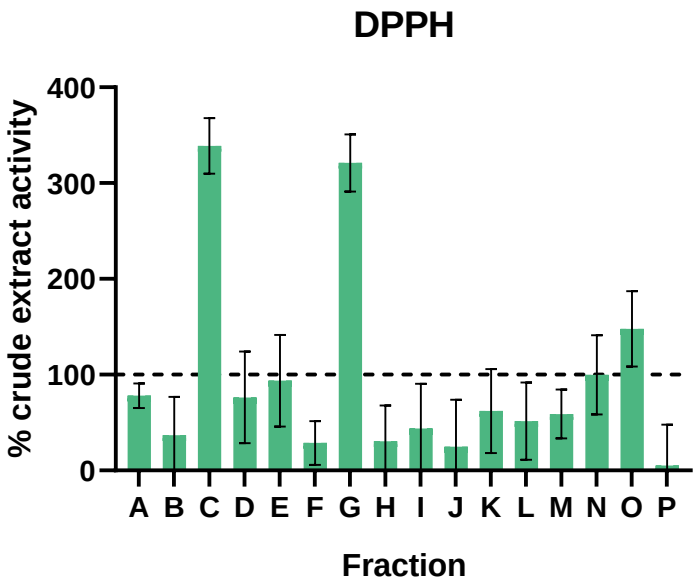


Figure 21. DPPH assay results for the fractions of the ethyl acetate extract. Fractions were tested at 1 mg/mL on triplicate during three days in a row (*n*=9). Error bars indicate 95% interval of confidence. Dotted line represents 100% of crude ethyl acetate extract activity, thus values over the dotted line correspond to fractions more concentrated in bioactive compounds than the ethyl acetate crude extract.

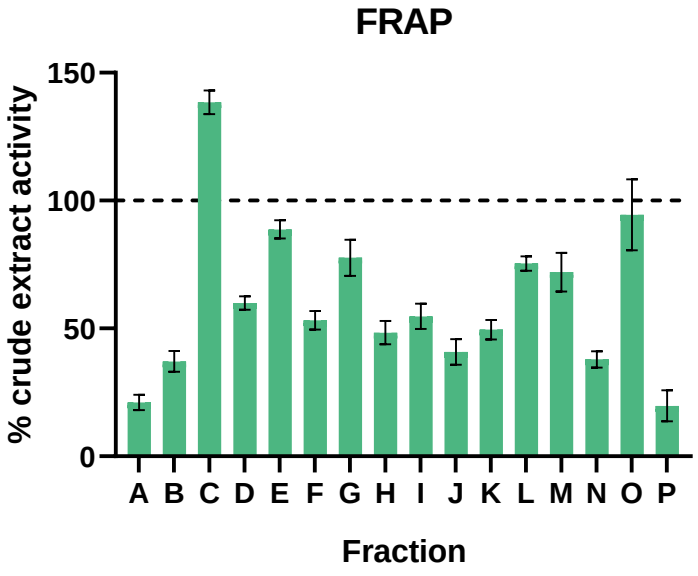


Figure 22. FRAP assay results for the fractions of the ethyl acetate extract. Fractions were tested at 1 mg/mL on triplicate during three days in a row (*n*=9). Error bars indicate 95% interval of confidence. Dotted line represents 100% of crude ethyl acetate extract activity, thus values over the dotted line correspond to fractions more concentrated in bioactive compounds than the ethyl acetate crude extract.

The ABTS assay highlighted fractions C and G as those with more antioxidant power than the crude extract, with 126 % and 131 %, respectively. The profile of the DPPH

assay results is very similar to the one from the ABTS, but with higher and more dispersed results. Fractions C and G are again highlighted as the most antioxidant reaching more than three times the antioxidant activity of the crude extract. O and N also surpassed the 100 % threshold. The confidence intervals for fractions D, E and K reveal the possibility of those fractions also being more active than the crude extract.

FRAP assay results confirmed that C and O are the most antioxidant fractions of the group. Interestingly, G and N show low values in this assay. This is probably due to the differences in mechanisms between the assays. FRAP works only through a SET mechanism, so it is likely that compounds in G and N are carotenoids, as they show their antioxidant activity mostly through hydrogen transfer mechanisms, as discussed before. C and O could be polyphenols or other groups of antioxidants that work through both mechanisms[43].

4.4.2 Anti-hypertensive activity

Fractions were analysed again through the ACE inhibition test (Figure 23). N showed the highest inhibition activity (129 %). C, D, E and O are also close to 100 %. This indicates a certain dispersion of ACE inhibitor compounds throughout the fractions, with a slightly higher concentration in fraction N.

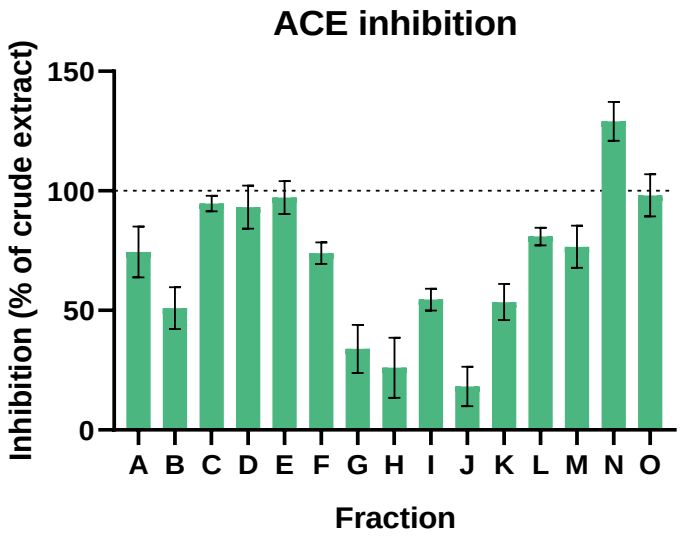


Figure 23. ACE inhibitory activity assay results for the fractions of the ethyl acetate extract. Fractions were tested at 1 mg/mL on triplicate during three days in a row (n=9). Error bars indicate 95% interval of confidence. Dotted line represents 100% of crude ethyl acetate extract activity, thus values over the dotted line correspond to fractions more concentrated in bioactive compounds than the ethyl acetate crude extract.

In light of the results described above, fractions C, O, G, and N were the fractions selected for further analysis, as they likely contain bioactive compounds, specifically antioxidants, antihypertensive or both. The characteristics of these fractions are summarized in Table 4.

Table 4. Summary of the results obtained for the interesting fractions of the ethyl acetate extract.

Fraction	Time (min)	Antioxidant activity[1]	Antihypertensive activity[2]	UV absorption	Colour
C	16-17	Yes	Yes	Yes (380-500 nm)	Orange
G	31-32	Yes	No	Yes (380-500 nm)	Brown
N	48-49	No	Yes	No	Green
O	56-61	Yes	Yes	No	Light green

Notes: [1]More than 100 % relative to the crude extract activity in at least two out of three antioxidant tests. [2]More than 100 % relative to the crude extract activity.

Fractions C and G are orange and brown-coloured and they present UV absorption in the 380-500 nm range. This may indicate the presence of carotenoids in those fractions, as those are two of their main characteristics. During a previous discussion, the presence of carotenoids in C was discarded because of high activity in the FRAP assay (carotenoids do not usually display antioxidant activity through SET mechanism, which is the most important in FRAP). However, some specific carotenoids have also been reported to show high FRAP activities[59]. C and G likely contain at least two different carotenoids, according to the differences in polarity based on the time of elution and the differences in colour.

N and O do not present the typical characteristics of carotenoids. They could be part of other groups of antioxidant or antihypertensive compounds with no UV-Vis light absorption in this range. For instance, poly-unsaturated fatty acids (PUFAs) are present in a wide variety of microalgal species, and they do not typically absorb UV-vis light in the studied range. Furthermore, certain PUFAs are known to be antioxidants and ACE inhibitors[60,61]. Also, the fractions with ACE inhibitory activity could contain biopeptides, that generally do not absorb UV-vis light in the studied range. The fact that N and O are in the last part of the chromatogram suggests that compounds might be of low polarity, while C and G eluded earlier, thus they must display higher polarity.

These four fractions were selected for further analysis in LC-MS to tentatively identify the individual compounds causing the bioactivities reported, with special focus on carotenoids and PUFAs, that would match the reported properties of the fractions.

4.5 LC-MS analysis

Along with fractions G, C, N and O, their adjacent fractions were also analysed. In this way, compounds found in both bioactive and non-bioactive fractions could be discarded, as they did not play a role in the detected bioactivity. A total of 16 compounds were tentatively identified (Table 5). A literature search was carried out for all the compounds, and no direct study on their exact structures could be found.

Fraction C contains rather large compounds, all of them above 700 g/mol. Compound 1 contains six carboxylic moieties. Natural carboxylic acids (Figure 24a) are known for their direct and indirect antioxidant activity. They can directly reduce ROS through chemical reactions, and they can also chelate metal ions, thus stopping their catalytic activity in oxidation processes [62]. Also, the presence of highly chelated metal ions in fraction C (compounds 2 and 3) may indicate that the fraction has abundancy of ligands capable of cancelling the catalytic effects of metal ions in oxidating processes. This could be contributing to the overall antioxidant properties of fraction C.

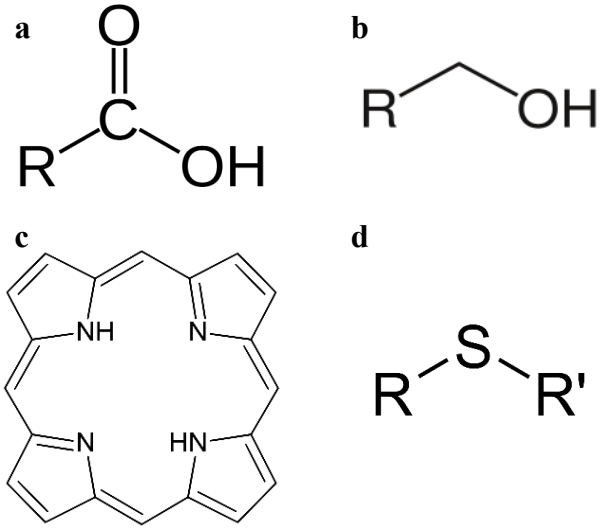


Figure 24. Antioxidant moieties present in tentatively identified compounds: carboxylic acids (a), hydroxymethyl groups (b), porphyrin rings (c) and sulphide links (d).

Compound 2 was tentatively identified as a possible rhenium complex. Rhenium is one of the rarest elements on earth and it has no known biological role. This could indicate that the tentative identification was wrong or that there was some contamination in the samples, as it is highly unlikely to find rhenium compounds in laboratory-grown algae [63]. This compound is also a bisborane. Boron compounds are not directly antioxidants but may play a role in the redox balance acting as a Lewis acid [64].

Compound 3 is possibly a nickel (II) complex with an hydroxymethyl moiety (Figure 24b), with well-known antioxidant properties. The hydroxy moiety is considered to be the strongest antioxidant chemical group present in biological systems [65]. Furthermore, this compound also has a porphyrin ring (Figre 24c). This heterocyclic macrocycle organic ligand has been reported to display antioxidant properties through ROS scavenging, metal chelation, and singlet oxygen quenching [66]. Also, porphyrin is the basic molecule of the chlorophyll[67], thus the presence of this ligand in the solution could be related with derivatives or degradation products of chlorophyll. All of them, including chlorophyll, are known antioxidants[68].

Compound 4 contains a carboxylic moiety that displays antioxidant properties as discussed above, and it also contains a tetrathiapentacyclo group. This group has four sulfide links (Figure 24d) in its structure, which also contribute to the antioxidant activity through chelation of metals, direct ROS scavenging, and regeneration of other antioxidants[69]. The presence of all these antioxidant moieties and chemical groups in Fraction C goes in agreement with the results of the antioxidant tests in which this fraction showed the highest antioxidant activity. Compounds present in Fraction G also contain antioxidant moieties such as a porphyrin ring in Compound 5, quinoline in Compound 8[70], and carboxylic acids in Compound 9. This could explain the activities observed in the tests of the fractions.

Seven compounds were identified in Fraction N and Fraction O. These fractions did not show the highest values for antioxidant activity, but they are the most antihypertensive fractions. In fact, they contain smaller and lighter compounds than C and G (all of them under 350 g/mol). Interestingly, some of the compounds present in these fractions have some similarities with the traditional ACE inhibitors (Figure 24).

Table 5. Tentatively identified compounds from fractions C, G, N, and O through LCMS analysis.

Compound number	Probability (%)	Proposed Formula	Name	Molecular weight (g/mol)	Library
Fraction C					
1	78.46	$C_{72}H_{60}N_2O_{10}$	N,N'-bis(2',5'-di-t-Butylphenyl)benzoperylene-1',2':3,4:9,10-hexacarboxylic-1',2'-anhydride-3,4:9,10-bis(imide)	1113.22	W10N14M2
2	98.70	$C_{34}H_{46}B_2O_3P_2Re$	(R,Sp)-2-(1-Dicyclohexylphosphinoethyl)diphenylphosphinocyrhetrene bisborane	773.267	W10N14M2
3	98.12	$C_{47}H_{50}N_4NiO_3$	Nickel(II)-2,8,12,18-Tetraethyl-5-[p-(hydroxymethyl)phenyl]-15-[2',5'-dimethoxyphenyl]-3,7,13,17-tetramethylporphyrin	776.323	W10N14M2
4	87.47	$C_{48}H_{40}O_2S_4$	Methyl 5,16,24,35-tetrathiapentacyclo [18.18.5.1(3,37).1(7,11).1(10,14).1(18,22).1(26,30).1(29,33).1(29,43)] pentaatriaconta-nonadecaene-50-carboxylate	776.191	W10N14M2
Fraction G					
5	98.72	$C_{60}H_{60}N_4NiO$	[2,3,7,8,12,13,17,18-Octaethyl-5-phenoxy-10,15,20-triphenylporphyrinato]nickel (II)	910.412	W10N14M2
6	97.72	$C_{32}H_{72}Al_8O_8$	Octi[tert-Butylaluminum(μ(3)-oxy]	800.375	W10N14M2
7	85.74	$C_{12}H_{20}BrO_3P$	1-Bromo-2-cyclohexylidene-1-(diethoxyphosphoryl)ethene	322.033	W10N14M2
8	85.93	$C_{17}H_{19}NO_3$	5-(N,2-Dimethyl-1',4'-dihydroquinoline)-2-methyl-1-oxacyclohexane-4,6-dione	285.136	W10N14M1
9	72.25	$C_{36}H_{27}Br_3N_2O_4$	N,N'-Dicyclohexyl-1,6,7-tribromoperylene-3,4:9,10-tetracarboxylic acid bisimide	787.952	W10N14M2
Fraction N					
10	87.67	$C_{18}H_{15}NOS$	2-(2-Thien-2-yl-1H-indol-3-yl)cyclohex-2-en-1-one	293.087	W10N14M1
11	82.61	$C_{12}H_{20}BrO_3P$	1-Bromo-2-cyclohexylidene-1-(diethoxyphosphoryl)ethene	322.033	W10N14M2
12	91.21	C_2H_2BrCl	1-bromo-2-chloro-ethene	139.902	W10N14M1
13	92.05	$C_{11}H_{20}O_2$	(5S,6S)-5-Methyl-6-pentyltetrahydropyran-2-one	184.146	W10N14M1
Fraction O					
14	90.41	$C_{15}H_{23}N_3O_3$	4-(Hexyloxy)vanillin-semicarbazone	167.094	W10N14M1
15	95.58	$C_{16}H_{22}NO_5$	3-[(Ethoxycarbonyl)methyl]-2-formyl-4-carbethoxyvinyl-1-methylpyrrole	308.390	W10N14M1
16	90.62	$C_9H_{13}NO_2$	Methyl (S)-1-Amino-3-vinyl-3-cyclopentene-1-carboxylate	167.094	W10N14M1

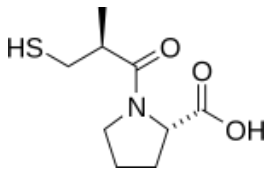
There are three main types of ACE inhibitors: inhibitors with a sulfhydryl group (captopril, rentiapril, and alacepril); inhibitors with a phosphinyl group (fosinopril); and inhibitors with carboxyl moieties (lisinopril, enalapril, quinapril and ramiapril). All of them are small molecules with peptide-like or amino acid-like structures, specifically, most of them show a proline-like structure [71]. ACE inhibition works by competitive non-permanent binding to the active site of the enzyme. Key moieties for this interaction are terminal carboxyl group, amide carbonyl groups and sulfur groups. Molecules containing these and similar structures could interact to some degree with the active site by competitive or non-competitive inhibition[72-74].

Compound 10 shows some similarities with the traditional ACE inhibitors containing sulfur groups such as captopril, alacepril and rentiapril. Although it does not have the proline-like structure with the characteristic carboxylic moiety of those inhibitors, it does have a heterocycle that gives it some structural similarity to the mentioned compounds that could explain some degree of interaction with ACE.

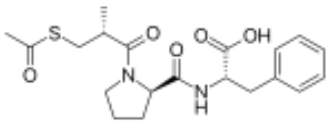
Also, compound 11 contains a phosphinyl group, which is characteristic of a well-known ACE inhibitor, fosinopril[75]. Compound 11 also lacks the carboxylic moiety and does not show much structural similarity. Despite that, the phosphinyl group could be interacting with ACE and causing some inhibition.

On another note, compounds 14, 15, and 16 all share some structural similarities with other described ACE inhibitors. Compound 15 contains a nitrogen heterocycle and carboxylate-like moieties. Compound 16 is the only one compound found in the fractions with an actual amino acid-like structure. These similarities could explain some ACE inhibition activity.

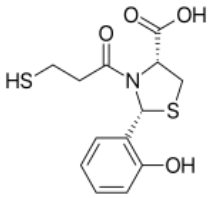
Additionally, Compound 14 is a vanillin semicarbazone substituted with a non-polar chain. Interestingly, semicarbazones are well known compounds with antioxidant, antifungal, antibacterial, anticonvulsant, anti-inflammatory and anticancer properties [76–78]. Specifically, vanillin semicarbazone has been described as a potent anticancer agent after *in vivo* tests against Ehrlich ascites carcinoma in albine Swiss mice[76,77].



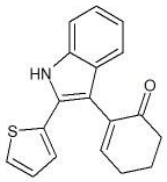
Captopril



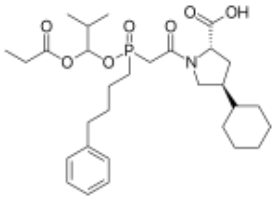
Alacepril



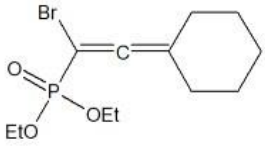
Rentiapril



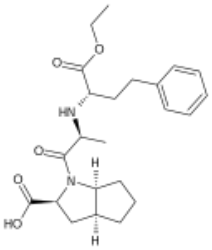
10



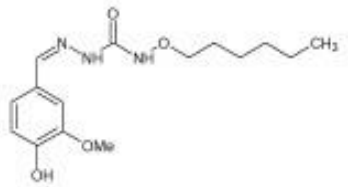
Fosinopril



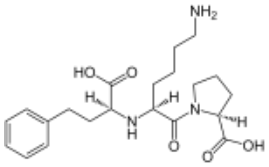
11



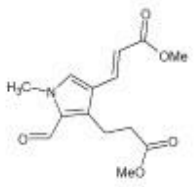
Ramipril



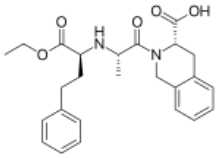
14



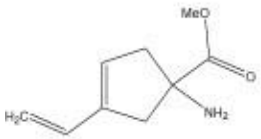
Lisinopril



15



Quinapril



16

Figure 25. Some of the traditional ACE inhibitors and tentatively identified compounds 10, 11, 14, 15, and 16.

Moreover, previous studies have described and tested the anticancer effects of ACE inhibitors. The inhibition of ACE reduces vascular endothelial growth factor levels in tumours and prevents construction of blood vessels to masses, leaving them nutrient-depleted and subsequently hindering their growth[78,79]. Compound 14 is a vanillin semicarbazone derivative; if sufficient similarity to simple vanillin semicarbazone is assumed, then it is likely that compound 14 also has some anticancer and antioxidant properties. Considering that anticancer activity is sometimes linked to the disruption of the renin-angiotensin metabolism by inhibition of ACE, as discussed above, compound 14 could be contributing to both the antioxidant and antihypertensive properties of fraction O. This has not been proven yet, and further research is needed in order to isolate and purify the compound, confirm its structure through Nuclear Magnetic Resonance analysis and test it.

In summary, large molecules with antioxidant properties were tentatively identified in fractions C and G, while small-medium molecules with similarities to ACE inhibitors were tentatively identified in fractions N and O. Furthermore, an interesting compound (Compound 14) was tentatively identified in fraction O. The backbone of the compound is vanillin semicarbazone, a substance with proven anticancer, antioxidant, anti-inflammatory, and other interesting biological activities.

Finally, it must be noted that none of the compounds expected to be present were identified in the samples, i.e., fatty acids, pigments, carotenoids, or ACE inhibiting biopeptides. This could be explained by different factors: degradation of labile compounds during treatment and storage of the fractions, mistakes during the analysis; or the need of other type of analysis, for instance, tandem mass spectrometry is the proper technique used to detect biopeptides, and not simple mass spectrometry. Also, the chromatograms of the LC-MS showed a high number of peaks and background noise, implying that the fractions were still complex mixtures with a high variety of compounds.

5. Conclusions and next steps

The goal of this work was to tentatively identify antioxidant and antihypertensive compounds from extracts of *T. striata* CTP4. The three extracts obtained (ethanol, ethyl acetate, and hexane) were tested for the mentioned bioactivities and all three showed bioactivity in some degree. The results revealed that the ethyl acetate extract of *T. striata* CTP4 was the most interesting extract because of showing the highest bioactivities.

Then, the extract was further divided in fractions and tested again, and then four interesting fractions in which bioactive were concentrated were identified. Finally, the fractions of interest were analysed by LC-MS and a total of 16 compounds were tentatively identified. Some of them displayed moieties like porphyrin or carboxylate groups that justified their antioxidant properties, others had similar structures to ACE inhibitors, thus potentially explaining their antihypertensive activity. Furthermore, a particularly interesting compound (Compound 14) was tentatively identified. The backbone structure of the compound is known for showing different bioactivities and potent anticancer behaviour. Making it an excellent subject of study for further research.

This work confirmed that the extracts of *T. striata* CTP4 have antioxidant activity and revealed that the extracts also contain ACE inhibitors. It also identified the fractions where the bioactive compounds are in the extracts and identified different compounds that possibly contribute to their bioactivity. Although the main goal was partially achieved, there are several steps left down the discovery pipeline to definitively describe the bioactive compounds present in this strain of microalgae.

Now that tentative identification of compounds was carried out, new fractionations and LC-MS analysis should be performed in order to isolate the potentially bioactive compounds. Then, definitive identification through nuclear magnetic resonance should be done, and, lastly, the purified molecules should undergo different bioactivity tests to confirm its properties, mode of action, efficacy *in vitro* and *in vivo*, toxicity assessment, etc. The last steps in the innovation pathway would be to find a commercial application for the compounds, for instance as a drug or nutraceutical, and pass through the mandatory trials to get market authorisation. Eventually, bioengineering could be applied to *T. striata* to further optimize and industrially produce the tentatively identified bioactive compounds.

In conclusion, the results of this work expanded the available knowledge of *T. striata* CTP4 and opened the door for the identification and isolation of antioxidant, antihypertensive, and even anticancer compounds in *T. striata* CTP4 extracts.

6. Bibliography

(1) Avila-Roman, J.; Garda-Gil, S.; Rodriguez-Luna, A.; Motilva, V.; Talero, E. Anti-Inflammatory and Anticancer Effects of Microalgal Carotenoids. *Mar. Drugs* **2021**, *19* (10), 1–49. https://doi.org/10.3390/md19100531.

(2) Levasseur, W.; Perré, P.; Pozzobon, V. A Review of High Value-Added Molecules Production by Microalgae in Light of the Classification. *Biotechnol. Adv.* **2020**, *41* (March), 107545. https://doi.org/10.1016/j.biotechadv.2020.107545.

(3) Musa Ardo, F.; Wei Lim, J.; Ramli, A.; Kee Lam, M.; Kiatkittipong, W.; Alaaeldin Abdelfattah, E.; Kashif Shahid, M.; Usman, A.; Wongsakulphasatch, S.; Tasnim Sahrin, N. A Review in Redressing Challenges to Produce Sustainable Hydrogen from Microalgae for Aviation Industry. *Fuel* **2022**, *330* (August), 125646. https://doi.org/10.1016/j.fuel.2022.125646.

(4) Yap, J. K.; Sankaran, R.; Chew, K. W.; Halimatul Munawaroh, H. S.; Ho, S. H.; Rajesh Banu, J.; Show, P. L. A Comprehensive Review on the Potential Application of Microalgae Biomass. *Chemosphere* **2021**, *281* (February), 130886. https://doi.org/10.1016/j.chemosphere.2021.130886.

(5) Guedes, A. C.; Amaro, H. M.; Malcata, F. X. Microalgae as Sources of High Added-Value Compounds-a Brief Review of Recent Work. *Biotechnol. Prog.* **2011**, *27* (3), 597–613. https://doi.org/10.1002/btpr.575.

(6) Fu, W.; Nelson, D. R.; Yi, Z.; Xu, M.; Khraiwesh, B.; Jijakli, K.; Chaiboonchoe, A.; Alzahmi, A.; Al-Khairy, D.; Brynjolfsson, S.; Salehi-Ashtiani, K. Bioactive Compounds From Microalgae: Current Development and Prospects. *Stud. Nat. Prod. Chem.* **2017**, *54*, 199–225. https://doi.org/10.1016/B978-0-444-63929-5.00006-1.

(7) Moreira, J. B.; Santos, T. D.; Duarte, J. H.; Bezerra, P. Q. M.; de Morais, M. G.; Costa, J. A. V. Role of Microalgae in Circular Bioeconomy: From Waste Treatment to Biofuel Production. *Clean Technol. Environ. Policy* **2021**, No. 0123456789. https://doi.org/10.1007/s10098-021-02149-1.

(8) Spolaore, P.; Joannis-Cassan, C.; Duran, E.; Isambert, A. Commercial Applications of Microalgae. *J. Biosci. Bioeng.* **2006**, *101* (2), 87–96. https://doi.org/10.1263/jbb.101.87.

(9) Sarkar, S.; Manna, M. S.; Bhowmick, T. K.; Gayen, K. Priority-Based Multiple Products from Microalgae: Review on Techniques and Strategies. *Crit. Rev. Biotechnol.* **2020**, *40* (5), 590–607. https://doi.org/10.1080/07388551.2020.1753649.

(10) García, J. L.; de Vicente, M.; Galán, B. Microalgae, Old Sustainable Food and Fashion Nutraceuticals. *Microb. Biotechnol.* **2017**, *10* (5), 1017–1024. https://doi.org/10.1111/1751-7915.12800.

(11) Milledge, J. J. Commercial Application of Microalgae Other than as Biofuels: A Brief Review. *Rev. Environ. Sci. Biotechnol.* **2011**, *10* (1), 31–41. https://doi.org/10.1007/s11157-010-9214-7.

(12) Borowitzka, M. A.; Moheimani, N. R. Energy from Microalgae: A Short History. *Algae for Biofuels and Energy* **2013**, 1–288. https://doi.org/10.1007/978-94-007-5479-9.

(13) Rizwan, M.; Mujtaba, G.; Memon, S. A.; Lee, K.; Rashid, N. Exploring the Potential of Microalgae for New Biotechnology Applications and beyond: A Review. *Renew. Sustain. Energy Rev.* **2018**, *92* (March 2017), 394–404. https://doi.org/10.1016/j.rser.2018.04.034.

(14) Becker, E. W. Microalgae for Human and Animal Nutrition. In *Handbook of Microalgal Culture: Applied Phycology and Biotechnology*; Richmond, A., Hu, Q., Eds.; Blackwell Publishing Ltd, 2013; pp 465–483.

(15) Silva, M. V. M. Establishment of Micoalgae as Functional Food with Antioxidant and Anti-Inflammatory Properties. 2021.

(16) Chen, C.; Tang, T.; Shi, Q.; Zhou, Z.; Fan, J. The Potential and Challenge of Microalgae as Promising Future Food Sources. *Trends Food Sci. Technol.* **2022**, *126* (July), 99–112. https://doi.org/10.1016/j.tifs.2022.06.016.

(17) Costa, J. A. V.; Lucas, B. F.; Alvarenga, A. G. P.; Moreira, J. B.; de Morais, M. G. Microalgae Polysaccharides: An Overview of Production, Characterization, and Potential Applications. *Polysaccharides* **2021**, *2* (4), 759–772. https://doi.org/10.3390/polysaccharides2040046.

(18) De Morais, M. G.; Vaz, B. D. S.; De Morais, E. G.; Costa, J. A. V. Biologically Active Metabolites Synthesized by Microalgae. *Biomed Res. Int.* **2015**, *2015*. https://doi.org/10.1155/2015/835761.

(19) Halliwell, B. How to Characterize an Antioxidant: An Update. *Biochem. Soc. Symp.* **1995**, *61*, 73–101. https://doi.org/10.1042/bss0610073.

(20) Demidchik, V. Mechanisms of Oxidative Stress in Plants: From Classical Chemistry to Cell Biology. *Environ. Exp. Bot.* **2015**, *109*, 212–228. https://doi.org/10.1016/j.envexpbot.2014.06.021.

(21) Coulombier, N.; Jauffrais, T.; Lebouvier, N. Antioxidant Compounds from Microalgae: A Review. *Mar. Drugs* **2021**, *19* (10). https://doi.org/10.3390/md19100549.

(22) Pizzino, G.; Irrera, N.; Cucinotta, M.; Pallio, G.; Mannino, F.; Arcoraci, V.; Squadrito, F.; Altavilla, D.; Bitto, A. Oxidative Stress: Harms and Benefits for Human Health. *Oxid. Med. Cell. Longev.* **2017**, *2017*, 8416763. https://doi.org/10.1155/2017/8416763.

(23) Aruoma, O. I. Free Radicals, Oxidative Stress, and Antioxidants in Human Health and Disease. *J. Am. Oil Chem. Soc.* **1998**, *75* (2), 199–212. https://doi.org/https://doi.org/10.1007/s11746-998-0032-9.

(24) Zhao, C.; Wu, Y.; Yang, C.; Liu, B.; Huang, Y. Review Hypotensive , Hypoglycaemic and Hypolipidaemic Effects of Bioactive Compounds from Microalgae and Marine. **2015**, 1705–1717. https://doi.org/10.1111/ijfs.12860.

(25) Jiang, Q.; Chen, Q.; Zhang, T.; Liu, M.; Duan, S.; Sun, X. The Antihypertensive

Effects and Potential Molecular Mechanism of Microalgal Angiotensin I-Converting Enzyme Inhibitor-Like Peptides : A Mini Review. **2021**.

(26) Ejike, C. E. C. C.; Collins, S. A.; Balasuriya, N.; Swanson, A. K.; Mason, B.; Udenigwe, C. C. Trends in Food Science & Technology Prospects of Microalgae Proteins in Producing Peptide-Based Functional Foods for Promoting Cardiovascular Health. *Trends Food Sci. Technol.* **2017**, *59*, 30–36. https://doi.org/10.1016/j.tifs.2016.10.026.

(27) Xie, J.; Chen, X.; Wu, J.; Zhang, Y.; Zhou, Y.; Zhang, L.; Tang, Y.-J.; Wei, D. Antihypertensive Effects, Molecular Docking Study, and Isothermal Titration Calorimetry Assay of Angiotensin I-Converting Enzyme Inhibitory Peptides from Chlorella Vulgaris. *J. Agric. Food Chem.* **2018**, *66* (6), 1359–1368. https://doi.org/10.1021/acs.jafc.7b04294.

(28) van der Spiegel, M.; Noordam, M. Y.; van der Fels-Klerx, H. J. Safety of Novel Protein Sources (Insects, Microalgae, Seaweed, Duckweed, and Rapeseed) and Legislative Aspects for Their Application in Food and Feed Production. *Compr. Rev. Food Sci. Food Saf.* **2013**, *12* (6), 662–678. https://doi.org/https://doi.org/10.1111/1541-4337.12032.

(29) Fleurence, J.; Levine, I. A. Chapter 14 - Antiallergic and Allergic Properties. In *Microalgae in Health and Disease Prevention*; Levine, I. A., Fleurence, J., Eds.; Academic Press, 2018; pp 307–315. https://doi.org/https://doi.org/10.1016/B978-0-12-811405-6.00014-1.

(30) Markou, G.; Chentir, I.; Tzovenis, I. Chapter 9 - Microalgae and Cyanobacteria as Food: Legislative and Safety Aspects. In *Cultured Microalgae for the Food Industry*; Lafarga, T., Acién, G., Eds.; Academic Press, 2021; pp 249–264. https://doi.org/https://doi.org/10.1016/B978-0-12-821080-2.00003-4.

(31) Lam, T. P.; Lee, T.-M.; Chen, C.-Y.; Chang, J.-S. Strategies to Control Biological Contaminants during Microalgal Cultivation in Open Ponds. *Bioresour. Technol.* **2018**, *252*, 180–187. https://doi.org/https://doi.org/10.1016/j.biortech.2017.12.088.

(32) EABA. Algae as Novel Food in Europe . **2021**, No. April.

(33) Pereira, H.; Gangadhar, K. N.; Schulze, P. S. C.; Santos, T.; De Sousa, C. B.; Schueler, L. M.; Custódio, L.; Malcata, F. X.; Gouveia, L.; Varela, J. C. S.; Barreira, L. Isolation of a Euryhaline Microalgal Strain, Tetraselmis Sp. CTP4, as a Robust Feedstock for Biodiesel Production. *Sci. Rep.* **2016**, *6* (October), 1–11. https://doi.org/10.1038/srep35663.

(34) Pereira, H.; Silva, J.; Santos, T.; Gangadhar, K. N.; Raposo, A.; Nunes, C.; Coimbra, M. A.; Gouveia, L.; Barreira, L.; Varela, J. Nutritional Potential and Toxicological Evaluation of Tetraselmis Sp. CtP4 Microalgal Biomass Produced in Industrial Photobioreactors. *Molecules* **2019**, *24* (17), 1–18. https://doi.org/10.3390/molecules24173192.

(35) Schulze, P. S. C.; Carvalho, C. F. M.; Pereira, H.; Gangadhar, K. N.; Schüler, L. M.; Santos, T. F.; Varela, J. C. S.; Barreira, L. Urban Wastewater Treatment by Tetraselmis Sp. CTP4 (Chlorophyta). *Bioresour. Technol.* **2017**, *223*, 175–183.

https://doi.org/https://doi.org/10.1016/j.biortech.2016.10.027.

(36) Pereira, H.; Páramo, J.; Silva, J.; Marques, A.; Barros, A.; Maurício, D.; Santos, T.; Schulze, P.; Barros, R.; Gouveia, L.; Barreira, L.; Varela, J. Scale-up and Large-Scale Production of Tetraselmis Sp. CTP4 (Chlorophyta) for CO2 Mitigation: From an Agar Plate to 100-M3 Industrial Photobioreactors. *Sci. Rep.* **2018**, *8* (1), 5112. https://doi.org/10.1038/s41598-018-23340-3.

(37) Silva, M.; Kamberovic, F.; Uota, S. T.; Kovan, I. M.; Viegas, C. S. B.; Simes, D. C.; Gangadhar, K. N.; Varela, J.; Barreira, L. Microalgae as Potential Sources of Bioactive Compounds for Functional Foods and Pharmaceuticals. *Appl. Sci.* **2022**, *12* (12). https://doi.org/10.3390/app12125877.

(38) Pereira, H.; Sardinha, M.; Santos, T.; Gouveia, L.; Barreira, L.; Dias, J.; Varela, J. Incorporation of Defatted Microalgal Biomass (Tetraselmis Sp. CTP4) at the Expense of Soybean Meal as a Feed Ingredient for Juvenile Gilthead Seabream (Sparus Aurata). *Algal Res.* **2020**, *47*, 101869. https://doi.org/https://doi.org/10.1016/j.algal.2020.101869.

(39) Schüler, L. M.; Bombo, G.; Duarte, P.; Santos, T. F.; Maia, I. B.; Pinheiro, F.; Marques, J.; Jacinto, R.; Schulze, P. S. C.; Pereira, H.; Barreira, L.; Varela, J. C. S. Carotenoid Biosynthetic Gene Expression, Pigment and n-3 Fatty Acid Contents in Carotenoid-Rich Tetraselmis Striata CTP4 Strains under Heat Stress Combined with High Light. *Bioresour. Technol.* **2021**, *337* (June). https://doi.org/10.1016/j.biortech.2021.125385.

(40) Schüler, L. M.; Santos, T.; Pereira, H.; Duarte, P.; Katkam, N. G.; Florindo, C.; Schulze, P. S. C.; Barreira, L.; Varela, J. C. S. Improved Production of Lutein and β-Carotene by Thermal and Light Intensity Upshifts in the Marine Microalga Tetraselmis Sp. CTP4. *Algal Res.* **2020**, *45*, 101732. https://doi.org/https://doi.org/10.1016/j.algal.2019.101732.

(41) CEM. *EDGE Brochure*. January. https://cemcontenttype.s3.amazonaws.com/content/media-library/attachments/Broch_EDGE_B140_English_3.pdf (accessed 2023-06-15).

(42) Kinross, A. D.; Hageman, K. J.; Doucette, W. J.; Foster, A. L. Comparison of Accelerated Solvent Extraction (ASE) and Energized Dispersive Guided Extraction (EDGE) for the Analysis of Pesticides in Leaves. *J. Chromatogr. A* **2020**, *1627*, 461414. https://doi.org/https://doi.org/10.1016/j.chroma.2020.461414.

(43) Rumpf, J.; Burger, R.; Schulze, M. Statistical Evaluation of DPPH, ABTS, FRAP, and Folin-Ciocalteu Assays to Assess the Antioxidant Capacity of Lignins. *Int. J. Biol. Macromol.* **2023**, *233*, 123470. https://doi.org/10.1016/j.ijbiomac.2023.123470.

(44) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant Activity Applying an Improved ABTS Radical Cation Decolorization Assay. *Free Radic. Biol. Med.* **1999**, *26* (9–10), 1231–1237. https://doi.org/10.1016/s0891-5849(98)00315-3.

(45) BLOIS, M. S. Antioxidant Determinations by the Use of a Stable Free Radical.

Nature **1958**, *181* (4617), 1199–1200. https://doi.org/10.1038/1811199a0.

(46) Megías, C.; Pastor-Cavada, E.; Torres-Fuentes, C.; Girón-Calle, J.; Alaiz, M.; Juan, R.; Pastor, J.; Vioque, J. Chelating, Antioxidant and Antiproliferative Activity of Vicia Sativa Polyphenol Extracts. *Eur. Food Res. Technol.* **2009**, *230* (2), 353–359. https://doi.org/10.1007/s00217-009-1178-x.

(47) Sentandreu, M. A.; Toldrá, F. A Fluorescence-Based Protocol for Quantifying Angiotensin-Converting Enzyme Activity. *Nat. Protoc.* **2006**, *1* (5), 2423–2427. https://doi.org/10.1038/nprot.2006.349.

(48) Goiris, K.; Muylaert, K.; Fraeye, I.; Foubert, I.; De Brabanter, J.; De Cooman, L. Antioxidant Potential of Microalgae in Relation to Their Phenolic and Carotenoid Content. *J. Appl. Phycol.* **2012**, *24* (6), 1477–1486. https://doi.org/10.1007/s10811-012-9804-6.

(49) Gouveia, L.; Empis, J. Relative Stabilities of Microalgal Carotenoids in Microalgal Extracts, Biomass and Fish Feed: Effect of Storage Conditions. *Innov. Food Sci. Emerg. Technol.* **2003**, *4* (2), 227–233. https://doi.org/https://doi.org/10.1016/S1466-8564(03)00002-X.

(50) Widowati, I.; Zainuri, M.; Kusumaningrum, H. P.; Susilowati, R.; Hardivillier, Y.; Leignel, V.; Bourgougnon, N.; Mouget, J.-L. Antioxidant Activity of Three Microalgae Dunaliella Salina, Tetraselmis Chuii and Isochrysis Galbana Clone Tahiti. *IOP Conf. Ser. Earth Environ. Sci.* **2017**, *55* (1), 12067. https://doi.org/10.1088/1755-1315/55/1/012067.

(51) Custódio, L.; Soares, F.; Pereira, H.; Barreira, L.; Vizetto-Duarte, C.; Rodrigues, M. J.; Rauter, A. P.; Alberício, F.; Varela, J. Fatty Acid Composition and Biological Activities of Isochrysis Galbana T-ISO, Tetraselmis Sp. and Scenedesmus Sp.: Possible Application in the Pharmaceutical and Functional Food Industries. *J. Appl. Phycol.* **2014**, *26* (1), 151–161. https://doi.org/10.1007/s10811-013-0098-0.

(52) Santhakumaran, P.; Ayyappan, S. M.; Ray, J. G. Nutraceutical Applications of Twenty-Five Species of Rapid-Growing Green-Microalgae as Indicated by Their Antibacterial, Antioxidant and Mineral Content. *Algal Res.* **2020**, *47*, 101878. https://doi.org/https://doi.org/10.1016/j.algal.2020.101878.

(53) Nova, P.; Cunha, S. A.; Barros, D.; Ramos, C.; Castro, I.; Vaz-Velho, M.; Pintado, M.; Gomes, A. Anti-Hypertensive Activity of Horse Mackerel Pulverized with Three Different Extracts from Marine Origin. *Millenium* **2021**, *2* (ed espec nº8), 111–111.

(54) Hayes, M.; Bastiaens, L.; Gouveia, L.; Gkelis, S.; Skomedal, H.; Skjanes, K.; Murray, P.; García-Vaquero, M.; Hosoglu, M. I.; Dodd, J.; Konstantinou, D.; Safarik, I.; Zittelli, G. C.; Rimkus, V.; del Pino, V.; Muylaert, K.; Edwards, C.; Laake, M.; da Silva, J. G. L.; Pereira, H.; Abelho, J. Microalgal Bioactive Compounds Including Protein, Peptides, and Pigments: Applications, Opportunities, and Challenges During Biorefinery Processes. In *Novel Proteins for Food, Pharmaceuticals and Agriculture*; John Wiley & Sons, Ltd, **2018**; pp 239–255. https://doi.org/https://doi.org/10.1002/9781119385332.ch12.

(55) Samarakoon, K. W.; O-Nam, K.; Ko, J.-Y.; Lee, J.-H.; Kang, M.-C.; Kim, D.; Lee, J. B.; Lee, J.-S.; Jeon, Y.-J. Purification and Identification of Novel Angiotensin-I Converting Enzyme (ACE) Inhibitory Peptides from Cultured Marine Microalgae (Nannochloropsis Oculata) Protein Hydrolysate. *J. Appl. Phycol.* **2013**, *25* (5), 1595–1606. https://doi.org/10.1007/s10811-013-9994-6.

(56) Wu, H.; Xu, N.; Sun, X.; Yu, H.; Zhou, C. Hydrolysis and Purification of ACE Inhibitory Peptides from the Marine Microalga Isochrysis Galbana. *J. Appl. Phycol.* **2015**, *27* (1), 351–361. https://doi.org/10.1007/s10811-014-0347-x.

(57) Suetsuna, K.; Chen, J.-R. Identification of Antihypertensive Peptides from Peptic Digest of Two Microalgae, Chlorella Vulgaris and Spirulina Platensis. *Mar. Biotechnol.* **2001**, *3* (4), 305–309. https://doi.org/10.1007/s10126-001-0012-7.

(58) Kautzmann, B. Bioprospecting Anti-Inflammatory Compounds in Three Diatoms, Cylindrotheca Closterium, Cylindrotheca Fusiformis and Nanofrustulum Shiloi. 2022.

(59) Müller, L.; Fröhlich, K.; Böhm, V. Comparative Antioxidant Activities of Carotenoids Measured by Ferric Reducing Antioxidant Power (FRAP), ABTS Bleaching Assay (ATEAC), DPPH Assay and Peroxyl Radical Scavenging Assay. *Food Chem.* **2011**, *129* (1), 139–148. https://doi.org/https://doi.org/10.1016/j.foodchem.2011.04.045.

(60) Pekkoh, J.; Phinyo, K.; Thurakit, T.; Lomakool, S.; Duangjan, K.; Ruangrit, K.; Pumas, C.; Jiranusornkul, S.; Yooin, W.; Cheirsilp, B.; Pathom-Aree, W.; Srinuanpan, S. Lipid Profile, Antioxidant and Antihypertensive Activity, and Computational Molecular Docking of Diatom Fatty Acids as ACE Inhibitors. *Antioxidants (Basel, Switzerland)* **2022**, *11* (2). https://doi.org/10.3390/antiox11020186.

(61) Borghi, C.; Cicero, A. F. G. Omega-3 Polyunsaturated Fatty Acids: Their Potential Role in Blood Pressure Prevention and Management. *Heart Int.* **2006**, *2* (2), 98. https://doi.org/10.4081/hi.2006.98.

(62) Godlewska-żyłkiewicz, B.; Świsłocka, R.; Kalinowska, M.; Golonko, A.; Świderski, G.; Arciszewska, Ż.; Nalewajko-Sieliwoniuk, E.; Naumowicz, M.; Lewandowski, W. Biologically Active Compounds of Plants: Structure-Related Antioxidant, Microbiological and Cytotoxic Activity of Selected Carboxylic Acids. *Materials (Basel).* **2020**, *13* (19), 1–37. https://doi.org/10.3390/ma13194454.

(63) Xiong, Y.; Xu, J.; Shan, W.; Lou, Z.; Fang, D.; Zang, S.; Han, G. A New Approach for Rhenium(VII) Recovery by Using Modified Brown Algae Laminaria Japonica Adsorbent. *Bioresour. Technol.* **2013**, *127*, 464–472. https://doi.org/https://doi.org/10.1016/j.biortech.2012.09.099.

(64) Turkez, H.; Arslan, M. E.; Tatar, A.; Mardinoglu, A. Promising Potential of Boron Compounds against Glioblastoma: In Vitro Antioxidant, Anti-Inflammatory and Anticancer Studies. *Neurochem. Int.* **2021**, *149*, 105137. https://doi.org/https://doi.org/10.1016/j.neuint.2021.105137.

(65) Al-Mamary, M. A.; Moussa, Z. Antioxidant Activity: The Presence and Impact of

Hydroxyl Groups in Small Molecules of Natural and Synthetic Origin. In *Antioxidants*; Waisundara, V., Ed.; IntechOpen: Rijeka, 2021. https://doi.org/10.5772/intechopen.95616.

(66) Antonova, N. A.; Osipova, V. P.; Kolyada, M. N.; Movchan, N. O.; Milaeva, E. R.; Pimenov, Y. T. Study of the Antioxidant Properties of Porphyrins and Their Complexes with Metals. *Macroheterocycles* **2010**, *3* (2–3), 139–144. https://doi.org/10.6060/mhc2010.2-3.139.

(67) Hosikian, A.; Lim, S.; Halim, R.; Danquah, M. K. Chlorophyll Extraction from Microalgae: A Review on the Process Engineering Aspects. *Int. J. Chem. Eng.* **2010**, *2010*, 391632. https://doi.org/10.1155/2010/391632.

(68) Lanfer-Marquez, U. M.; Barros, R. M. C.; Sinnecker, P. Antioxidant Activity of Chlorophylls and Their Derivatives. *Food Res. Int.* **2005**, *38* (8), 885–891. https://doi.org/https://doi.org/10.1016/j.foodres.2005.02.012.

(69) Wu, Z.-Q.; Li, K.; Ma, J.-K.; Huang, Q.; Tian, X.; Li, Z.-J. Antioxidant Activity of Organic Sulfides from Fresh Allium Macrostemon Bunge and Their Protective Effects against Oxidative Stress in Caenorhabditis Elegans. *J. Food Biochem.* **2020**, *44* (11), e13447. https://doi.org/https://doi.org/10.1111/jfbc.13447.

(70) Orhan Püsküllü, M.; Tekiner, B.; Suzen, S. Recent Studies of Antioxidant Quinoline Derivatives. *Mini Rev. Med. Chem.* **2013**, *13* (3), 365–372. https://doi.org/10.2174/138955713804999793.

(71) Hanif, K.; Bid, H. K.; Konwar, R. Reinventing the ACE Inhibitors: Some Old and New Implications of ACE Inhibition. *Hypertens. Res.* **2010**, *33* (1), 11–21. https://doi.org/10.1038/hr.2009.184.

(72) Arora, P. K.; Lifesciences, N.; Chauhan, A. ACE Inhibitors : A Comprehensive Review. *Int. J. Pharm. Sci. Res.* **2014**, No. June.

(73) Tu, M.; Wang, C.; Chen, C.; Zhang, R.; Liu, H.; Lu, W.; Jiang, L.; Du, M. Identification of a Novel ACE-Inhibitory Peptide from Casein and Evaluation of the Inhibitory Mechanisms. *Food Chem.* **2018**, *256*, 98–104. https://doi.org/https://doi.org/10.1016/j.foodchem.2018.02.107.

(74) Andrews, P. R.; Carson, J. M.; Caselli, A.; Spark, M. J.; Woods, R. Conformational Analysis and Active Site Modeling of Angiotensin-Converting Enzyme Inhibitors. *J. Med. Chem.* **1985**, *28* (3), 393–399. https://doi.org/10.1021/jm00381a021.

(75) DeForrest, J. M.; Waldron, T. L.; Harvey, C.; Scalese, B.; Rubin, B.; Powell, J. R.; Petrillo, E. W.; Cushman, D. W. Fosinopril, a Phosphinic Acid Inhibitor of Angiotensin I Converting Enzyme: In Vitro and Preclinical in Vivo Pharmacology. *J. Cardiovasc. Pharmacol.* **1989**, *14* (5), 730–736. https://doi.org/10.1097/00005344-198911000-00009.

(76) Singhal, M.; Paul, A.; Singh, H. P.; Dubey, S. K.; Songara, R. K. Synthesis and Evaluation of Antioxidant Activity of Semicarbazone Derivatives Synthesis and Evaluation of Antioxidant Activity of Semicarbazone Derivatives. *IJPSDR* **2011**, *3* (August 2016), 150–154.

(77) Verma, T.; Shekhar Sharma, C.; HemendraPratap, S. Semicarbazone: A Versatile

Therapeutic Analogue and Its Pharmacological Evaluation. *Pharmatutor - Pharm. Infopedia* **2013**.

(78) Pandeya, S. Semicarbazone - a Versatile Therapeutic Pharmacophore for Fragment Based Anticonvulsant Drug Design. *Acta Pharm.* **2012**, *62*, 263–286. https://doi.org/10.2478/v10007-012-0030-1.

(79) Lindberg, H.; Nielsen, D.; Jensen, B. V; Eriksen, J.; Skovsgaard, T. Angiotensin Converting Enzyme Inhibitors for Cancer Treatment? *Acta Oncol.* **2004**, *43* (2), 142—152. https://doi.org/10.1080/02841860310022346.