MÉLANIE VANESSA MARTINS SILVA

ESTABLISHMENT OF MICROALGAE AS FUNCTIONAL FOOD WITH ANTIOXIDANT AND ANTI-INFLAMMATORY PROPERTIES

ESTABELECIMENTO DE MICROALGAS COMO ALIMENTOS FUNCIONAIS COM AÇÃO ANTIOXIDANTE E ANTI-INFLAMATÓRIA

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Master's in Food Technology

Work under the supervision of:

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Authorship Statement

I hereby declare to be the author of this work, which is original and unpublished. Authors and papers consulted are duly cited in the text and are listed in the included references.

(Mélanie Vanessa Martins Silva)

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"If Nothing Changes, Nothing Changes."

~Unknown

"Let food be thy medicine, and let medicine be thy food."

~ Hippocrates

RESUMO

As doenças crónicas não-transmissíveis (DCNT), tais como doenças cardiovasculares, cancros, doenças respiratórias e diabetes, são a principal causa de mortalidade e morbilidade a nível mundial, sendo consideradas um dos maiores problemas de saúde pública da atualidade pela Organização Mundial de Saúde. Para além das DCNT estarem relacionadas com o aparecimento de diversas comorbilidades, suspeita-se que ampliem os impactos da pandemia por COVID-19, diminuindo a qualidade de vida dos infetados. A maior parte dos fatores de risco para desenvolvimento de DCNT são preveníeis e modificáveis através de mudanças de estilo de vida, como a prática de atividade física e a adoção de uma alimentação saudável.

Recentemente tem-se verificado uma maior consciencialização dos consumidores relativamente ao papel da alimentação na modulação da saúde, levando à alteração nos padrões de consumo. Atualmente, os consumidores preocupados com a saúde reconhecem a alimentação saudável como um pilar da prevenção de doenças crónicas e procuram alimentos que para além do seu valor nutricional apresentem também benefícios para a sua saúde. Quando inseridos num estilo de vida saudável, os alimentos funcionais para além de fornecer macronutrientes necessários à sobrevivência, influenciam funções específicas do organismo através dos seus compostos ativos atuando a nível da promoção da saúde e/ou prevenção da doença. Para além das fontes tradicionais de compostos ativos (frutas, vegetais, cereais integrais, entre outros), a necessidade de satisfazer as expetativas dos consumidores tem estimulado a indústria alimentar a investigar fontes alternativas de alimentos funcionais. As microalgas apresentam um elevado potencial como alimentos funcionais devido ao seu perfil nutricional de boa qualidade (elevado teor de proteínas, minerais, vitaminas, ácido gordos polinsaturados, entre outros) e abundância de compostos como atividades biológicas benéficas para o seu humano (atividade antibacteriana, antioxidante anti tumoral, antifúngica, antivírica, anti-inflamatória, entre outros). Para além de taxa de crescimento elevada e baixo custo de produção, as algas podem ser induzidas a produzir compostos bioativos de valor acrescido específicos, por manipulação das condições de cultivo.

Compostos com atividade antioxidante e anti-inflamatória são de particular interesse, uma vez que o dano oxidativo prolongado e inflamação crónica estão na base da maior parte das patologias.

Nesta dissertação de mestrado o principal objetivo foi a análise da atividade anti-inflamatória e antioxidante de extratos de três espécies de microalgas (*Tisochrysis lutea*, *Tetraselmis chui*, *Tetraselmis striata* CTP4) preparados com quatro solventes (água, etanol, acetato de etilo e hexano). Estes solventes cobriram uma ampla faixa de índices de polaridade, variando desde 0.1 para hexano, 4.4 para acetato de etilo, 5.2 para etanol, até 10.2 para água para maximizar o espectro de compostos extraídos. O tipo de solvente e a polaridade são aspetos fundamentais na extração seletiva de compostos, enquanto os solventes apolares, como hexano, são conhecidos por terem maior recuperação de compostos apolares ou neutros (como terpenos, óleos essenciais e alcanos), os solventes polares como água têm maior recuperação de lipídios neutros e compostos polares. Água, etanol e acetato de etilo são solventes de extração considerados *food grade*, ou seja, que podem ser utilizados durante o processamento de alimentos segundo a Diretiva 2009/32/CE do Parlamento Europeu e do Conselho. Hexano embora não seja um solvente *food-grade*, encontra-se incluído na categoria " solventes de extração cujas condições de utilização são especificadas", por ser um composto altamente volátil que pode ser utilizado na utilização de extração de composto, desde que sejam cumpridos os limites máximos especificados de resíduos nos géneros alimentícios ou nos ingredientes alimentares extraídos.

A atividade antioxidante foi determinada usando ensaios de capacidade de captação dos radicais DPPH e ABTS, de atividade quelante de cobre e de ferro e de atividade redutora de ferro. Depois de determinados os IC_{50} dos respetivos ensaios, os extratos com maior potencial antioxidante foram os extratos etanólicos de *Tisochrysis lutea*, *Tetraselmis chui*, *Tetraselmis striata* CTP4 e o extrato de hexano de *Tetraselmis striata* CTP4.

Adicionalmente, investigou-se o efeito de diferentes técnicas de extração (lise celular auxiliada por *bead-beater*, extração auxiliada por micro-ondas e extração automática auxiliada por temperatura e pressão - EDGE) no rendimento e atividade antioxidante (avaliada através de ensaios de capacidade de captação de radicais DPPH e ABTS) dos extratos de etanol e hexano da *Tetraselmis striata* CTP4. O rendimento nos extratos etanólicos foi superior na extração com *bead-beater* (6.71%) (ANOVA, p <0,001), comparativamente com extratos obtidos com as outras duas técnicas. No entanto, é de realçar que o rendimento obtido no *bead-beater* foi obtido após 6 ciclos de extração, enquanto com os outros equipamentos foram feitos, no máximo, 4 ciclos de extração. Os rendimentos dos extratos de hexano foram significativamente menores com a extração

assistida por micro-ondas (ANOVA, p <0,05), mas não foram encontradas diferenças de rendimento entre os extratos obtidos por técnica de extração automática auxiliada por temperatura e pressão e extratos obtidos com *bead-beater*. Os extratos com hexano tiveram os maiores rendimentos a 50 ºC com o EDGE (2,38% e 1,64% por 1 ou 2 ciclos de 10 minutos, respetivamente), seguido pelo *bead-beater* (6 ciclos, 1,48%). A atividade antioxidante foi superior nos extratos de hexano em ambos os ensaios DPPH e ABTS. Em ambos os ensaios *bead-beater* mostra diferenças significativas (ANOVA, p <0,05) na atividade antioxidante em comparação com EDGE e MW. No ensaio DPPH, todos os extratos produzidos no extrator MW apresentaram menor atividade antioxidante (máx. 15,2%) do que aqueles produzidos no extrator EDGE (máx. 23,9%) ou no *bead-beater* com esferas de vidro (20,3%). No ensaio ABTS, todos os extratos produzidos no extrator EDGE apresentaram menor atividade antioxidante (máx. 9,3%) do que aqueles produzidos no extrator MW (máx. 18,4%) ou no *bead-beater* com esferas de vidro (26,9%). Portanto, em geral, temperaturas de extração mais elevadas geraram rendimentos mais elevados devido à disrupção celular mais eficiente, mas significativamente impactam a atividade antioxidante dos extratos, possivelmente devido à degradação térmica dos compostos bioativos.

A atividade anti-inflamatória de extratos foi determinada pelos ensaios de atividade inibitória da enzima conversora da angiotensina (ECA) e atividade inibitória da cicloxigenase. No ensaio inibitório da ECA, os valores obtidos foram relativamente baixos, sendo que na concentração de 1 mg/mL em DMSO o extrato etanólico de *Tetraselmis chui* exibiu maior atividade inibitória (5,8%) seguido de *Tysochrysis lutea* (2,9%), os extratos de *Tetraselmis striata* CTP4 apresentaram a inibição mais baixa, com 0,79% e 2,29% para o extrato etanólico e de hexano, respetivamente. No ensaio de inibição da cicloxigenase os extratos etanólicos à concentração de 1 mg/mL foram os que apresentaram maior percentagem de inibição (44,5 ± 3,7%; 32,2 ± 7.7%; 22,2 ± 4.3%, para *Tisochrysis lutea*, *Tetraselmis chui*, *Tetraselmis striata* CTP4, respetivamente), o extrato de hexano de *Tetraselmis striata* CTP4 apenas apresentou uma inibição de 6%.

A identificação dos componentes voláteis e fenólicos dos extratos mais bioativos (extratos etanólicos de *Tisochrysis lutea* e de *Tetraselmis chui*) foi efetuada por cromatografia gasosa acoplada a espetrometria de massa (GC-MS), tendo os compostos encontrados sido identificados por comparação dos seus espetros de massa com os presentes na biblioteca de espetros da NIST. O extrato de *Tisochrysis lutea* apresentou

ácido mirístico (6,06 ± 1,69%), ácido palmítico (5,02 ± 0,38%), norleucina (0,68 ± 0,05%) e ácido esteárico (0,22 ± 0,07%) enquanto o de *Tetraselmis chui* apenas ácido palmítico (17,5 ± 2,6%) e esteárico (5,97 ± 1,48%).

A composição nutricional da biomassa de *Tysochrysis lutea* revelou um teor de proteína de 36.7 ± 0.4%, um teor de humidade de 5.83 ± 0.33% e um teor de fibra de 15.3 ± 0.1%. Em termos de minerais a biomassa demonstrou altos teores de zinco, manganês, cálcio, ferro, potássio, fósforo e sódio (210 ± 3; 95 ± 3; 8736 ± 32; 2912 ± 48; 11875 ± 150; 9944 ± 315; 10873 ± 37 µg/g de biomassa seca, respetivamente).

Os resultados obtidos sugerem que *Tisochrysis lutea, Tetraselmis chui* e *Tetraselmis striata* CTP4 possuem potencial antioxidante e, apesar dos extratos analisados não apresentarem muita atividade anti-hipertensiva (avaliada através do ensaio de inibição da enzima conversora da angiotensina), esses extratos podem ter propriedades anti-inflamatórias (avaliadas através do ensaio de inibição da COX) que podem ser interessantes de continuar a ser investigados. Com base nestes dados encorajadores, existe suporte para prosseguir com mais pesquisas para estabelecimento destas microalgas ou os seus extratos como alimentos funcionais para complementar um estilo de vida saudável, possibilitando um envelhecimento saudável e mitigando doenças, podendo atuar a nível preventivo ou de complemento ao tratamento de doença.

De uma maneira geral, esta dissertação permitiu aumentar o conhecimento da atividade biológica de três espécies de microalgas e o impacto de diferentes metodologias de extração na composição de extratos e a sua bioatividade, foi também demonstrado que algumas das algas analisadas podem ter diversos ingredientes bioativos com potenciais aplicações em alimentos funcionais.

Este estudo esteve inserido no projeto H2020 Algae4IBD, que pretende desenvolver produtos alimentares funcionais para a prevenção e tratamento de Doenças Inflamatórias do Intestino utilizando recursos biológicos aquáticos.

Palavras-chave: microalgas; nutrição; antioxidante; anti-inflamatório; promoção de saúde; alimentos funcionais

ABSTRACT

Recently, consumers have become more aware of the relationship between health and nutrition, recognizing healthy diets as cornerstones for health promotion and disease prevention, demanding and seeking functional foods. Functional foods not only provide nutritional value but also promote healthy aging and prevent diseases due to their various biological properties (e.g., antioxidant, anti-inflammatory, antitumorigenic). Microalgae represent a promising opportunity as functional food due to their production of valuable bioactive ingredients, with several health-promoting effects, including anti-oxidative, anti-inflammatory, antimicrobial, and anti-cancer effects. The aim of this study was to assess the antioxidant and anti-inflammatory potential from bioactive compounds present in the extracts from 3 microalgal species (*Tisochrysis lutea, Tetraselmis chui, Tetraselmis striata* CTP4) and perform a preliminary phytochemical analysis on the most bioactive extracts. Antioxidant activity was determined via DPPH, ABTS, copper chelating, iron chelating and ferrous reducing activity assays. Extracts with highest overall antioxidant activity were identified as ethanolic extracts of *Tisochrysis lutea, Tetraselmis chui* and *Tetraselmis striata* CTP4 and hexane *Tetraselmis striata* CTP4 extract. Additionally, effects of different extraction techniques on yield and bioactivity of *Tetraselmis striata* CTP4 extracts were investigated. These included milling, which had best yields and antioxidant activity comparing with automated fast solvent extraction and microwave assisted extraction. Anti-inflammatory activity assays were performed regarding ACE and COX inhibitory activity. The highest anti-inflammatory activity was observed for the ethanolic extract of *Tetraselmis chui* (5.80% for ACE inhibition and 32.2 ± 7.69% COX inhibition) followed by ethanolic *T. lutea* extract (2.90% for ACE inhibition and 44.5 ± 3.70% COX inhibition). Their phytochemical screening identified palmitic, myristic and stearic acids as the most abundant compounds. The proximate composition of *Tisochrysis lutea* revealed high fiber and mineral content.

Overall, this thesis demonstrated that some of the analyzed algae could have several bioactive ingredients with potential applications as functional food.

This study is a part of the Algae4IBD project, that intends to develop commercial functional food products for Inflammatory Bowel Disease (IBD) prevention and treatment using aquatic natural biological resources.

Key words: microalgae; nutrition; antioxidant; anti-inflammatory; health benefits; functional foods.

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Abbreviations

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

ABTS• - 2,2′-Azinobis (3-ethylbenzothiazoline-6 sulfonic acid) radical

ACE – Angiotensin Converting Enzyme

AChE – Acetylcholinesterase

ASE - Accelerated Solvent Extraction

BHT - Butylated Hydroxytoluene

COX – Cyclooxygenase

CVD – Cardiovascular Diseases

DHA - Docosahexaenoic Acid

DMSO – Dimethyl Sulfoxide

DPPH - 2,2-Diphenyl-1-picrylhydrazyl

DPPH• - 2,2-Diphenyl-1-picrylhydrazyl radical

EDGE - Energized Dispersive Guided Extraction

EDTA - Ethylenediaminetetraacetic acid

ELISA - Enzyme-Linked Immunosorbent Assay

EPA - Eicosapentaenoic Acid

EtOH – Ethanol

FDA - Food and Drug Administration

GC – Gas Chromatography

GCMS - Gas Chromatography- Mass Spectrometer

GRAS – Generally Recognized as Safe

Hx - Hexane

IC_{50} - Half Maximal Inhibitory Concentration

IL – Interleukin

MPAES- Microwave Plasma-Atomic Emission Spectrometer

MS – Mass Spectrometer

MW – Microwave

Mw - Molecular weight

NCDs - Non-Communicable Diseases

ND - Neurodegenerative Diseases

NIST - National Institute of Standards and Technology

NO - Nitric Oxide

OH• - Hydroxyl radical

PG - Prostaglandin

PUFA - Polyunsaturated Fatty Acids

ROS – Reactive Oxygen Species

RT - Retention Time

TNF - Tumor Necrosis Factor

Background

The high prevalence of non-communicable diseases (NCDs) is a worldwide concerning phenomena and is the main overall death cause, accounting for 71% of all deaths, equivalent to 41 million people annually, besides accounting for about 80% of all premature deaths. The main NCDs are cardiovascular diseases (such as heart attacks and strokes), cancers, chronic respiratory diseases, and diabetes. NCDs are known to increase the occurrence of other diseases (such as diabetes and are suspected to amplify impacts of COVID-19), further decreasing life quality. Most NCDs risk factors can be easily prevented by making lifestyle changes, such as practicing moderate physical activity and adopting a healthy diet [1,2] .

Despite or maybe because of the increase of health issues that can be prevented or managed with diet, a recent shift in consumption patterns in some parts of industrialized societies has been observed. Health-conscious consumer trends reveal that consumers are becoming increasingly aware about health aspects of purchased food and food products, taking greater responsibility for their own health and well-being by proactively searching for healthy foods. Nowadays health-conscious consumers recognize healthy diets as being cornerstones to prevent chronic diet related diseases and seek foods that not only provide nutritional value but also promote an increased overall well-being [3,4].

A way to meet consumers expectations is by creating foods that, besides the nutritional value, also have additional health benefits, such as health promotion or disease management. Functional food influence specific functions of the organism and can therefore help reduce the risk of noncommunicable diseases, while simultaneously providing a new way of expressing healthiness through food choices. With the growing consumer demand for healthy food (about 8% annually), the functional food market is growing steadily worldwide and with continuous innovations, many new products are launched, increasing availability [5–7].

Consumer's demand for novel sources of functional food ingredients is constantly increasing, and several sources of bioactive compounds have been under investigation. Microalgae are rich in bioactive molecules with unique properties and a huge biotechnological potential. Microalgae are largely untapped resources with a wide range of bioactive compounds suitable as novel source of functional foods with unique properties [8–10].

1. Introduction and Literature Review

1.1. Functional foods and Nutraceuticals

1.1.1. Historical overview and definitions

Although the terms "*functional foods"* and "*nutraceuticals*" have recently gained popularity (mainly due to growing consumer concern for improving health and life quality) and are used worldwide, there is no consensus on their meaning, as no official or legal definition exists to this date (neither one is legally defined in the current EU and worldwide food regulatory framework) [6]. The boundary between nutraceuticals and functional foods is not so clear and most of the consumers and industries use it interchangeably [11].

The first written evidence of the existence of functional foods traces back to 1000 BC in China and a similar concept – "special foods"- appears again around AD 1000 in the same region. Regarding the western part of the world, the first time a similar concept was recorded was by Hippocrates, who lived in 4th and 5th centuries BC, with a sentence that is (still nowadays) considered the basis of the paradigm of functional foods "Let food be your medicine and medicine your food." [12]

Specific cases of "functional food" were first established in Japan in the early 1980s by the Ministry of Health and Welfare, as an attempt to counteract the rising healthcare costs related to malnutrition and improving the health of the nation's aging population. A regulatory system was then created to approve "food containing an ingredient with functions for health and officially approved to claim their physiological effects on the human body". In 1991, Japan created a legal and working definition for these types of food, creating a new product category, and labeling the concept "foods for specified health use" (FOSHU). This law set the first milestone for the appearance of health claims on food labelling, and slowly the concept of "functional foods" started to spread to the rest of the world [13,14].

Shortly after, the United States of America also developed the first health claim act, however, no formal definition of "functional food" was made. The U.S. Food and Drug Administration (FDA)'s does not recognize 'functional food' as a distinct regulatory category. The Food and Nutrition Board of the National Academy of Sciences defines

"functional food" as food containing potentially valuable compounds, including "any modified food or food ingredient that may provide a health benefit beyond that of the traditional nutrients it contains" [15,16]. Health Canada, the department of the Government of Canada responsible for national health policy, defines functional foods as "products that resemble traditional foods but possess demonstrated physiological benefits" [17].

A working definition adopted in a European Consensus document in 1999 states that a food ingredient is considered "functional food" if, besides its nutrition capacity, it has a scientifically proven benefit for one or more functions of the human organism, improving the state of health or well-being or reducing the risk of disease. Additionally, functional foods must remain foods (not transformed in pills or capsules) and must demonstrate their benefic health effects in amounts that can normally be expected to be consumed in the diet. Functional foods do not cure or prevent illnesses on their own and are not essential to the diet, instead are part of a normal food pattern [12,18].

In Europe, functional foods may be (indirectly) covered by Regulation (EC) no. 2015/2283 of the European Parliament and of the Council of 25 November 2015, which is the most recent legislation that regulates "novel foods" (defined as "any food that was not used for human consumption to a significant degree within the Union before 15 May 1997", such as "food consisting of, isolated from or produced from microorganisms, fungi or algae"). These novel foods shall not present risks to the consumer, mislead the consumer, nor differ nutritionally from the foods they are intended to replace [19].

As a practical definition approach, functional foods are similar to conventional food and are consumed as part of the normal diet but also improve health or reduce disease, beyond primary nutritional function [20].

To some extent, all foods can be considered functional as all foods provide taste, aroma, and nutritive value. However, some foods may be particularly beneficial in improving health and/or reducing the risk of acquiring a disease, representing a promising indirect opportunity to improve public health [13,16,20].

A term than often is used interchangeably with "functional foods", although it is less appealing for consumers, is "nutraceuticals". Nutraceuticals have received international recognition as potentially benefitting health when consumed regularly as part of a diverse diet and in adequate amounts [21].

Nutraceuticals is a hybrid term between “nutrition” and “pharmaceutical”, created by the Foundation for Innovation in Medicine in 1989, referring to “almost any food, food ingredient or bioactive compound that delivers health benefits, including the prevention and/or treatment of a disease"[21].

The Canadian government’s definition of nutraceutical is a “product isolated or purified from foods that is generally sold in medicinal forms not usually associated with food. A nutraceutical is demonstrated to have a physiological benefit or provide protection against chronic disease” [22].

In a report commissioned by the European Commission, nutraceuticals are set as equal to supplements: “dietary supplements that are used to enhance health [...] are also called nutriceuticals and nutraceuticals. The ‘nutri’ type contains mixtures of essential primary nutrients, while the ‘nutra’ type consists of non-essential secondary nutrients. Bioactivity for essential nutrients is not in doubt, but still has to be proven for the nutraceuticals” [23].

As a practical definition, nutraceuticals can be defined as a group of products that are more than food but less than pharmaceuticals [24]. So, nutraceuticals are seen as special or more bioactive sort of food supplement (in formulated and dosage form, such as capsules, tinctures, or tablets) – and the definition does not cover functional food. On the other hand, functional foods provide their health benefits while maintaining the look of traditional foods [23].

1.1.2. Examples of functional foods

Functional foods can be of plant or animal origin and can be categorized into three broad categories: 1) Conventional foods naturally containing bioactive food compounds that provide benefits beyond basic nutrition (such as vegetables, fruits, grains, dairy, and fish); 2) Modified foods where bioactive components were added to the food to provide additional health benefits for the consumer, either through enrichment or fortification; 3) Food ingredients that are synthesized, such as indigestible carbohydrates (oligosaccharides or resistant starch), which offer prebiotic benefits [14,25].

Conventional functional foods include nuts, whole grains, fruit, and vegetables. Extensive epidemiologic evidence shows that a plant-based diet contains a wide range of compounds that are associated with a reduced risk of developing chronic diseases, such as heart disease, cancer, diabetes, hypertension, and a variety of other medical conditions.

For example, evidence shows that whole grains have many different compounds (e.g., dietary fiber, antioxidants, trace minerals and phenolic compounds) that may provide protection against several diseases and carrots and tomatoes have known active compounds (beta carotene and lycopene, respectively) that have a significant role in cell metabolism [26].

Modified functional foods, also called fortified foods, are foods that have nutrients added which are not otherwise present. Fortified foods are cost-effective strategies meant to improve nutrition and provide health benefits. Foods are most often fortified with antioxidants, multivalent cationic minerals (such as calcium, iron, magnesium, and aluminum) and vitamins, (like C, D, E, and B-complex). Most common fortified foods are fortified juices, fortified dairy products (such as milk and yogurt), fortified milk alternatives (such as almond, rice, coconut, and cashew milk) and fortified eggs. Fortification of milk alternatives with vitamin D and calcium are cost-effective strategies to reduce bone loss related with aging, mainly in elderly population in risk of bone fractures. Another common example are margarine spreads and eggs enriched with omega 3 fatty acids, to promote cardiovascular health [27,28].

Prebiotics, which are inserted in the third functional food category, are non-digestible food ingredients that beneficially affect gut health, thus improving host health. The most well-known prebiotics are galactooligosaccharides (GOS), fructooligosaccharides (FOS) and inulin, which can be incorporated in dairy products, biscuits, juices, among others. Prebiotics improve the host's health by selectively stimulating the proliferation and/or activity of desirable bacterial populations in the gut. In addition, prebiotics can inhibit the multiplication of pathogens, ensuring additional health benefits [29].

1.1.3. Health benefits of functional foods

Functional foods have many physiological effects in the body depending on the specific featured bioactive compounds, with specific biological properties (antioxidant, anti-inflammatory, antitumorigenic, etc.) promoting healthy aging and disease prevention, including cardiovascular disease, cancer, cardiometabolic syndrome, and neurodegenerative diseases [30].

Functional foods can reduce CVD risk by several potential mechanisms, such as lowering blood lipid levels, decreasing the plaque formation (due to anti-inflammatory activity), reducing lipoprotein oxidation (due to antioxidant activity and radical scavenging activity), and improving arterial compliance. CVD-benefic functional foods are low in saturated fat, cholesterol and rich in phytosterols. Antioxidant-rich foods, such as cocoa, berries, grapes, coffee and soy, have a special potential cardiovascular protection by inhibiting low-density lipoproteins oxidation and plaque development [12,20,30,31].

Cancer is a large group of diseases in which body cells grow uncontrollably and may spread to other parts of the body (metastasis). Carcinogenesis is a highly complex and long-term process, involving inherited and environmental risk factors. However, it is estimated that eighty to ninety percent of cancer cases are caused by environmental factors, including dietary habits [12,20,30]. Many studies showed that diet is one of the most important modifiable risk factors, which can modify carcinogenesis and has a huge impact on mortality, it is even estimated that about one-third of all cancer deaths in developed countries could be avoided through appropriate dietary formulations [12,20,30]. Recently a new concept has emerged – "chemoprevention"- a phenomenon where carcinogenesis can be suppressed via natural products (such as foods) or synthetic drugs. The main strategy with the natural route is through the consumption of bioactive substances, as scientific evidence proves that these compounds in food, even in very low concentrations, can have a huge impact in the regulation of cancer mechanisms. Bioactive foods can exhibit anticancer properties through several mechanisms: inhibiting metastasis; inhibiting proliferation; inducing apoptosis; inhibiting angiogenesis; neutralizing free radicals and ROS; inhibiting matrix metalloproteinases and inducing DNA methylation [30,32,33]. Most of these mechanisms are attributed to fruits and vegetables, mainly due to phytochemicals (such as carotenoids, phenolic compounds, organosulfur compounds) and phytosterols, although the exact mechanisms by which these compounds offer protection from cancer is unknown. Specific functional foods that have attributed anti-cancer properties are tomatoes, cauliflower, and brussels sprouts, which have carotenoids exhibiting potent antioxidant ant anti-inflammatory properties. Other functional foods with attributed anti-cancer properties are teas, garlic, citrus fruits, soybean and berries [30,32,34].

Obesity is a chronic, complex, multifactorial, and largely preventable disease that is strongly related to the development of other pathologies. The prime strategy to deal with this disease is to ensure weight loss, developing good eating habits and having an active lifestyle, which are already widely recognized for bringing major health benefits, such as increased life expectancy and decrease obesity-related complications. Therefore, dietary components play a major role in the development of obesity, and functional foods have the chance to prevent and aid treatment against obesity or reduce comorbidities [12,35]. The ideal functional food for prevention of obesity should be able to regulate appetite and satiety (while ensuring adequate energy consumption) and simultaneously suppress the of growth of adipose tissue by modulation of adipocyte metabolism. Although some functional foods appear to be promising in the fight against obesity, their exact mechanism of action is not fully understood. Polyphenols, that are primarily found in fruits and vegetables, have a strong antioxidant and inflammatory activity, which can neutralize ROS and somehow aid in the inflammation related to obesity, they are also believed to have a role in the induction of lipolysis and apoptosis in adipose tissue. Long-chain polyunsaturated fatty acids n-3 series (n-3 LC-PUFAs), particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are believed to have some similar antioxidant and inflammatory properties. Fiber-rich foods such as whole grains, are believed to aid in weight management and help reduce obesity problems by retarding gastric emptying and increasing satiety [26,30].

Diabetes is a disease that occurs when blood glucose is too high. The main clinical characteristic of diabetes is hyperglycemia but, when untreated, can cause complications in organs such as the eyes and kidneys. Diabetes is closely related to overweight and physical inactivity, and both these factors increase the risk of developing diabetes type 2, so intervention should be endorsed with weight reduction and physical activity. The ideal functional food for type 2 diabetes prevention would be foods or ingredients that influence either insulin secretion or insulin resistance. Known functional foods ingredients are dietary fibers, phytochemical compounds, and antioxidant compounds, which have anti-diabetic properties [21,26].

The gut microbiota is essential in the fermentation of non-digestible dietary fibers and synthesize vitamins which the host is incapable of producing, such as vitamin K. A healthy gut microbiome plays a very important role in an organism’s overall health by

helping control digestion and producing metabolites with various mechanisms, benefiting immune system and many other aspects of health, such as heart health, brain health, improved mood and healthy sleep, may even help in cancer prevention and autoimmune diseases [36,37]. As diet has such a huge impact on the composition of gut microbiome and directly influences the gut health and all associated benefits, the gut is a clear target for the development of functional foods. Four strategies of functional foods have been followed to promote healthier microbiota: fiber-rich foods, prebiotics, probiotics and synbiotics [36,37]. Dietary fibers can be either soluble or insoluble, both being crucial for health, digestion, and preventing diseases. Soluble dietary fiber are fermentable compounds that are fermented by gut microbiome and produce short chain fatty acids which have various health benefits. Some dietary fibers are non-digestible and can also be classified as prebiotic, which stimulate growth, modify the metabolic activity of one or several bacterial species benefic to gut health or the overall microbiome composition in the colon. The main known prebiotics are inulin-type fructans (such as native inulin, enzymatically hydrolyzed inulin or oligofructose) and synthetic fructooligosaccharides, which can be found in dairy products, breads, and meat products. Inulin and oligofructose have several known health benefits such as, including modulation of the gut microbiota composition, prevention of pathogen adhesion and colonization, anti-inflammatory activity and modulation of bowel habits [12,30,36,37].

Neurodegenerative diseases (ND) are a heterogeneous group of disorders that involve the progressive deterioration or death of nervous system cells in the brain and spinal cord, leading to memory impairment, locomotor dysfunction, cognitive defects, emotional and behavioral problems (such as anxiety, agitation, and mood changes). Some of the diseases included in the category of neurodegenerative diseases are Alzheimer's disease, Parkinson's disease, multiple sclerosis, Huntington's disease, and amyotrophic lateral sclerosis [38,39]. The main mechanism of action which triggers neurodegenerative diseases is oxidative stress, as the nervous system is the place in the human body with the highest amount of oxygen, being especially susceptible to oxidative stress. Besides oxidative stress, other factors influencing ND-pathogenesis are inflammatory response and altered cell signaling [12,30]. Dietary factors seem to affect brain physiology and may play a preventive role in determining whether the brain ages successfully or experiences a neurodegenerative disorder. The most well-known dietary strategy to prevent ND is the Mediterranean diet[40], which is rich in phenolic compounds, flavonoid-rich foods, and

omega 3 fatty acids. Flavonoids (present mainly in fruits and vegetables) are believed to have protective and regenerative activity for neurons, due to their antioxidant and anti-inflammatory capacity, also potentially modulating cell signaling pathway. Omega 3 fatty acids (present in fish, seafood, nuts, seeds, and plants oil with unsaturated fats), especially EPA and DHA, have been widely associated with better cognitive performance, decreased risk of developing dementia and reducing neuroinflammation. Another food component that is associated with improved cognitive function is selenium (present in whole grains, dairy products, fish) a trace element that has antioxidant activity which protects neurons from ROS and is known to play an important role in brain physiology and pathophysiology [12,30].

1.2. Valuable biocompounds

Valuable biocompounds, also called bioactive compounds, are primary and secondary metabolites, naturally found in small quantities in food, that are physiologically active in the human body, providing health benefits beyond the basic nutritional value of the product. Bioactive compounds provide the *functional* aspect to functional foods [41,42].

The most well-established bioactive compounds are vitamins, omega-3 fatty acids, nucleosides, nucleotide, carotenoids, phenolic compounds, terpenoids, peptides, and saponins. Bioactive compounds may naturally be found in various foods, both of animal and plant origin, the most known bioactive-rich foods include fruits, vegetables, fish, nuts, herbs, and whole grains [8,9].

The development of new functional foods with health benefits is a current topic and represents an attractive opportunity for the food and/or pharmaceutical industries to improve human health and prevent diseases. Recently, novel sources of bioactive compounds have been investigated, such as secondary metabolites from novel marine microbes, endophytic fungi, marine invertebrates and algae, which have huge biotechnological potential [8–10].

Bioactive compounds have very diverse polarity, solubility, molecular size, bioavailability, metabolic pathways, chemical structure and function, so they can trigger health benefits through various regulating biological mechanisms, such as antioxidant, anticarcinogenic, anti-inflammatory, and antimicrobial activity [10,43,44].

1.2.1. Antioxidant compounds

Reactive oxygen species (also called free radicals) are unstable metabolic by-products generated by organisms during oxidation reactions in normal cell activity, as shown in figure 1. The factors that can increase the production of free radicals in the body can be either internal (such as inflammation), or external (as pollution). The most well studied ROS are superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), and singlet oxygen ($^1O_2$) [45–48].

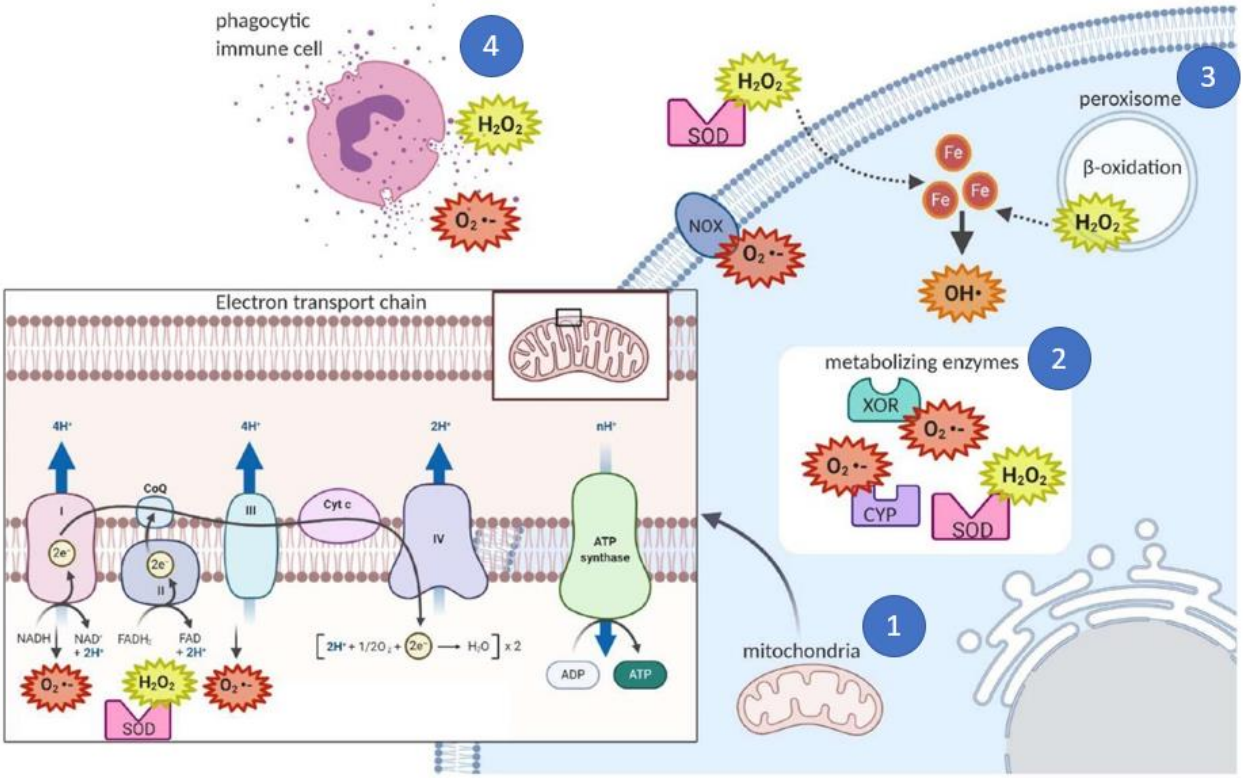

Figure 1- Production of reactive oxygen species in various cell components.
ROS can be produced either intracellularly (**1-** mitochondrial electron transport chain, **2-** cytoplasm metabolism reactions, **3-** peroxisome) or extracellularly (respiratory burst from phagocytic immune cells), for example when phagocytic immune cells release ROS to attack pathogens. Adapted from: Shields et al.[49]

Antioxidants, also called "free-radical scavengers", are defined as molecules capable of slowing down or preventing the oxidation of other molecules by either eliminating or stabilizing ROS. Biological antioxidants are defined as "substances that, when present at low concentrations compared to those of an oxidizable substrate, significantly delay or prevent oxidation of that substrate" [45,46].

In normal conditions, cells have an appropriate oxidant:antioxidant balance, however if an imbalance occurs, oxidative stress develops. Oxidative stress in an organism is a complex process that is characterized by an imbalance between the production of ROS and the body's ability to eliminate these reactive species using antioxidants. When oxidative stress persists over time, ROS activated chain reactions result in damages in various cell components, such as nucleic acids, DNA, proteins and sometimes even leading to cell death and eventual development of diseases over time [47,48].

The delay of propagation of ROS or inhibition of ROS production by antioxidants can be achieved by several biological mechanisms, depending on the reactivity and chemical structure of ROS: 1) scavenging species that can initiate peroxidation reactions, 2) chelating metal ions so that they are unable to generate reactive species or decompose lipid peroxides, 3) quenching O_2^- preventing the formation of peroxides, 4) breaking autoxidative chain reactions, and 5) reducing localized O_2 concentrations. Thus, the existing methods for the evaluation of antioxidant properties of a sample can be classified based on the three main types of reaction (figure 2). Either way, antioxidants are believed to prevent or delay some types of cell damage and development of future diseases [47].

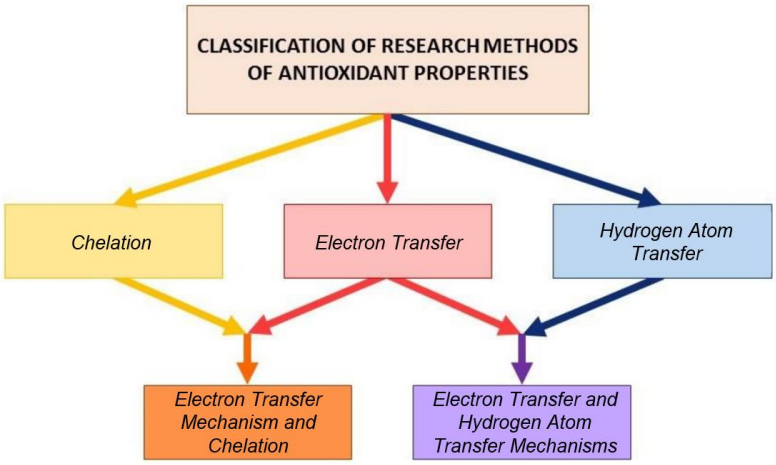

Figure 2- Classification of research methods for evaluation of antioxidant properties. Adapted from: Ivanova et al.[50]

Antioxidant systems exist in the cells to protect them against ROS, but when ROS production is increased, antioxidant self-production sometimes is compromised and, consequently, fail to guarantee a complete protection of the cells. The antioxidants produced by the own body are known as endogenous antioxidants, which can be enzymes (such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase), or non-enzymatic compounds (such as bilirubin, ascorbate, glutathione, and albumin). When the

internal antioxidant system is compromised, the body can use alternative and external antioxidant sources such as food or nutritional supplements, to compensate the deficit, in these case antioxidants are called exogenous [48].

Oxidative stress is a key factor in cell injury (damaging membranes, lipids, proteins, lipoproteins, and DNA) and, if not managed and strictly controlled, can induce several diseases and disorders (both chronic and degenerative), premature aging and acute pathologies, such as strokes (figure 3) [51,52]. Depending on the specific disease, oxidative stress's role is variable: in some diseases, oxidative stress plays a central role whereas in others, it is an important but secondary factor. Oxidative stress can induce diseases (cancer, arteriosclerosis, myocardial infarction, diabetes, inflammatory diseases, central nervous system disorders, and cell aging) through various mechanisms, such as mutagenesis and cell transformation [53].

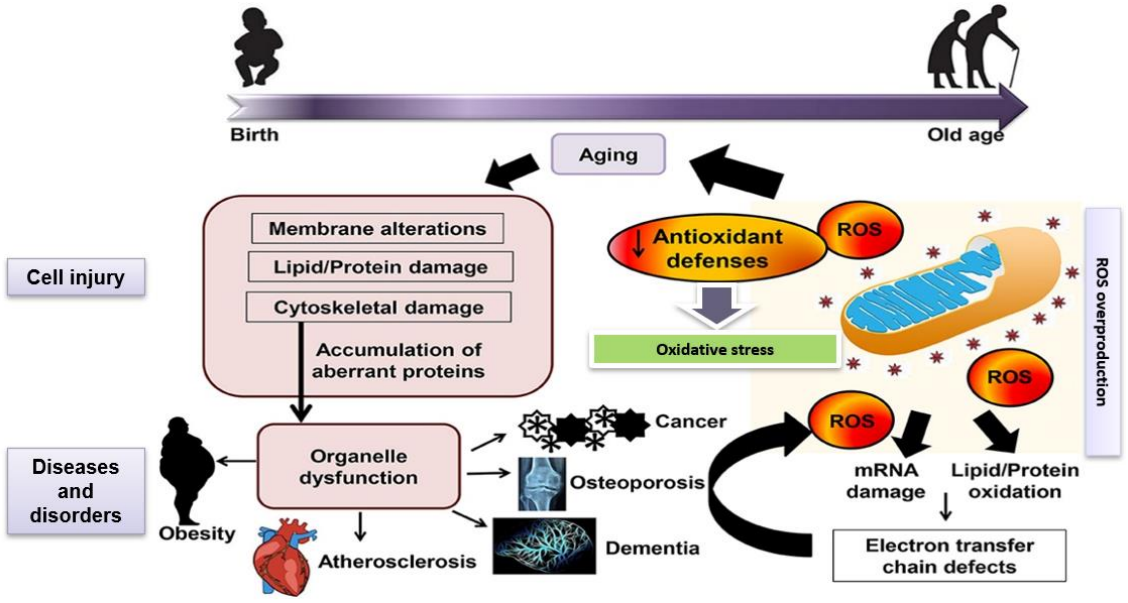

Figure 3 – ROS overproduction leading to oxidative stress. The maintenance of oxidative stress over time leads to cell injuries and age-related diseases. Adapted from: Tan et al.[54]

Hydroxyl radical (OH•**)** is a highly reactive molecule which can react with lipids, sugars, proteins, and DNA. When OH• interacts with DNA, severe irreversible damage occurs to the DNA molecule and a wide range of products is formed, some of which are mutagenic. Oxidative DNA damage is one of the exogenous *stimuli* responsible for cancer development. Another cell component which is at risk to suffer oxidation by ROS are lipids; lipid peroxidation causes further DNA damage besides also resulting progressively

in protein malfunction, and eventually leading to alterations in tissue functioning due to cytotoxicity, cell dysfunction, inflammation, and apoptotic cell death. Lipid peroxidation is implicated in the underlying mechanisms of several disorders and diseases, such as cardiovascular disease, atherosclerosis, diabetes, cancer, neurodegenerative diseases, and aging [46–48,51,52]. Oxidative stress can also cause chronic inflammation due to various mechanism, mainly due to modifications of DNA and proteins and overall ROS-induced damage, all leading to the signaling to activate inflammatory pathway. Prolonged oxidative stress is likely to lead to chronic inflammation associated disorders [51,52].

The main role of antioxidants, both from endogenous and exogenous origin, is to act as free radical scavengers preventing and repairing damages caused by ROS and stabilizing the ROS molecules, avoiding them to react with other molecules and preventing chronic inflammatory states (figure 4). Antioxidants can neutralize free radicals via accepting or donating electron(s) to remove the unpaired status of the radical. Due to the implication of oxidative stress in the etiology of several chronic and degenerative diseases, antioxidant therapy and/or supplementation represents a promising path for prevention and/or treatment of diseases [46–48,51,52].

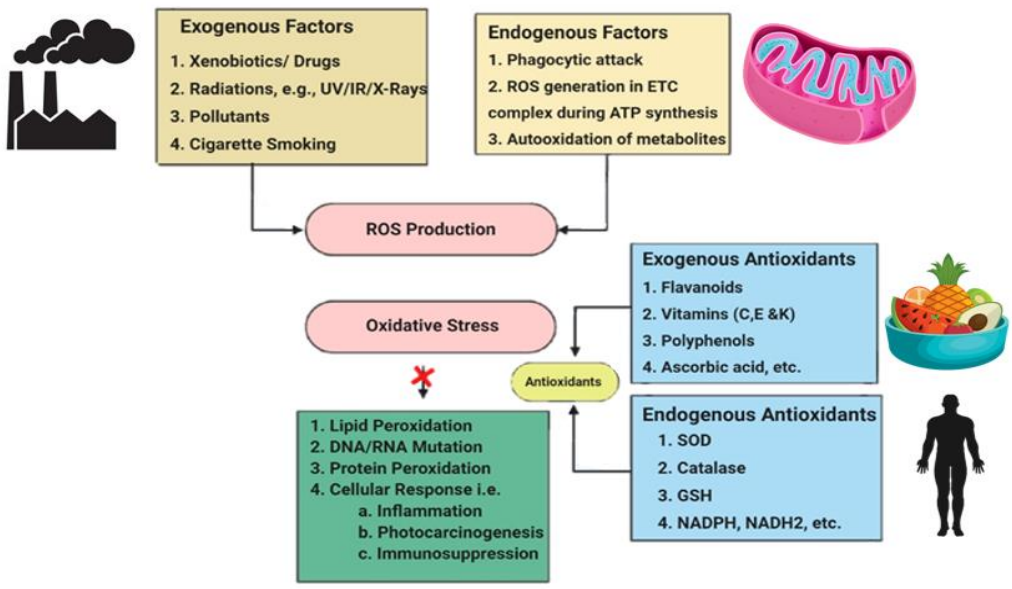

Figure 4 - Sources of oxidative stress and antioxidants and their role in their health benefits. Adapted from: Rani et al.[55]

The endogenous antioxidant defense system can be enhanced by dietary antioxidants, also called exogenous antioxidants, including vitamin C, selenium, zinc, beta-carotene, and

taurine. Dietary antioxidants play a key role in the inhibition of oxidation processes not only in foods but also in the human body, improving redox homeostasis and preventing premature aging. Dietary antioxidants can have either natural or synthetic origin and can interact among themselves and result in synergistic, additive, and antagonistic interactions. Naturally occurring antioxidants react with radicals and convert them into more stable products and are classified as chain-breaking antioxidants. Each nutrient is unique in terms of its structure and antioxidant function (free radical scavengers, retarding chain initiation; repair of damaged biomolecules) but the overall effect is to oppose ROS action and limit oxidative stress. Most natural antioxidants have plant origin such as antioxidant minerals, antioxidant vitamins and phytochemicals with phenolic structures [56].

Antioxidant micronutrients and minerals, such as selenium, copper, iron, zinc, and manganese, act mainly as enzyme co-factors. Iron, copper, zinc, and manganese are cofactors for the antioxidant enzymes catalase and superoxide dismutase. Selenium is a cofactor of glutathione transferase enzyme. Superoxide dismutase protect cells from radical attack by transforming the superoxide radical (generated in tissues as a by-product of oxygen metabolism) into hydrogen peroxide and molecular oxygen, however hydrogen peroxide can cause cell damages if in high concentrations, to avoid that catalase breaks down peroxide into water and molecular oxygen, limiting free radical-induced damage, as shown in figure 5. Glutathione Peroxidase also breakdowns hydrogen peroxides (H_2O_2) into water and lipid peroxides to their corresponding alcohols. The cofactors (iron, copper, zinc, selenium and manganese) are vital for both enzyme's activity [57]. Selenium rich foods are Brazil nuts, seafoods, and organ meats. The other co-factors may be found in whole grains, clams, oysters, fish, nuts, legumes, rice, leafy vegetables, coffee and tea [58].

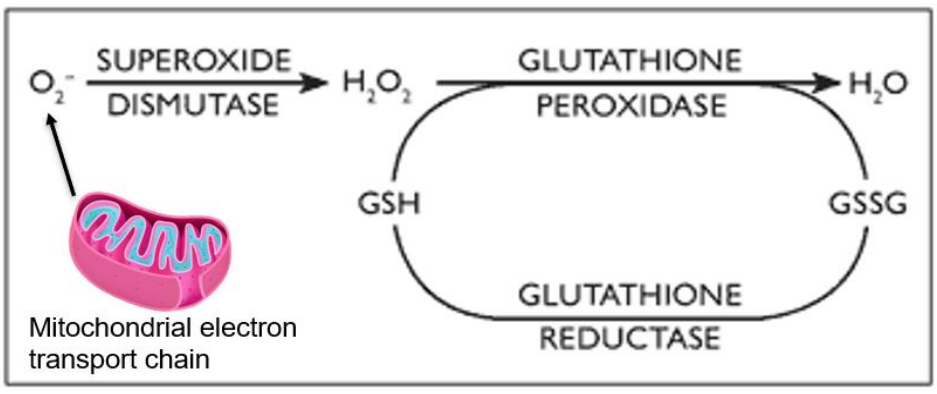

Figure 5 – The role of superoxide dismutase and glutathione peroxidase in oxidative stress. Adapted from: Pinto et al.[59]

Antioxidant vitamins, such as, vitamin C and E, are important and required for most body metabolism functions. Vitamin C, also called ascorbic acid, is a water-soluble vitamin with antioxidant, anti-atherogenic, anti-carcinogenic and immunomodulator activity. Vitamin C reacts synergistically with vitamin E in the quenching of ROS and promoting the conversion into a poorly reactive chemical species, suppressing free radicals and oxidative stress. Natural sources of vitamin C are fruits and vegetables, particularly strawberries, citrus, kiwi, brussels sprouts, tomato, and cauliflower. Ascorbic acid is a thermolabile molecule, therefore it may be lost during cooking [46,51,56]. Vitamin E (α-tocopherol) is a fat-soluble vitamin and is the most important membrane-bound antioxidant, acting mainly in the protection against lipid peroxidation and inhibiting inflammatory reactions, protecting cell membranes from damage by free radicals. Vitamin E is considered to act by two mechanisms: quenching of ROS and chain breaking due to absorption of electrons and/or energy. Main food sources of Vitamin E are green leafy vegetables (e.g., spinach); nuts, seeds; vegetable oils, especially sunflower; fruits; eggs; poultry and meat [46,56,60].Phytochemicals are a group of various plant secondary metabolites and are found naturally in many fruits and vegetables that may provide desirable health benefits beyond basic nutrition, due to their antioxidant potential (e.g. polyphenols, flavonoids, terpenoids, carotenoids, limonoids, phytosterols, and fibers) [61].

Phenolic compounds are largely distributed phytochemicals almost exclusively found in plant tissues, although they also can be synthetized by some microorganisms in response to stress. Phenolic compounds are neither vitamins nor minerals which have an aromatic ring with one or more hydroxyl groups and act as antioxidants, such as simple phenolics, phenolic acids, flavonoids, carotenes, coumarins, stilbenes, tannins, lignans, and lignins. Phenolic compounds exhibit their antioxidant activity by acting as reducing agents, hydrogen donators, singlet oxygen quenchers, and as metal chelators. It has been reported that the flavones and catechins seem to be the most powerful flavonoids for protecting the body against reactive oxygen species. Phenolic-rich foods berries (catechins), carrots (carotenoids), apples, onions, tea (catechins and flavonoids), red wine (flavonoids), some herbs (parsley, thyme) citrus fruits, grapes and cherries [51,60–62].

1.2.2. Anti-inflammatory compounds

Inflammation has been described as a general, complex, and crucial biological process for maintaining the body's homeostasis by involving the immune system in response to external harmful challenges (pathogens or allergens) or tissue damage. Inflammatory responses involve complex interactions between blood cells, immune cells, and molecular mediators such as pro- and anti-inflammatory cytokines, and other inflammatory mediators directed toward eradicating invaders and promoting healing (figure 6). Inflammation is an indispensable mechanism for the body to restore structural and functional integrity to damaged tissue and without inflammatory response wounds would never heal [47,63]. An acute inflammatory response occurs immediately upon injury or other external stimuli, usually only lasting a few days until the damaged tissue is completely repaired. Acute inflammatory response is initiated when mediators like cytokines (such as IL-6 or TNF-alpha), acute phase proteins (C-reactive protein, for example) and chemokines (CCL2 and CXCL8, or prostaglandins) promote the migration of neutrophils and macrophages to the site of inflammation by increasing vasodilatation and angiogenesis (formation of new blood vessels to enable more inflammatory cells to migrate).

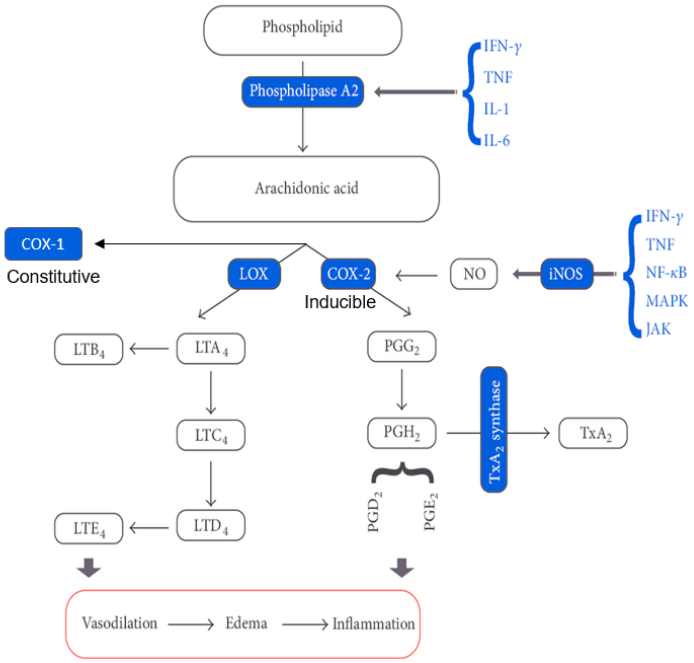

Figure 6 - Inflammation pathway mediated by pro-inflammatory cytokines and the role of cyclooxygenase in the inflammation process.
Adapted from: Ghasemian et al.[64]

Another type of signaling molecule and mediator of inflammation is ROS (figure 7), which are produced by phagocytic as part of the body defense system. After penetration of leukocytes to the infection site, pathogens are destroyed in a process in which ROS are involved, followed by the repair of damaged tissue. However, despite inflammation being a natural and beneficial response of the body, when inflammation is not closely monitored for a long time, chronic inflammation may occur (longer than 6 weeks). Chronic inflammation in tissue usually happens when inflammatory responses are in the absence of an actual stimulus and are often attributed to an excessive production of pro-inflammatory mediators. Examples of pro-inflammatory mediators which may be elevated are cytokines, chemokines, cyclooxygenase-2, prostaglandins (PGs), nitric oxide synthase (iNOS) and nitric oxide (NO) [65,66].

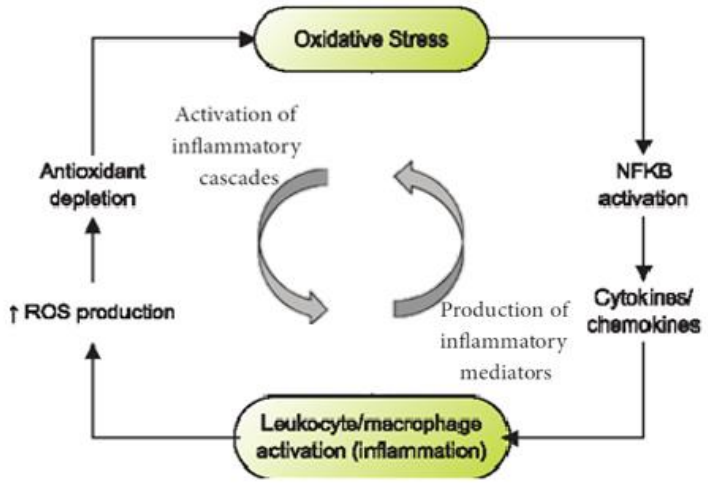

Figure 7 - Link between oxidative stress and inflammation. Oxidative stress-induced inflammatory cascades cause the inflammation, which in turn increases oxidative stress and ROS is a mediator of inflammation, turning into a vicious cycle. ROS indicates Reactive Oxygen Species and NFKB, Nuclear Factor-Kappa B. Adapted from: Lee et al.[67] and Vaziri et al.[68]

Excessive amounts of pro-inflammatory mediators and factors are involved in numerous chronic diseases occurrence such as cancer, neurodegenerative disorders, multiple sclerosis, diabetes, atherosclerosis, arthritis, and cardiovascular diseases, as shown in figure 8. In fact, dysregulated inflammation has been identified in the genesis of almost all types of human diseases or disorders. Also, chronic inflammation due to endogenous factors is associated with a higher production of ROS, which have the primary function of killing pathogens, but when produced excessively or the antioxidant:ROS balance is compromised, cell damage and modifications of DNA can occur due to oxidative stress,

leading to the signaling to activate inflammatory pathway. ROS are also produced in inflammatory process, mainly due to phagocytic cells using NADPH oxidase system and leukocytes when consuming oxygen, generating superoxide ion, inducing a chain reaction, and creating other ROS molecules. Another mechanism that increases ROS is the body's cell production of nitric oxide radical by the action of nitric oxide-synthase. The reaction of oxygen and nitric oxide radical creates ONOO which induced lipid peroxidation, being associated in various diseases such as auto-immune diseases. Thus, an effective anti-inflammatory substance should be able to inhibit the development of inflammation without interfering in normal homeostasis [47,63,66,69]

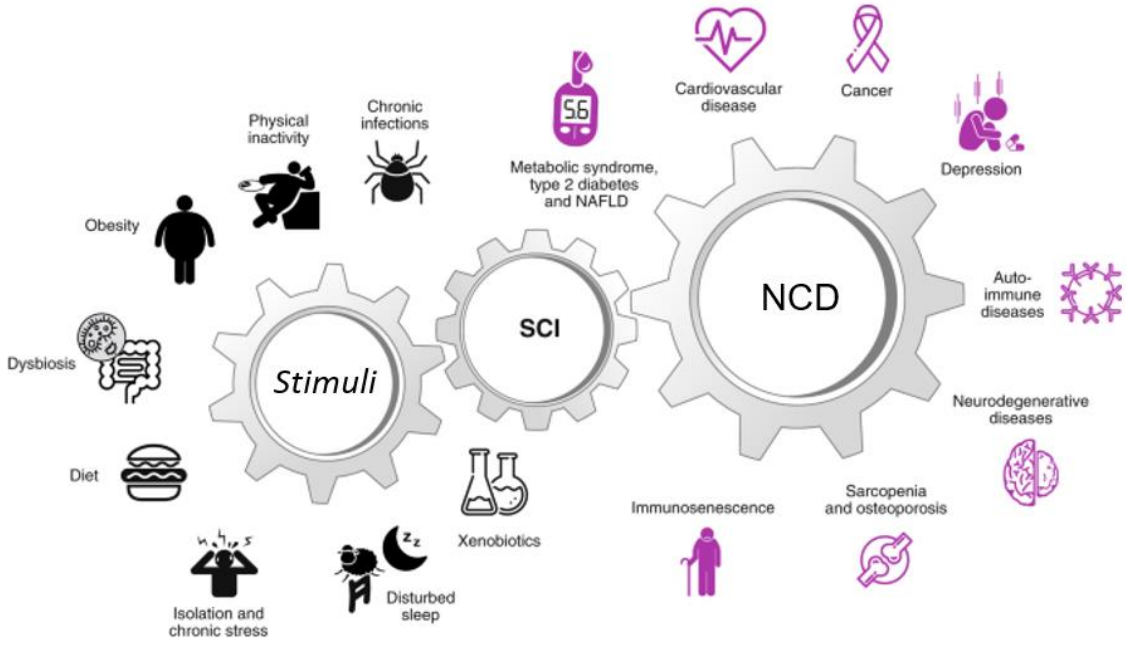

Figure 8 - Causes (left) of low-grade systemic chronic inflammation (SCI) and their consequences (right), such as non-communicable diseases (NCD's).
Adapted from: Furman et al.[70]

Around 15%–20% of all cancer cases are preceded by infection and chronic inflammation. Depending on the specific cancer type, inflammation might either precede or accompany tumor development. Cancer-related chronic inflammation facilitates unlimited replicative potential, independence of growth factors, resistance to growth inhibition, escape of programmed cell death, enhanced angiogenesis, tumor extravasation, and metastasis. Inflammation promotes all stages of tumorigenesis and usually is linked to the site where chronic infection or inflammation occurs, so for

example, the inflammatory diseases colitis, inflammatory bowel disease, pancreatitis, and hepatitis, are linked to a greater risk of colon, colorectal, pancreatic, and liver cancers, respectively. If inflammation is induced by obesity, hyperglycemia, or excessive lipid accumulation, it is considered a "low-grade chronic inflammation" and generally is of systemic nature, increasing the overall risk of cancer development, including breast, colon, and liver cancers. The main goal of anti-inflammatory compounds is the inhibition of prostaglandin synthesis, known mediators of inflammation, by blocking the enzyme cyclooxygenase (COX). During inflammation, prostaglandins increase vasodilatation and microvascular permeability, which lead to the classical signs of redness and swelling and facilitate the angiogenesis, which is a major component regarding cancer cell survival and replication. By inhibiting this inflammatory pathway, with anti-inflammatory compounds, cell damage is minimized (decreased ROS production) and cancerous cell survival is minimized [71–74].

Inflammation also plays a critical role in the genesis, progression, and manifestation of CVD, such as atherosclerosis. The unique microenvironment of the atherosclerotic plaque and its surrounding tissue is characterized by repeated inflammatory and reparative reaction, which is initiated and amplified by several mediators (such as cytokines and interleukins). The inflammatory cascade plays and important role in the development, modulation, and progression of atherosclerotic plaque, as it involves upregulation of cytokines and interleukins, and production of reactive oxygen species. Inflammation also plays a key role in determining the functional stability of complex atherosclerotic plaques by influencing the formation and destabilization of collagen in the fibrous cap. Together with lipid core growth, thinning of the fibrous cap leads to plaque instability with increased risk for rupture and possible acute events such as stroke [75].

Inflammation of the central nervous system is associated with many neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and multiple sclerosis. Inflammation and changes in microglia morphology are induced by signaling through Toll-like receptors, activating transcription factors, and leading to the production of ROS. These, amplify the expression of inflammatory mediators such as cytokines, amplifying even more proinflammatory signals that induce neurotoxic effects. Apoptosis and necrosis of neurons result in the release of ATP, which further activates microglia, inducing the production of various factors, such as nitric oxide

(NO), ROS, proinflammatory cytokines (e.g., TNF-α, IL-1β, IL-6), chemokines (e.g., IL-18), and prostaglandins (e.g., PGE_2), that promote further neuronal death. Natural anti-inflammatory compounds specifically target microglial cells to avoid their activation, blocking off the beginning of the chain reaction that would lead to the inflammation of the central nervous system, and therefore can be an effective therapy to prevent neurodegenerative and neuroinflammatory diseases [76,77] .

Obesity is characterized by a broad chronic low-grade inflammatory response and is associated with a cluster of metabolic disorders such as insulin resistance, type-2 diabetes, hypertension, and atherosclerosis. In obesity the levels of proinflammatory cytokines are increased. A characteristic of obesity and other inflammatory states are the elevated plasma lipid levels, which are partially responsible for inducing peripheral tissue insulin resistance and dyslipidemia and contributes to the development of atherosclerosis. Oxidative stress may also play an important role in the inflammation pathway, as reactive oxygen species are increased by hyperglycemia and free fatty acids, which translates into oxidative damage and initiation of inflammatory signals. Hyperglycemia stimulates the production of ROS in adipocytes, which leads to an increase in proinflammatory cytokine production, producing inflammation. The main target for anti-inflammatory compounds in obesity and diabetes is TNF-α, so TNF- α antagonists lead to an improvement of glycemia control and decreased risk for developing diabetes [78,79].

Several foods can be a good source of anti-inflammatory compounds when included in an overall healthy diet, aiding in the reduction of inflammation in the body. A good example of an anti-inflammatory diet is the Mediterranean Diet with high consumption of fruits, vegetables, nuts, whole grains, and fish. Whereas some unhealthy food choices (high amounts of fat, alcohol and processed foods) contain ingredients that can trigger or worsen inflammation [52,80].

Polyphenol-rich foods such as fruits and vegetables have a potential anti-inflammatory effect as they promote inhibition of enzymes involved in the production of eicosanoids, and therefore reduce inflammation (figure 9). Examples of polyphenols are flavonoids such as, flavones, isoflavones, anthocyanidins, resveratrol, curcumin, tannins, lignans, and phenolic acids. Besides fruits (such as grapes, apple, pear, cherries, and berries and their byproducts), other food sources are rich in polyphenols such as red wine, leafy greens, tea, coffee, cereals, herbs, and dry legumes. These foods are also rich in

antioxidant compounds, which act synergistically with the anti-inflammatory compounds to decrease oxidative stress and inflammation. Antioxidant activity reduces oxidative stress by increasing superoxide dismutase (SOD), decreasing TNF-α levels and inhibiting the cell signaling pathways for inflammatory response [52,63].

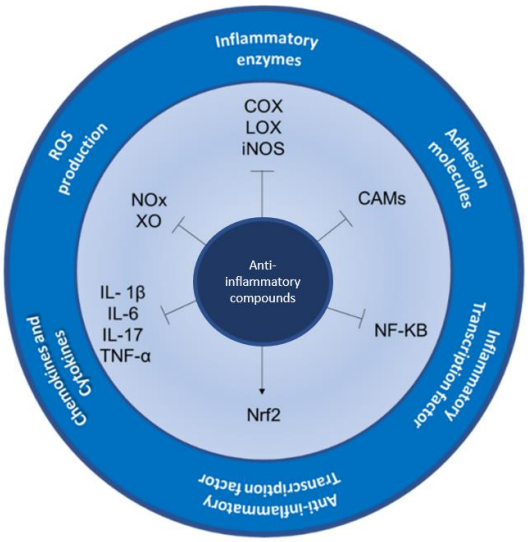

Figure 9 - Anti-inflammatory mechanisms in which anti-inflammatory compounds may be involved. Arrows and bar-headed lines mean activation and inhibition of the pathway respectively. Adapted from: Stromsnes et al.[81]

Probiotics and synbiotics in dairy products reduce inflammatory response by decreasing levels of pro-inflammatory cytokines (such as IL-12 and TNF-α), while upregulating the secretion of regulatory cytokines like IL-10 and TGF-β. Probiotics also lead to the regulation of gut microbiota and show anti-inflammatory and antioxidant activity [52,63,80].

Omega-6 and omega-3 fatty acids are polyunsaturated fatty acids (PUFA) which are present in a variety of food, while omega-6 has been linked with pro-inflammatory activity, omega-3 fatty acids (especially EPA and DHA), have anti-inflammatory properties and, therefore, might be useful in the management of inflammation. Omega 3 FA, more specifically EPA competes with arachidonic acid (an omega-6 fatty acid) for prostaglandin and leukotriene synthesis at the cyclooxygenase and lipoxygenase level. Thus, an increased consumption of omega-3 FA rich foods, such as fish and other seafood (especially cold-water fatty fish, such as salmon, mackerel, tuna, herring, and sardines), nuts and seeds (flaxseed, chia seeds, and walnuts), plant oils (such as flaxseed oil, soybean oil, and canola oil) and fortified foods (such as certain brands of eggs, yogurt, juices, milk, soy beverages) may have an anti-inflammatory role in the body by modulating prostaglandin metabolism and decreasing triglycerides [82].

1.3. Microalgae

Microalgae are a huge group of unicellular organisms comprising eukaryotic protists, prokaryotic cyanobacteria, and blue-green algae, with over 25,000 identified and isolated species. Microalgae together with cyanobacteria are also called phytoplankton. Microalgae is a very heterogeneous group of microorganisms in terms of biodiversity, morphology (for example, with flagellate, coccoid and cysts stage), reproduction (sexual reproduction or asexual reproduction) and size. Regarding the energy and carbon sources used for their metabolism, microalgae can grow in three different ways: autotrophy, heterotrophy, and mixotrophy. Although, most microalgae are autotrophic, and use carbon dioxide (atmospheric or marine origin) as carbon source and sunlight as energy source to produce oxygen by photosynthesis. The photosynthetic efficiency of microalgae is about ten times higher than terrestrial plants and are responsible for 50% of the overall oxygen production [83,84].

Microalgae can be found in both marine (seas and oceans) and freshwater environments, such as estuaries, rivers, and lakes and are capable of survival in many environmental conditions, including high and low pH, temperature, salinity, and light conditions, whereas the optimal growth conditions depend upon species. Growth conditions (such pH, carbon source, nutrient availability, light exposure, salinity) and type of cultivation strongly influence microalgal metabolism, conditioning growth rate, biomass productivity and biochemical composition [85–87]

Due to the wide spectrum of biologically active compounds found in microalgal biomass, microalgae have been highlighted from the biotechnological point of view for various applications. Microalgae fit within a circular economy by being able to bioremediate nutrient waste and as a source of biomass for several commercial applications. The main industrial applications are functional foods, animal feed, pharmaceutical industry, fertilizers, as well as tools for wastewater treatment and biofuel production [88], as shown in figure 10.

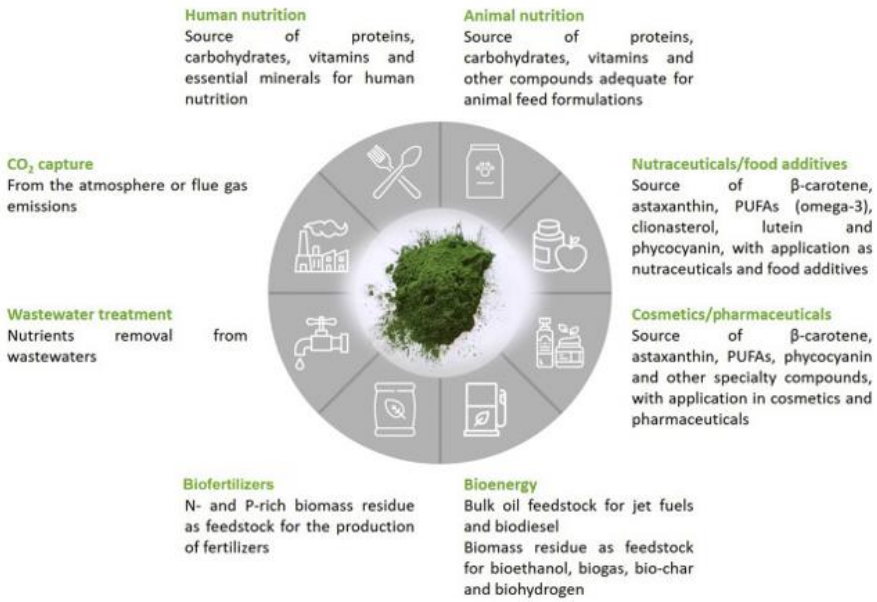

Figure 10- Main applications described for microalgae.
Adapted from: Moreira et al.[89]

1.3.1. Microalgae for human nutrition

Microalgae for human consumption is not a new practice, in fact the first evidence of algae consumption by humans dates back 14,000 years ago in Chile and records show that it has been continued to be consumed throughout the world for the last centuries [90].

Generally, marine algae are rich in dietary fiber, minerals, lipids, proteins, omega-3 fatty acids, pigments, essential amino acids, polysaccharides, and vitamins A, B, C, and E which have attributed several health-promoting effects, including anti-oxidative, anti-inflammatory, antimicrobial, and anti-cancer effects. However, the specific compounds and their amount depend of microalgae species and growing conditions[90], as shown in figure 11.

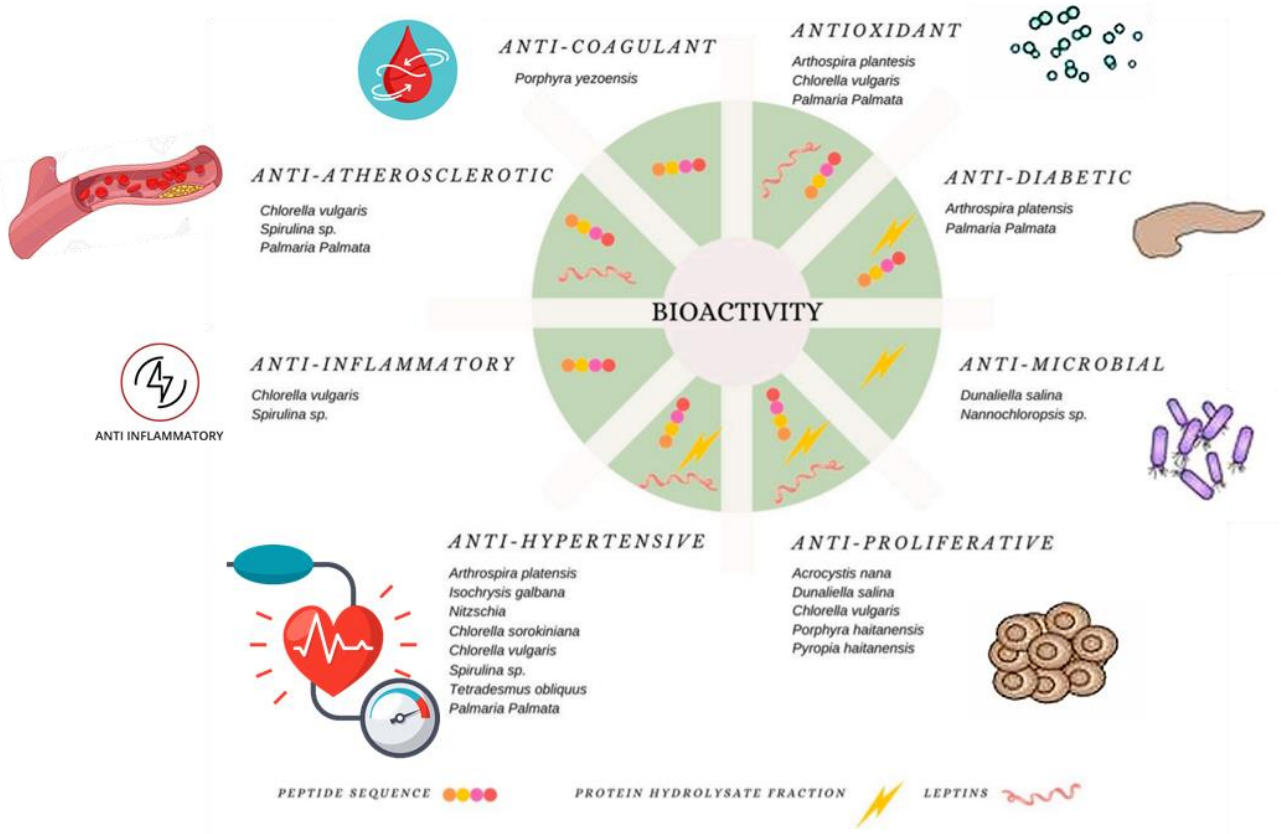

Figure 11 - Micro- and macroalgae bioactive compounds and some of their health-promoting effects. Adapted from: Geada et al.[91]

However, only a few microalgae have GRAS status recognized by the FDA, including *Arthrospira platensis, Chlamydomonas reinhardtii, Auxenochlorella protothecoides, Chlorella vulgaris, Dunaliella bardawil,* and *Euglena gracilis* [92]. In Europe, even fewer species (eleven in total) are authorized for human consumption *Ulkenia striata* (algal oil); *Haematococcus pluvialis* (astaxanthin-rich oleoresin), *Fucus vesiculosus* (fucoidan extract), *Undaria pinnatifid* (fucoidan extract), *Odontella aurita, Schizochytrium sp* (oil), *Chlorella vulgaris, Chlorella luteoviridis, Chlorella pyrenoidosa, Arthrospira platensis (Spirulina)* and *Tetraselmis chui*[93]. For new algae or algae derived products to be approved for human consumption in the EU, an application for authorization of a "novel food" must be submitted to the European Commission, when accepted, an implementing act authorizing the placing on the market of a novel food is issued and the novel food is updated into the Union list. To be authorized and included as novel food in the Union list several requirements must be clarified, such as specifications, conditions of use (maximum amounts, specified food category), additional specific labelling requirements

(specific additional requirements for the labelling of a novel food may apply, to properly inform the consumer) and post-market monitoring requirements, if applicable [94].

The nutritional composition of microalgae varies significantly between algal species and growth environment. Important nutritional components to consider are protein and lipid content, as well as vitamin and mineral content, all of which are known to positively impact human health [92]. Microalgae are generally protein-rich foods, with an average value of protein content of about 40%, remarkably *Arthrospira platensis* has been reported to have up to 70% of their biomass as protein content. All GRAS microalgae have all essential amino acids, which cannot be produced by the human body, making microalgae high quality protein-rich foods. The amino acid profiles of microalgae are similar to other conventional protein sources such as eggs and soybean [92]. Average algae lipid values vary a lot depending on species and cultivation conditions usually varying between 30-50%, values up to 70% have been reported for *Auxenochlorella protothecoides*. From the total lipid content, the most interesting compounds are docosahexaenoic acid and eicosapentaenoic acid which have been proven to have a positive impact on human health [92]. Fatty acid production in microalgae can be increased by manipulating various environmental factors such as oxygen level, temperature, light exposure, pH as well as limiting nutrient supplementation [95]. Carbohydrates, with focus on polysaccharides, fibers and oligosaccharides are promising compounds with potential health benefits, and can have prebiotic applications [92]. Besides proteins, lipids and carbohydrates, microalgae are source of several valuable compounds with health benefits such as essential minerals, and vitamins whereas the specific composition is extremely variable according to many factors including species, geographic area, season of the year, and environmental parameters. For example, a single spoonful (7 g) of dried biomass of Spirulina contains almost 4 g of protein, 1 g of fat including PUFAs like omega-3 and omega-6 fatty acids and 11%, 15% and 4% of Required Daily Allowance (RDA) of Vitamin B1, B2 and B3, respectively. It also constitutes for 21% and 11% of the RDA of copper and iron, respectively. Algae also have good amounts of magnesium, potassium, manganese, zinc, selenium, which have anti-oxidant and anti-inflammatory properties and are also important as co-factors for several enzymes [90,96–98]

Overall, the bioactive compounds of microalgae biomass have several health benefits with antimicrobial, anti-inflammatory, anti-aging, aggregative, vasoconstricting,

hypocholesterolemic, antioxidant, immunosuppressive, photoprotective, antiviral and neurotransmitting activities [99].

Presently, most of the commercially available microalgal biomass is marketed as pills and capsules (*Arthrospira* and *Chlorella* are commonly consumed as food supplements, *Tetraselmis chui* as a seafood flavoring agent, and the diatom *Odontella aurita* is consumed as a food supplement due to its high EPA content). However, novel functional foods enriched with microalgae are also on the rise [92].

1.3.2. Microalgae as functional food

Given the nutrient and bio compound richness of algae (see above), it is understandable why microalgae are increasingly being marketed as "functional foods".

A recent event that boosted interest in natural functional foods is the COVID-19 pandemic, whereas consumers demanded food ingredients that strengthened immune and digestive systems. Microalgae (mainly *Spirulina* and *Chlorella*) have several bioactive compounds which are considered to boost the human immune system, modulate the production of cytokines, stimulate the intestinal immune system, and have anti-viral activities. It has even been theorized that some algae-derived, biologically active molecules could be used against COVID-19 virus. Therefore, microalgae have been trending as functional foods [100].

Most of the commercially available microalgal biomass are sold in the form of food supplement (capsules, liquids, tablets, powders) mainly with PUFA, proteins and astaxanthin as bioactive compounds, with anti-inflammatory, anti-coagulation, and antioxidant activities. Microalgae extracts also have great potential as an alternative source of natural ingredients that can be incorporated into food products, enhancing their nutritional value [101].

However, food made with microalgae is still not widely available due to various factors such as, limited number of allowed species, market demand, nature of the food matrix (e.g., emulsion, gel, aerated dough systems) and the interactions with other food components. Other obstacles that limit the incorporation of the algal material into

conventional foods are sensatory/organoleptic: powder like consistency of the dried biomass; dark green color and its slight smell and intense taste [98].

It has been reported that the addition of microalgae (*Dunaliella striata, O. amphibian, A. platensis, A. fusiformis, Arthrospira sp., I. galbana, T. suecica, S. almeriensis)* to bread, pasta, noodles, and cookies can be done until only a certain percentage, otherwise dough consistency and taste became unpalatable and while cooking the color of the noodles changed into an unattractive brownish color. Nonetheless, these functional foods had increased content of protein, fiber, minerals, and omega 3 fatty acids and higher amounts of antioxidant activity [90,102].

Dairy products (such as yogurts, fermented milk, frozen yogurt, and cheese) can also be supplemented with microalgae (*Chlorella sp., A. maxima and H. pluvialis , A. maxima, C. vulgaris, D. vlkianum , A. platensis, Arthrospira sp.*) to deliver bioactive compounds. Some studies suggested a synergetic action between microalgae and probiotic bacteria, possibly due to the availability of trace elements, vitamins, and other bioactive compounds in microalgae powders that promote the development of desired bacteria, increasing the viability of the probiotics. The newest innovation is a “algae milk”, a microalgae-based milk alternative which is made from a signature microalgae protein flour homogenized with water, which claims to have nutritional value as cow milk [90,103].

Food emulsions such as mayonnaise enriched with certain microalgae species (*C. vulgaris green, C. vulgaris and H. pluvialis)* had enhanced antioxidant properties and improved color. Vegetarian dessert gels (pudding, jelly) were suggested as a vehicle to provide valuable microalgae-based compounds from *C. vulgaris, H. pluvialis, A. maxima* and *D. vlkianum*, and had improved content of antioxidants and omega-3 fatty acids and improved structure and color characteristics [90,102,103]

1.3.3. Microalgae species used in this study

1.3.3.1. *Tisochrysis lutea*

Tisochrysis lutea or *T. lutea*, (formerly known as *Isochrysis galbana*, *Isochrysis galbana* Parke, T-*Iso or Isochrysis galbana* Parke 1949*)* is a golden-brown photoautotrophic haptophyte microalga and was originally isolated from Tahiti, French Polynesia although, nowadays, *T. lutea* has a large coastal Atlantic distribution [104]. This microalga usually presents itself as a unicellular flagellate with two flagella (around 7 μm long) but can also be found in palmelloid stages in marine environments. *T. lutea* cells are small (5 to 6 μm long, 2 to 4 μm wide, and 2.5 to 3 μm thick), elongated, variable in shape and have an absence of rigid cell wall, and are encapsulated by plasma membranes, as shown in figure 12 [105–107].

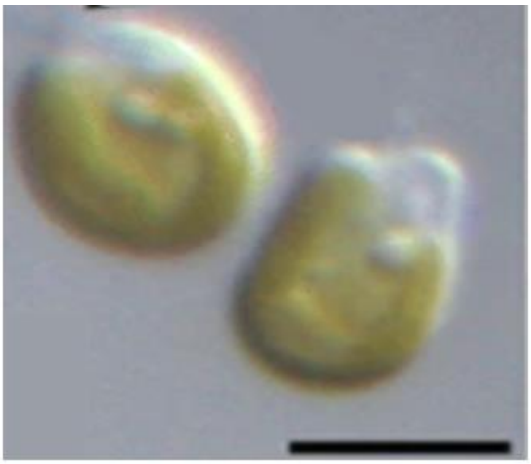

Figure 12 - Light electron micrographs of two *Tisochrysis lutea* motile cells. Scale bar = 5 μm. Adapted from: Bendif et al.[104]

Tisochrysis lutea is one of the most used microalgal species in aquaculture, principally as feed in the early stages of growth of mollusk larvae, fish, and crustaceans. The main characteristics than makes *T. lutea* so important in aquaculture are their small cell size; lack of cell wall (which makes it easy to be ingested by small invertebrates); and good nutritional qualities (especially high amount of PUFAs and DHA). *T. lutea* is easily cultivated, presents fast growth rates and wide physicochemical tolerance ranges, which makes this microalga an excellent candidate for mass cultivation [107–109].

The biochemical composition of *T. lutea* is dependent on many different factors, such as growth phase and culture conditions, these factors seriously influence the algal metabolism, thus directing the synthesis of valuable compounds [109]. *T. lutea* has a very

interesting nutritional composition with several important bio-molecules, namely polysaccharides, fatty acids, carotenoids (mainly fucoxanthin), chlorophylls, vitamins, and sterols (brassicasterol, stigmasterol, fucosterol) [110].

Many bioactivities are attributed to *T. lutea*, which has potential health benefits, with the capacity to improve the human health and have therapeutic effects against several diseases like cardiovascular disease, cancer, diabetes, infectious diseases, arthritis and cancer [109,110]. The high amount of polyunsaturated fatty acids (PUFAs), particularly EPA and DHA, play an important role in many metabolic activities, such as antimicrobial activity. This antibacterial activity may be due to ability of these fatty acids and their derivates to bring bacterial cell to lyses [109,110].

Other bioactivities that are attributed to *T. lutea* biomass are anticoagulant activity and immune-modulatory activities (possibly due to polysaccharide content). It also has free radical scavenging ability and high chelating ability of Fe^{2+} and Cu^{2+} [109,110].

All these compounds and resulting bioactivities are extremely interesting for the application in therapeutics, food, and pharmaceuticals industry. Currently, *T. lutea* is not approved for human consumption, however *T. lutea* extracts could be interesting for food enrichment (such as yogurts, bread, etc.) or as nutritional supplements (powder form, tablets and capsules) [111,112].

1.3.3.2. *Tetraselmis chui*

Tetraselmis chui Butcher (1959) (also known as *T. chui),* is a unicellular green marine microalga which belongs to the class Prasinophyceae and *Chlamydomonadaceae* family, which was first isolated off the coast of Great Britain in 1959 but has since been found around the world. Nowadays, *Tetraselmis chui* is the most abundant microalgae in European estuaries [113].

This alga has a characteristic slightly compressed oval cell shape (cell dimensions range from 10–25 μm in length, 7–20 μm in breadth and 6.5–18 μm in thickness) and four flagella emerging from a depression near the apex [114–116], as can be seen in figure 13. *T. chui* is a robust alga that has rapid growth rates and tolerates extremes of pH, salinity, and temperature, being especially suited for outdoor mass culture [114,117].

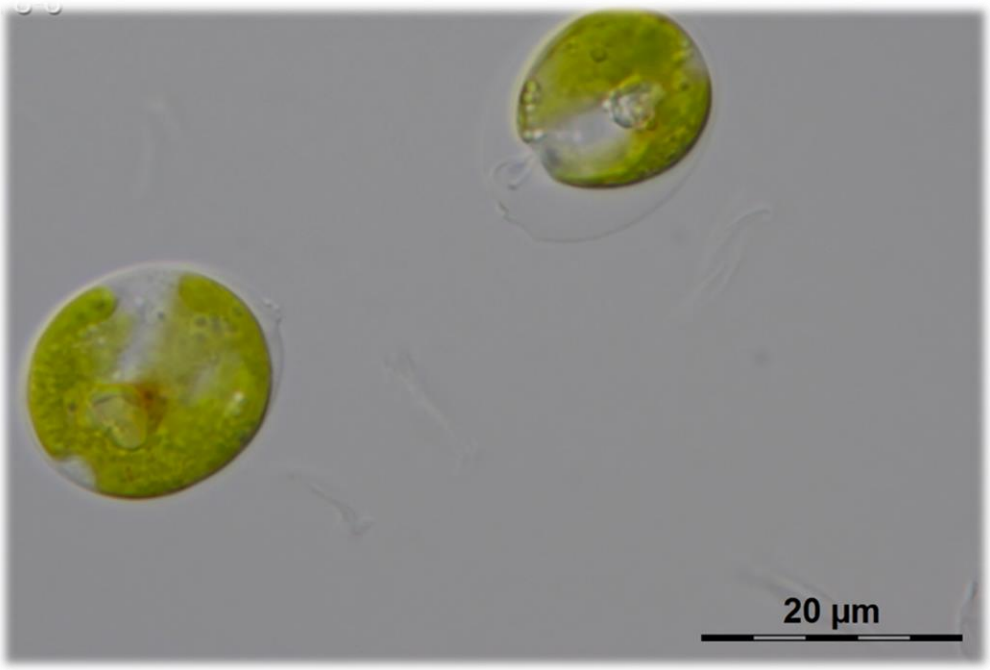

Figure 13 - *Tetraselmis chui (*SAG 8-6 *Tetraselmis chui).*
Scale bar = 20 µm. Adapted from: Georg-August-Universität Göttingen[118]

Tetraselmis chui has been used for decades in aquaculture, as it does not produce toxins, is harmless toward marine species and contains adequate amounts of proteins, lipids, carbohydrates, and fatty acids which are essential for the cultured organisms. The main applications in aquaculture are as feed for crustaceans and can also be used in the production of larvae of mollusks and adults, such as clams, oysters, and scallops [114,119].

T. chui also has an enormous potential as a novel dietary source, due to its high protein (35–40%), carbohydrate (30–35%), and mineral content. It may even be considered a source of sustainable protein of high nutritional quality, due to its ease of culture and presence of all essential amino acids (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine), making it a source of complete protein [120]. This species is also very rich in long-chain polyunsaturated fatty acids, especially EPA and DHA[120].

Besides the good nutritional profile in terms of macronutrients, *T. chui* also is a source of various bioactive compounds witch have antioxidant, anticancer and antimicrobial properties, such as α-tocopherol (vitamin E), carotenoids (e.g., fucoxanthin and β-carotene), phenolic compounds, and terpenes. These natural antioxidants found in microalgae additionally protect cells against oxidative damage [114,121,122]

T. chui has been determined to be Generally Recognized as Safe (GRAS) and was approved as novel food by the European Union (Article 3(1) of Regulation (EC) No 258/97)[123] in 2017, as a food supplement with maximum levels of 250 mg/day [120]. However, the current commercial food applications of *T. chui* in the EU is limited to sauces, condiments, and salts. Recently, several experimental works have been carried out regarding the incorporation of *T. chui* into food, some of the proposals are pasta, cookies and wheat bread [114,124].

1.3.3.3. Tetraselmis striata CTP4

Tetraselmis striata CTP4 is a recently isolated microalgae stain and belongs to the phylum Chlorophyta and to the class Chlorodendrophyceae which is considered to have nutritional and biotechnological potential, figure 14. *T. striata* CTP4 is a very fast growing and robust autochthonous strain from the Algarve, which can endure high temperatures (up to 40 ºC) and salinity (up to 100 ppm). Due to the relatively large cell size (9–22 µm) and lack of flagella, the cells usually sediment pretty easily [125,126].

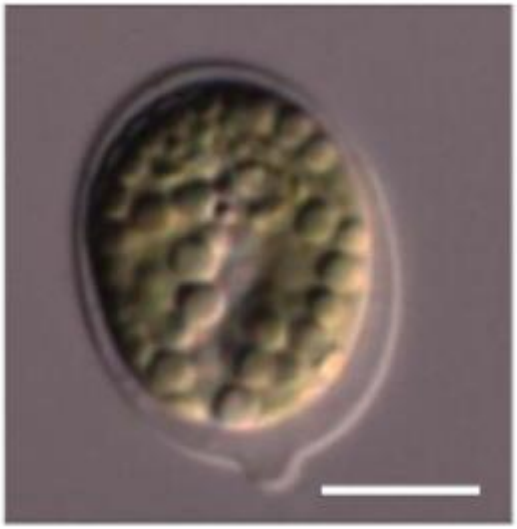

Figure 14 - *Tetraselmis striata* CTP4, seen under the microscope.
Scale bar = 5 µm. Adapted from: Pereira et al.[125]

T. striata CTP4 biomass has an interesting nutritional profile, especially in terms of protein (31.2 g/100 g) (high contents of leucine, valine, lysine, and phenylalanine), dietary fibers (24.6 g/100 g), (is known to be a source of starch-like polysaccharides), carotenoids and vitamins (ascorbic acid (79.2 mg/100 g); tocopherol (20.28 mg/100 g) and niacin (7.98 mg/100 mg), presenting a proximate composition similar to that of soybean. Another factor that makes *T. striata* CTP4 interesting is moderate antioxidant capacity, an extract of ethyl acetate demonstrated ABTS scavenging ability and both iron and copper chelating ability [126,127].

2. Aim and objectives

The main aim of this dissertation was to identify the antioxidant and anti-inflammatory potential of three microalgal species (*Tisochrysis lutea, Tetraselmis chui*, *Tetraselmis striata* CTP4) which might contribute for their establishment as novel functional foods.

To achieve this main objective several specific objectives were underlined:

I. Evaluate the antioxidant and anti-inflammatory properties of microalgae extracts produced with solvents of different polarities, thus maximizing the range of extracted compounds.
II. Identify known phytochemicals in the most active extracts that may be responsible for the observed bioactivities.
III. Compare different extraction techniques (conventional extraction *vs.* Automated Accelerated Solvent Extraction Systems) on yield and antioxidant activity of *T. striata* CTP4 extracts.
IV. Evaluate the proximate composition and minerals and trace elements profile of *Tisochrysis lutea* biomass to complement the knowledge on its nutritional composition.

Overall, the present dissertation is expected to contribute to the current knowledge on microalgae and identification of small molecules from microalgal origin with the ability to promote the treatment of inflammation, pain, and Inflammatory Bowel Disease, contributing to the establishment of these microalgae as functional food.

3. Materials and Methodology

3.1. Chemicals, kits, and drugs

Extracts were prepared with the following solvents: ethanol, Milli-Q water, hexane, and ethyl acetate. Solvents used were from HPLC grade (methanol and ethyl acetate), analytical reagent grade (DMSO and 96% ethanol) or commercial grade (hexane). Reagents were provided by different suppliers from Sigma-Aldrich (Munich, Germany; Trichloroacetic acid, ABTS, DPPH and sodium acetate; Acros Organics (Geel, Belgium) supplied glass beads, ferrozine, pyrocatechol and ferricyanide; VWR (Portugal) supplied Iron (II) chloride, ferric chloride; Potassium persulfate was from Biochem Chemopharm (Cosne Sur Loire, France) and EDTA from Fluka Chemika (Buchs, Switzerland).

Distilled water was produced with a Elix/Gradient Water Purification System (Millipore). All Milli-Q water was obtained through Milli-Q® Advantage A10 Ultrapure Water Purification System (Merck, Germany).

Cyclooxygenase (COX) Inhibitor Screening Assay Kit was obtained from Cayman Chemical (Cayman Chemical, Ann Arbor, MI, USA). The positive control used was Ibuprofen (≥98% (GC)) from Sigma–Aldrich (Munich, Germany).

Angiotensin I Converting Enzyme (ACE) Activity Assay Kit and Angiotensin Converting Enzyme from rabbit lung (≥2.0 units/mg protein (modified Warburg-Christian)) were purchased at Sigma–Aldrich (Munich, Germany). The positive control used was Enalapril maleate salt powder (≥98% (TLC)) from Sigma–Aldrich (Munich, Germany).

3.2. Algal material

A total of three biomass samples were used in this study: *Tisochrysis lutea, Tetraselmis chui* and *Tetraselmis striata* CTP4. Each sample was obtained from pure cultures and was provided by commercial producers. The freeze-dried biomass samples from *Tisochrysis lutea* and *Tetraselmis chui* were provided by Necton S.A. (Olhão, Portugal). *Tetraselmis striata* CTP4 biomass was spray dried and provided by Allmicroalgae - Natural Products, S.A (Pataias, Portugal).

3.3. Preparation of extracts

All the algal extracts were obtained by solvent-based methods, as solvent-based extraction methods are essential for the separation of analytes prior to their identification by chromatographic analysis, being an intermediate step in the purification of extracts. Solvent extraction has proved to be the most workable method when it comes to algal and plant extractions [128]. As conventional manual extraction method a bead beater-assisted solvent-based extraction was performed, whereas the bead beater acts as a "pre-treatment" for the actual extraction, mixing the solution and facilitating the disruption of biological cells, allowing a better solvent access to intracellular components and a more efficient extraction. As non-conventional extraction methods, such as Accelerated Solvent Extraction (ASE), promote an increase in temperature and/or pressure and generate a massive amount of pressure on the cell walls, which eventually leads to cell wall rupture, decreasing the need of a pre-treatment. Each of these methods has certain advantages and disadvantages, so usually they are combined to obtain improved extraction results [128].

3.3.1. Conventional Extraction Methods

Conventional extraction refers to the recovery of bioactive compounds from plants or algae using conventional solvents, where the microalgae matrix is initially homogenized and soaked in a solvent, often under a constant agitation, thereby the desired molecules are extracted due to cell disruption. These methods generally are very simple, but have some significant downsides, such as longer extraction times, need to evaporate big amounts of solvent, poor efficiency, low extraction selectivity and high solvent consumption. All these drawbacks may lead to an increase of process costs and decrease of environmental sustainability [97,129].

3.3.1.1. Extraction with disruptive equipment (*bead beater*)

The microalgae biomass was extracted at a 1:10 biomass/solvent ratio with four different solvents: ethanol, water, hexane, and ethyl acetate, to maximize the spectrum of extracted compounds. Approximately 150 mg of the microalgae biomass was added into 2 ml Eppendorf™ Snap-Cap Microcentrifuge Safe-Lock™ Tubes, together with glass beads (ACROS ORGANICS, 500-750 μm) and 1.5 mL of solvent. Glass beads were added as

they are particularly helpful in the cell disruption of the microalgae cells as the work from [130] shows. The racks of the used *bead beater* ((MM400, Retsch GmbH, Germany)) were kept on ice or in the freezer prior to each extraction cycle and in between extractions cycles, to avoid any loss or degradation of bioactive compounds from the samples while milling.

Each extraction cycle of the *bead beater* was programmed to last five minutes at a frequency of 30.0 s^{-1} and the extractions were performed at room temperature. When removing the racks with the samples from the *bead beater*, the samples were immediately put in ice to avoid any loss or degradation of bioactive compounds and the racks were put into the freezer. The extracts were then centrifuged at 3 ºC and 215 *g* for 3 min in high-speed micro centrifuge (HITACHI KOKI Himac CT15E). After centrifugation, the supernatant of the tubes was collected and kept in a box with ice. To the remaining *pellet* in each tube, 1.5 ml of solvent was again added, and the previously mentioned procedure repeated. At the end of extraction cycles, the collected supernatants were combined. The number extraction cycles varied, being repeated until it was possible to see a clear discoloration of the pellet.

In the case of the extracts made from organic solvents (ethanol, hexane, and ethyl acetate), the combined amount of collected supernatant was evaporated in a rotary vacuum evaporator (IKA®, RV 10 Digital FLEX Rotary Evaporator), until the final volume of about 5 mL was obtained. The remaining volume was filtered with a syringe Teflon filter (Whatman®, 0.2 μm pore size) using a glass syringe (FORTUNA OPTIMA®). After filtration, the extracts were transferred into previously weight amber vials and then dried to total dryness using a gentle nitrogen flow.

In the case of the aqueous extracts, a freeze dryer (model Lyoalfa 15, Telstar, SA, Barcelona, Spain) was used to dry the extracts. Aqueous extracts were left in the freeze dryer for about 48 h. In either methodology (rotary evaporator or freeze dryer), to make sure that total dryness was achieved, two subsequent weightings had to present the same mass. The dried extracts were resuspended in dimethyl sulfoxide (DMSO) at concentrations of 20 mg/mL (stock solution), 10 mg/mL; 5 mg/mL; 1 mg/mL (3 working solutions) and stored at -18 ºC until usage. The whole extraction process is summarized in figure 15.

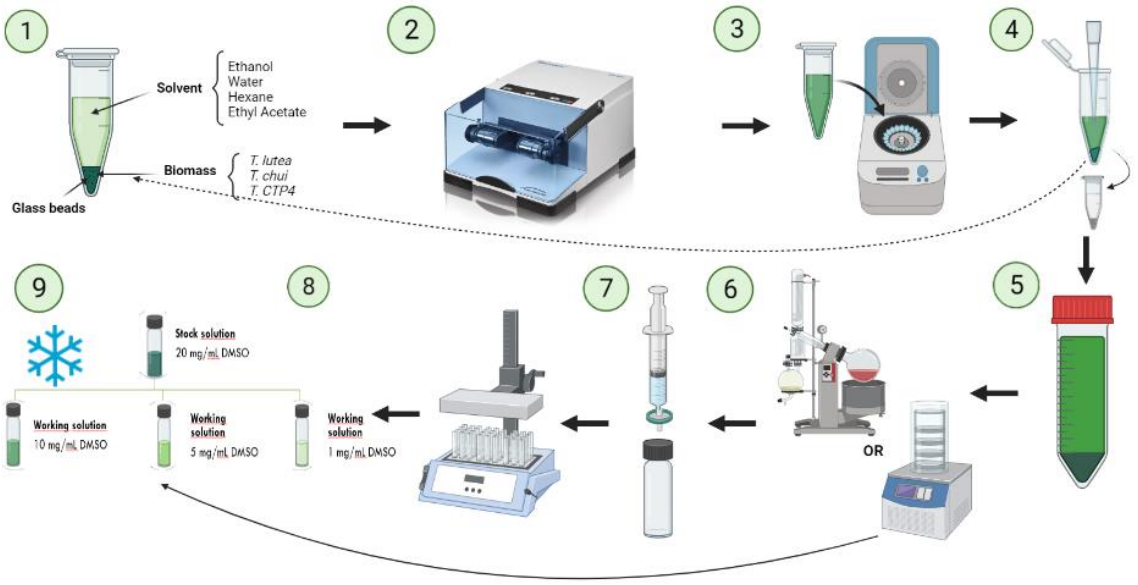

Figure 15- Flowchart of extraction process with disruptive equipment.
1- Addition of solvent to glass beads and biomass to Eppendorf tube. 2- *Bead beater*. 3- Microcentrifugation. 4- Collection of supernatants. 5- Total supernatant collected. 6- Evaporation of solvent (rotary evaporator or dry freezer). 7- Syringe filtration .8- Nitrogen drying of extracts. 9- Suspension in dimethyl sulfoxide (DMSO)

The yield of extractable substances (Y, %) was expressed as a percentage by dividing the total weight of the extract (W_E) by the amount of dried biomass used for extraction (W_B), as shown in equation 1.

$$Y = \frac{W_E}{W_B} \times 100\% \qquad (1)$$

3.3.2. Emerging extraction techniques - Automated Fast Solvent Extraction systems

Although conventional extraction methods (as milling, for example) are already employed in largescale productions, they often need large amounts of solvents, being expensive and not very environmental-friendly besides also requiring several additional steps to recover the extract, these processes frequently are very demanding in terms of energy and time [131,132]. Therefore, researchers are exploring alternative extraction methods which are more environment friendly, use less energy and solvent while generating higher yields [129] .

Non-conventional (also called emerging) extraction techniques use specific processing aids/energy inputs to improve the extraction efficiency and/or selectivity. Examples of emerging extracting techniques are pulsed electric field (PEF), supercritical fluid extraction (SFE), subcritical fluid extraction, pressurized liquid extraction (PLE), microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), enzyme-assisted extraction, and accelerated solvent extraction (ASE), just to mention a few. Among these alternatives, accelerated solvent extraction (ASE) is one of the most promising techniques [97,133–135]. In the Automated Accelerated Solvent Extraction (ASE) systems, the extraction process is carried out at temperatures exceeding the boiling point of a solvent what implies that the pressure inside the extraction cell must be kept high to maintain the solvent in a liquid state. Both temperature and pressure influence the process efficiency [133–136]. By applying new alternative extraction methods, it is possible to accelerate extraction of various biomolecules (decreasing extraction time), lower the amount of energy and solvent that are consumed, and increase the extraction yield. Depending on the specific methods it is possible to increase the extraction efficiency by selectively or non-selectively increase the number of biomolecules that are extracted from the intracellular space of microalgae. The effectiveness depends on the selected operating conditions, such as temperature, pressure, time and other factors [131,136].

3.3.2.1. Energized Dispersive Guided Extraction -EDGE

The EDGE is a new disruptive extraction equipment for sample preparation. The EDGE is an automated solvent extractor which combines two different extraction methods: pressurized fluid extraction and dispersive solid phase extraction. The pressurized fluid extraction component is gained as the reaction chamber walls are heated, the pressure in

the reaction chamber increases, forcing the solvent to disperse into the sample. The dispersive effect is also obtained due to the addition of solvent from both top and bottom. EDGE is also able to perform Supported Liquid Extraction and Liquid-Liquid Extraction. A scheme of the EDGE process is exhibited in figure 16.

Based on these principles, with EDGE it should be possible to "reduce extract preparation time and obtain fast, simple, and efficient extractions", with applicability in various fields – environmental, food, pharmaceutical, consumer products and polymers [137].

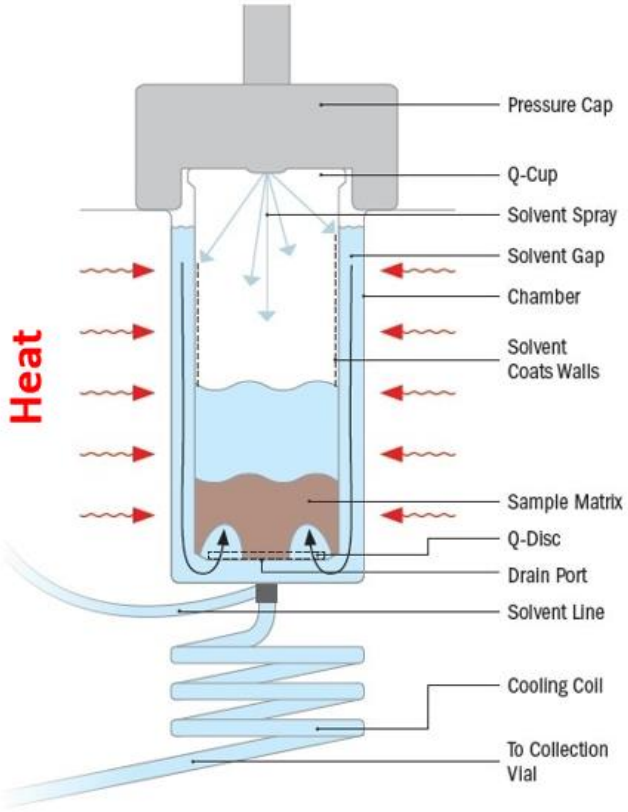

Figure 16- Schematics of EDGE extraction process.
Adapted from: AZO Materials (2018)[138]

The maximum temperature of the equipment is 200 ºC and maximum pressure will be solvent dependent as the pressure in the vessel chamber is a result of heating the solvent. Each extraction can consist of an extraction, rinse, and wash. In between extractions, the pressurized chamber, where the reaction happens, cleans itself with the solvent that is going to be used in the next extraction to avoid cross contamination [137].

The extraction is made in a Q-cup (extraction vessel), with two detachable parts, where in between both parts is placed a *filter sandwich* (S1 *Q-disc*) set together from 2 cellulose filters (high retention capacity but low structural capacity) and a glass filter (high structural capacity), forming a universal filter (to avoid any grease to block lines). On the rack, next to each Q-cup, exists a collection vial. The complete system hardware is presented in figure 17.

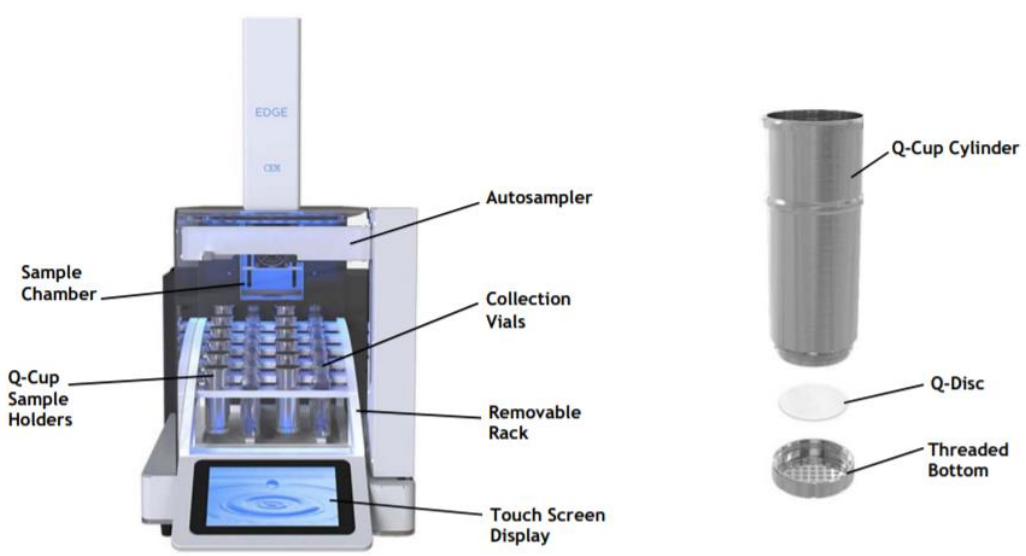

Figure 17- System Hardware of EDGE.
Retrieved from: https://cem.com/edge/

The EDGE is fully operated by a touchscreen, whereas the user defines maximum temperature, solvent, cycle time, number of cycles (maximum amount is five cycles) and cooling temperature. For each rack position it is possible to designate a different method, if so desired. After selecting and starting an extraction method, the robotic arm from EDGE picks up the selected Q-cup and brings it to the reaction chamber, the pressure cap then creates a pressurized seal on the top of the Q-Cup. When reaction chamber closes, the correspondent solvent is added, and the extraction starts with the set temperature, time, and cycle number. The formed extract is automatically conducted into the corresponding collection vial. Afterwards, the robotic arm will put the Q-cup back to its initial position [137].

Algal biomass was extracted using EDGE (CEM, US) accelerated solvent extraction (ASE) system. Spray-dried *T. striata* CTP4 algal biomass (1 g) was extracted with a total of 10 ml solvent (ethanol or hexane) per cycle, at 3 temperatures (30 ºC, 50 ºC and 100

°C) for either 5 or 10 minutes per cycle. The number of cycles varied from one to four. The extraction conditions of the 15 extracts obtained with EDGE are summarized in Table 1.

Table 1- Extraction conditions (solvent, temperature, time per cycle, number of cycles) of *Tetraselmis striata* CTP4 with EDGE

Solvent	Temperature (ºC)	Time per cycle (minutes)	Number of cycles
Ethanol	**30**	**5**	**1**
	30	**5**	**2**
	30	**10**	**1**
	30	**10**	**2**
	50	**5**	**2**
	50	**10**	**1**
	50	**10**	**2**
	50	**10**	**3**
	50	**10**	**4**
	100	**5**	**2**
	100	**10**	**2**
	100	**10**	**1**
Hexane	**50**	**10**	**1**
	50	**10**	**2**
	50	**10**	**3**

In between sample conditions, the lines were rinsed with the next to be used solvent. At the end of the extraction, the collection vial was immediately removed from the rack and put in a box with ice.

The extracts were transferred into previously weighted amber vials and dried with a gentle nitrogen flow until constant weight was obtained. The dried extracts were resuspended in dimethyl sulfoxide (DMSO) at concentrations of 20 mg/mL (stock solution), 10 mg/mL; 5 mg/mL; 1 mg/mL (3 working solutions) and stored in the freezer at -18 ºC until usage. The whole extraction process is summarized in figure 18.

The yield of extractable substances was expressed as a percentage calculated using equation 1.

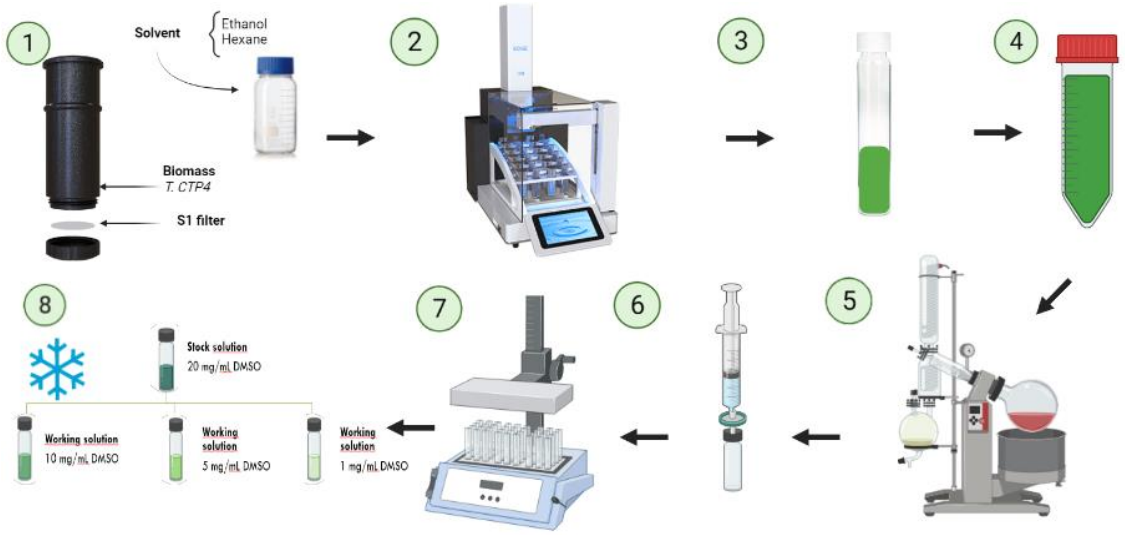

Figure 18- Flowchart of extraction process with EDGE equipment.
1- Addition of solvent to the biomass in the Q-cup. 2- EDGE extractor. 3- Collection of extract from collection vial. 4- Total supernatant collected. 5- Evaporation of solvent. 6- Syringe filtration. 7- Nitrogen drying of extracts. 8- Suspension in dimethyl sulfoxide (DMSO)

3.3.2.2. Microwave-assisted Extraction

Microwaves are non-ionizing electromagnetic waves of frequency between 300 MHz to 300 GHz. These electromagnetic waves are generated by two oscillating fields: magnetic field and electric field, which are perpendicular to each other [139]. The base principle for microwave technology is linked to the heating capacity of solvents by dielectric heating effects using microwave energy to heat solvents containing samples. Dielectric heating mainly operates by two mechanisms: dipolar polarization and ionic conduction [140] (figure 19). For dipolar polarization to occur, the solvent must possess a dipole moment (being part of the molecule negative charged and the other part positively charged), otherwise the molecule will not be able to generate heat when exposed to microwaves, this condition is true for both solvent and sample. Hence, microwave heating efficiency is bound to the dielectric constant of the solvent [140–142].

The main advantage of microwave heating in comparison to conventional heating is the ability to, due to dielectric heating, heat the whole sample simultaneously, significantly reducing extraction time, while presenting increased yields, in cases of conventional heating, which depend on conduction and convection phenomenon, the heating up is

uneven due to bigger amounts of heat and energy loss to the surrounding environment, as can be observed in figure 19 [140–142].

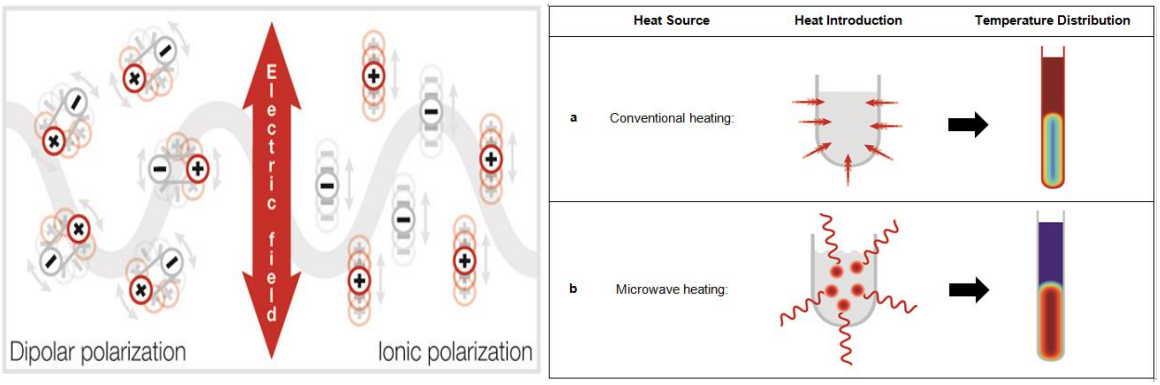

Figure 19- Microwave dielectric heating due to dipolar polarization and ionic polarization (on the left). Comparison of temperature distribution between conventional heating and microwave heating (on the right).
Retrieved from: https://wiki.anton-paar.com/en/microwave-assisted-synthesis/,

The main target of microwave heating is the moisture inside the sample matrix, as every extraction sample contains at least trace amounts of moisture in its matrix. When the temperature inside the cell increases due to microwave heating, this matrix moisture evaporates and generates a massive amount of pressure on the cell walls, which eventually leads to cell wall rupture, allowing the solvent to access the active substances in the cell interior. The microwave-induced temperature increase can also compromise cells walls integrity by dehydration process, which lead to weakened cell walls, enabling an easier solvent access to desired cell interior compounds [142]. The main advantages of using microwave-assisted extraction equipment are even temperature distributions, overall decreased process time, savings in solvent usage, high reproducibility, and software-controlled processing. This type of microwave-assisted extraction (MAE) technique has already been successfully applied for isolation of bioactive compounds from both plant and algae sources [143,144].

The Monowave 450 (Graz, Austria) (single-mode 2.45 GHz microwave reactor) is a microwave synthesis reactor, allowing for a vast sample variety, from environmental analysis (sediments, soil, waste), food testing, pharmaceutical analysis to standard test methods. The equipment has a maximum operation temperature of 300°C and maximum operation pressure of 30 bar. Reaction parameters are continuously controlled by an external infrared sensor for temperature and an integrated pressure sensor, as showed in figure 20. The Monowave 450 also has a built-in camera to monitor in real time the

extraction process, allowing to adjust stirring speed, time, or temperature. The reaction mixture is continuously stirring by a magnetic stirring bar in order to promote homogenous heating [145]. In case of an apolar solvent (such as hexane) is used, a passive heating element made of sintered silicon carbide must be added. The heating element possesses a high microwave absorbance and heat capacity so, it is used to efficiently heat low-absorbing solvents in a microwave environment [146]. The Monowave 450 is fully operated by a touchscreen user interface, where personalized methods can be created and saved, defining maximum temperature, cooling temperature, cycle time, and agitation speed. The adjustable stirring velocity ensures proper mixing and accelerates the extraction [140].

The Monowave 450 can be converted into an Automated Accelerated Solvent Extraction (ASE) by adding a MAS 24 autosampler, allowing unattended sequential operation of up to 24 vials. Each vial was closed with a polytetrafluoroethylene (PTFE)-coated silicon septum and a snap cap. This sealed-vessel technology and the efficient heating enables chemical processes well above the boiling point of the solvent, reducing extraction times [140].

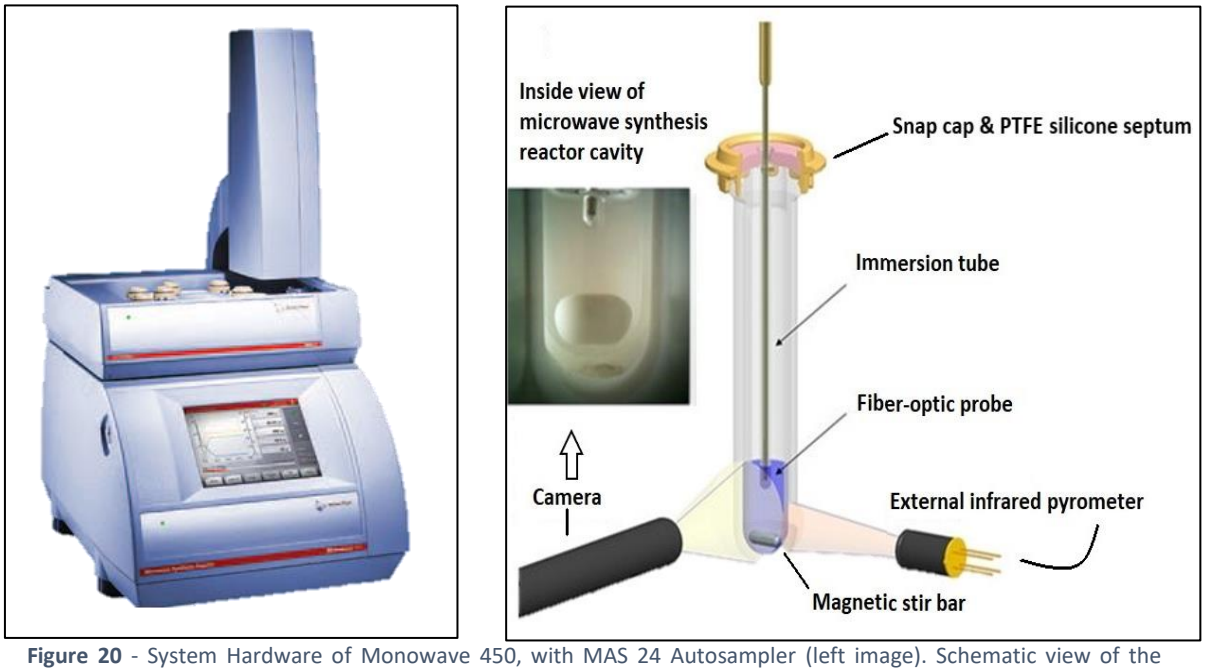

Figure 20 - System Hardware of Monowave 450, with MAS 24 Autosampler (left image). Schematic view of the microwave cavity and image obtained by camera (right image).
Adapted from: Kappe et al.[145]; Anton Paar et al.[145]

Algal biomass was extracted using the Monowave 450 Microwave synthesis reactor with its autosampler MAS24 (Anton Paar, Austria).Spray-dried *T. striata* CTP4 algal biomass

(1 g) was extracted with a total of 10 ml solvent (ethanol or hexane) at different temperatures (30 ºC, 50 ºC and 100 °C, 120 ºC, 160 ºC) for either 30 seconds, 1 or 10 min per cycle. Number of cycles varied from 1 to 2 cycles. As the microwaves are only activated in high temperatures, higher extraction temperatures were chosen and lower cycles duration. The exact extraction conditions of the 11 extracts obtained with Monowave 450 are summarized in Table 2.

For every extraction cycle, cooling temperature was set to 30 ºC, with *heat as fast as possible* mode and agitation of 600 rpm.

Table 2 - Extraction conditions (solvent, temperature, time per cycle, number of cycles) of *T. striata* CTP4 with Monowave 450.

Solvent	Temperature (ºC)	Time per cycle (min)	Number of cycles
Ethanol	**30**	**10**	**1**
	50	**10**	**1**
	100	**10**	**1**
	120	**0.5**	**1**
	120	**1**	**1**
	160	**0.5**	**1**
Hexane	**50**	**10**	**1**
	50	**10**	**2**
	120	**0.5**	**1**
	160	**0.5**	**1**

Into each reaction vial, *T. striata* CTP4 biomass, solvent and a magnetic stirrer were added. When the extraction solvent was hexane, a passive heating element was used. Each vial was closed with a polytetrafluoroethylene (PTFE)-coated silicon septum and snap cap. The extracts were transferred into previously weighted amber vials and dried with a gentle nitrogen flow until total dryness. The dried extracts were resuspended in dimethyl sulfoxide (DMSO) at concentrations of 20 mg/mL (stock solution), 10 mg/mL; 5 mg/mL; 1 mg/mL (3 working solutions) and stored in the freezer at -18 ºC until usage, as can be seen in figure 21.

The yield of extractable substances was expressed as a percentage calculated using equation 1.

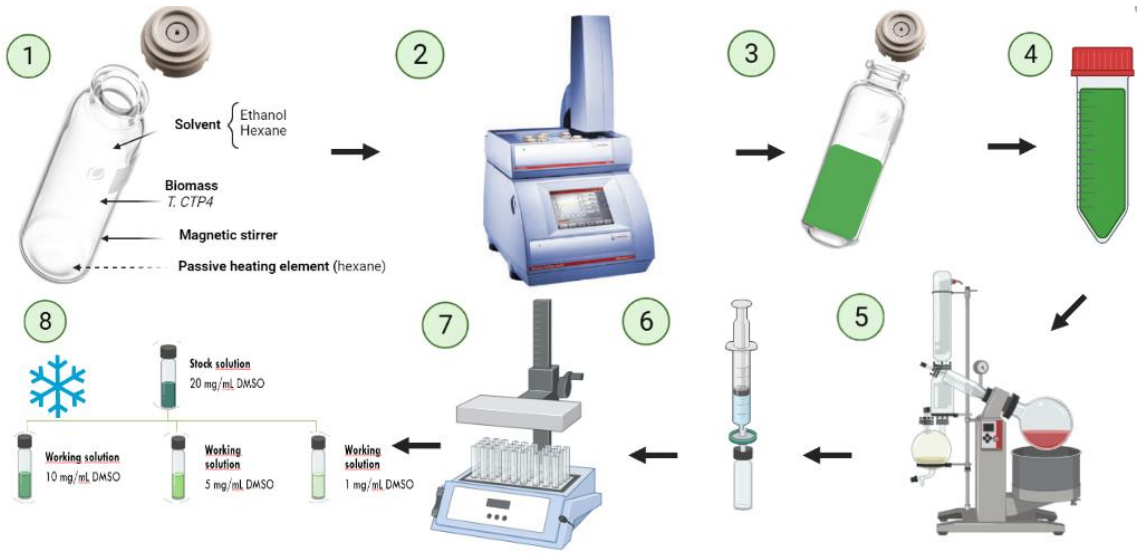

Figure 21 - *Flowchart of extraction process with Monowave 450 equipment.*
1- Addition of solvent and magnetic stirrer to the biomass in the reaction vial (plus passive heating element when solvent is hexane). 2- - Microwave synthesis reactor. 3- Collection of extract from collection vial. 4- Total supernatant collected. 5- Evaporation of solvent. 6- Syringe filtration. 7- Nitrogen drying of extracts. 8- Suspension in dimethyl sulfoxide

3.4. Bioactivity assays

The measurement of the bioactivity of food extracts can be assessed by various techniques. Each of this methods acts through different mechanisms and relates to the generation of different radicals [147].

Upon extraction with solvents of different polarities, several in vitro assays were performed to determine the antioxidant activity of extracts. Extracts obtained with Automated Accelerated Solvent Extraction systems, with ethanol and hexane as solvents, were also analyzed with DPPH and ABTS assays to understand the influence on bioactivity due to different extraction techniques.

3.4.1. Antioxidant activity assays

All experiments were performed in 96-well microplates, in sextuplicate, at room temperature. A Synergy HT MultiDetection Microplate Reader (BioTec Instruments, Inc., USA) was used for all spectrophotometric measurements.

3.4.1.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay

The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay is often used in the determination of antioxidant activity of biological substrates such as fruits or algae, as this is an easy and accurate method [45].

The DPPH radical (DPPH•) is a stable molecule with a hydrogen acceptor capability from antioxidants. DPPH• has a characteristic purple/violet color when dissolved in a methanol solution and, in the presence of antioxidants, the purple coloration fades into shades of yellow, proportionately with the loss of the radical in exchange for a hydrogen atom (figure 22) [148,149].

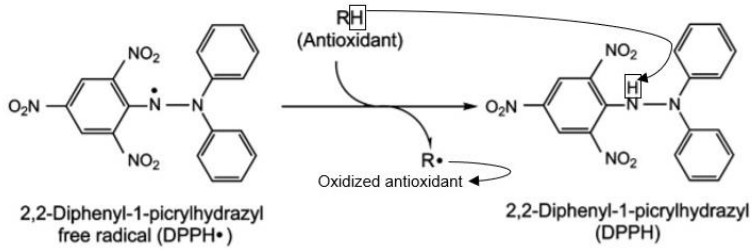

***Figure 22**- Reaction between DPPH• and antioxidant.*
Adapted from: Moon et al. [45]

The hydrogen atom donating ability of algae extracts was determined by the decolorization of methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) in 96 well plates by the method of Blois [150] with some modifications:

A solution of 0.1 mM DPPH in methanol was prepared. Per well, 200 µl of DPPH solution and 22 µl of sample (at concentrations of 1, 5 and 10 mg/mL) were added.

In case of the extracts obtained by Advanced Automated Accelerated Solvent Extraction systems, the sample concentration used was 1mg/mL DMSO.

Butylated hydroxytoluene (BHT, 1 mg/mL) was used as positive control in both situations. As color control 200 µl of methanol was added with 22 µl of sample. The reaction mixture was left in the dark at room temperature for 30 min. Discoloration was measured at 517 nm in sextuplicate and percentage DPPH radical scavenging activity (A_{DPPH}, %) was calculated by equation 2 [151]:

$$A_{DPPH} = \left(\frac{A_0 - A_1}{A_0}\right) \times 100\% \qquad (2)$$

where A_0 is the absorbance of the negative control, and A_1 is the absorbance of the extract corrected for the absorbance of the color control.

When radical scavenging activity was higher than 50% at a concentration of 5mg/mL DMSO), six serial dilutions were performed, and the IC_{50} was calculated. IC_{50} is the concentration of extract necessary to decrease the initial DPPH• concentration by 50%.

3.4.1.2. 2,2′-Azinobis (3-ethylbenzothiazoline-6 sulfonic acid) (ABTS) Assay

The 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonate (ABTS•) assay, also called the ABTS scavenging radical assay, is a widely used spectrophotometric method for the assessment of the antioxidant activity of different phenolic compounds in food. The ABTS assay is able to determine radical scavenging ability of antioxidants in both aqueous and lipid phases, even when present in highly complex biological matrix, such as plant or algae extracts [45,152].

The ABTS radical scavenging assay is an electron transfer-based assay, with the formation of the blue/green ABTS radical (ABTS•) which is reduced by an antioxidant into colorless ABTS, with a maximum absorbance at 405 nm (figure 23) [153,154].

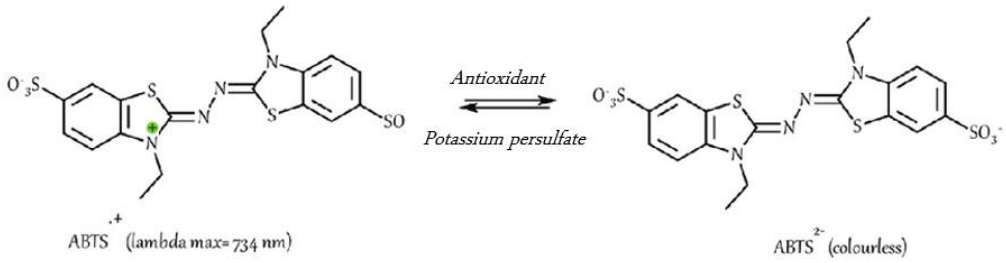

Figure 23 *- Reaction of ABTS • with antioxidant compounds.*
Adapted from: Santos-Sánchez et al.[47]

The ABTS• assay was performed by the method of Re et al.[155], where a 7 mM aqueous ABTS solution with a 2.45 mM potassium persulfate ($K_2S_2O_8$) solution was prepared. After storage in the dark, at 4°C, the radical cation solution was further diluted in water until the initial absorbance value of 0.7 at 734 nm was reached. The assay was performed in the microtiter plate with 190 µl of ABTS solution and 10 µl sample (at concentrations of 1, 5 and 10 mg/mL). In case of the extracts obtained by Advanced Automated Accelerated Solvent Extraction systems, the sample concentration used was 1mg/mL DMSO. Butylated hydroxytoluene (BHT, 1 mg/mL) was used as positive control in both cases. To calculate the percentage of ABTS radical scavenging activity ($A_{ABTS,}$ %) was calculated according to equation 3 [156]:

$$A_{ABTS} = \left(\frac{A_0 - A_1}{A_0}\right) \times 100\% \quad (3)$$

where A_0 is the absorbance of the negative control, and A_1 is the absorbance of the extract corrected for the absorbance of the color control.

When radical scavenging activity was higher than 50% at a concentration of 5mg/mL DMSO), six serial dilutions were performed, and the IC_{50} was calculated. IC_{50} is the concentration of extract necessary to decrease the initial ABTS• concentration by 50%.

3.4.1.3. Iron (II) chelating activity assay

The iron chelating activity is a common assay used to determine the secondary antioxidant activity of flavonoids, as metal ion-chelating capacity plays a significant role in antioxidant mechanism. Iron in biological matrix is known to generate free radicals through the Fenton and Haber-Weiss reactions, which are often implicated in diseases. In the presence of iron chelating agents and antioxidants, such as ferrozine, a stable iron (II) chelate molecule is formed, reducing free ferrous ion concentration, leading to the inhibition of OH• generation, and reduces the concentration of the catalyzing transition metal (ferrous ion) in lipid oxidation. So, compounds that exhibit a higher metal chelating activity can also, indirectly, be considered antioxidant compounds [157–161].

Ferrozine is commonly used for the determination of iron in biological samples, by forming red-colored complexes with ferrous iron, which strongly absorbs at 550 nm. In the presence of antioxidants and/or chelating agents, there is disruption of the Fe (II)–ferrozine complex, which leads to a decrease of the red color (figure 24). Measurement of color reduction allows an estimation of the chelating activity of the coexisting chelator [162,163].

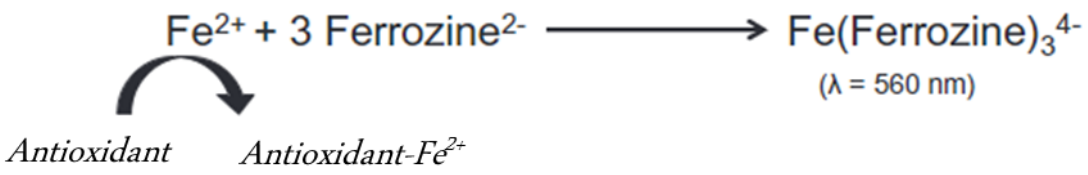

Figure 24 - Chemical reactions involved in iron(II) chelating ability assay.
Adapted from: Canabady-Rochelle et al.[161]

Iron (II) binding ability of the chelators was determined in microtiter plates according to the method of Dinis et al.[164], except that ferrous sulphate was substituted by ferrous chloride [165]. Briefly, into each well were added 200 µl of distilled water, 30 µl of extract working concentrations (at concentrations of 1, 5 and 10 mg/mL)) and 30 µl of ferrous chloride (0.8 mM) and incubated for 30 minutes at room temperature. Then, 12.5 µl ferrozine (40mM) per well was added to start the reaction. The resulting mixture was incubated for 10 min at room temperature. The absorbance of the solution was measured at 562 nm. The synthetic metal chelator EDTA was used as positive control (1 mg/mL).

The percentage of iron chelating activity ($A_{\text{Iron Chelating}}$) was calculated by equation 4 [166]:

$$A_{\text{Iron Chelating}} = \left(\frac{A_0 - A_1}{A_0}\right) \times 100\% \quad (4)$$

where A_0 is the absorbance of the negative control, and A_1 is the absorbance of the extract corrected for the absorbance of the color control.

When radical scavenging activity was higher than 50% at a concentration of 5mg/mL DMSO), six serial dilutions were performed, and the IC_{50} was calculated. TheIC_{50} concentration is the concentration that chelates 50% of the free metal ion.

3.4.1.4. Copper chelating activity assay

The copper chelating activity is considered a secondary mechanism to estimate the antioxidant activity of an extract, as metal ion-chelating capacity plays a significant role in antioxidant mechanism. Unbound copper ions participate in the formation of reactive oxygen species (ROS) due to the catalysis of the Fenton-like reaction which consequently causes damage to various biological structures and are involved in some non-communicable diseases [167,168].

Cu^{2+}-chelating activity was determined using pyrocatechol violet (PV) method used by Saiga et al.[169], where the complex of PV with $CuSO_4$ is blue, and the color turns into yellow when PV dissociates a Cu ion in the presence of chelating agents (figure 25), so the copper chelating activity can be estimated by the measurement of the rate of color reduction [167].

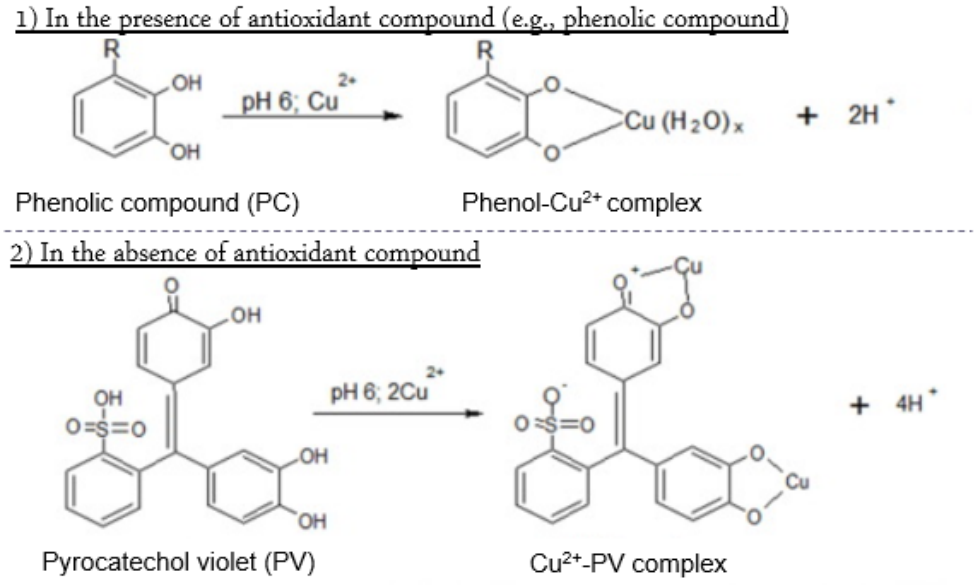

Figure 25 – *Copper complex formation in presence and absence of antioxidant compounds (Antioxidant- Cu^{2+} complex or Cu^{2}-PV complex, respectively).*
Adapted from: Santos et al.[167]

Briefly, samples (30 µL, at concentrations of 1, 5 and 10 mg/mL) were mixed in 96-well microplates with 200 µL of Na acetate buffer (50 mM, pH 6), 6 µL PV (4 mM) in above buffer and 100 µl $CuSo_4$. The synthetic metal chelator EDTA was used as positive control (1 mg/mL). The change in color of the solution was measured at 632 nm using a microplate reader.

The percent inhibition (%) of the formation of the PV–Cu^{2+} complex ($A_{CopperC\ Chelating}$, %) was calculated by equation 5 [167]:

$$A_{\text{Copper Chelating}} = \left(\frac{A_0 - A_1}{A_0}\right) \times 100\% \qquad (5)$$

where A_0 is the absorbance of the negative control, and A_1 is the absorbance of the extract corrected for the absorbance of the color control.

When radical scavenging activity was higher than 50% at a concentration of 5mg/mL DMSO), six serial dilutions were performed, and the IC_{50} was calculated. The IC_{50} concentration is the concentration that chelates 50% of the free metal ion.

3.4.1.5. Ferrous reducing antioxidant capacity assay

Ferrous reducing antioxidant capacity assay is a ferricyanide-based reduction–oxidation (redox) assay which is a simple, quick, and inexpensive assay to evaluate the antioxidant

properties of biological reducing agents. The ability to reduce Fe^{3+} to Fe^{2+} indicates the capacity of the samples to act as antioxidants by donating electrons [170].

Reducing power assay is a two-step reaction method (figure 26): the first step is based on the reduction of Fe^{3+} from ferric chloride ($FeCl_3 \cdot 6H_2O$) into Fe^{2+}. The second step is the reaction of the ferrous (Fe^{2+}) ion with potassium ferricyanide ($K_3[Fe(CN)_6]$), which generates the potassium ferric/ferrocyanide complex ($KFe^{3+}[Fe^{2+}(CN)_6]$) which has a strong blue color and an absorption maximum at 700 nm. Increased absorbance values indicates increased reducing power and the presence of antioxidants [171,172].

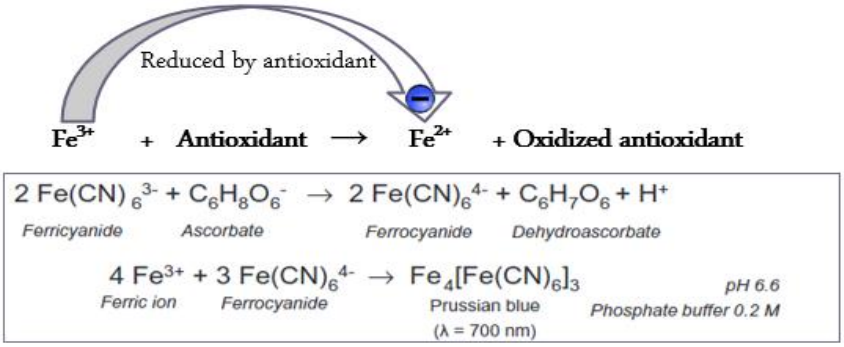

***Figure 26** - Chemical reactions involved in the reducing power assay and the role of antioxidant compounds.* Adapted from: Canabady-Rochelle et al.[161]

Ferrous reducing antioxidant capacity assay was performed in microtiter plates by the method of Oyaizu et al.[173] modified by Megías et al.[171]. Briefly, samples (50 µL, at concentrations of 1, 5 and 10 mg/mL), 50 µL 0.2 M phosphate buffer (pH 6.6) and 50 µL 1% potassium ferricyanide were added in 96-well microplate and incubated at 50 °C for 20 min. Plates were incubated for another 10 min at 50°C after addition of 50 µL 10% trichloroacetic acid and 10 µL 0.1% ferric chloride. Butylated hydroxytoluene (BHT, 1 mg/mL) was used as positive control. Absorbance was read at 700 nm.

The reducing capacity was expressed in percentage (R_{Fe}, %) and calculated by equation 6 [161]:

$$R_{Fe} = 100 - \left(\frac{A_0 - A_1}{A_0}\right) \times 100\% \quad (6)$$

where A_0 is the absorbance of the positive control, and A_1 is the absorbance of the extract corrected for the absorbance of the color control.

When radical scavenging activity was higher than 50% at a concentration of 5 mg/mL DMSO), six serial dilutions were performed, and the IC_{50} was calculated.

3.4.2. Anti-inflammatory assays

3.4.2.1. Angiotensin-I converting enzyme (ACE) Inhibitory Activity

Angiotensin converting enzyme (ACE, also known as peptidyl-dipeptidase A or kininase II) (kininase II; EC 3.4.15.1) belongs to the class of zinc metal proteases and acts as a key regulator in the Renin-angiotensin-aldosterone system that is essential for cardiovascular hemodynamics and has an important role in the development of cardiovascular diseases. Circulating ACE is mainly originated from endothelial cells and is present in biological fluids while ACE peptides can be found in diverse tissue types, such as brain, lung, kidneys, or heart [174].

The main role from ACE is to catalyze the Conversion of inactive angiotensin I into angiotensin II (Ang II), which plays a very important part in blood pressure control, as it is a powerful vasoconstrictor and promoter of sodium retention. An excessive ACE catalyzation can lead to hypertension and other cardiovascular diseases. Additionally, Ang II, *via* AT1 receptor, seems to play a key role in the cardiac inflammatory process, acting as an inflammatory mediator through several mechanisms (e.g., induce the production of IL-6, IL-1β, and TNF α). The determination of angiotensin-converting enzyme (ACE) activity represents a useful tool in the study of different health pathologies, such as hypertension and vascular inflammatory related diseases [174,175].

Therefore, in the case of hypertension, it can be benefic to suppress the renin-angiotensin-aldosterone system, since the inhibition of its activity leads to a decrease in the production of angiotensin II, and consequently prevents constriction of blood vessels, lowers blood pressure, and downregulates the production of inflammatory cytokines, as shown in figure 27. The inhibition of ACE can be achieved by the administration of angiotensin converting enzyme inhibitors (ACEI), ACE inhibitors administrated as hypertension treatment are usually of synthetic origin, such as captopril, enalapril, lisinopril and temocapril. ACE inhibitors also can be found in natural sources, such as specific plants and phytochemicals [176–181].

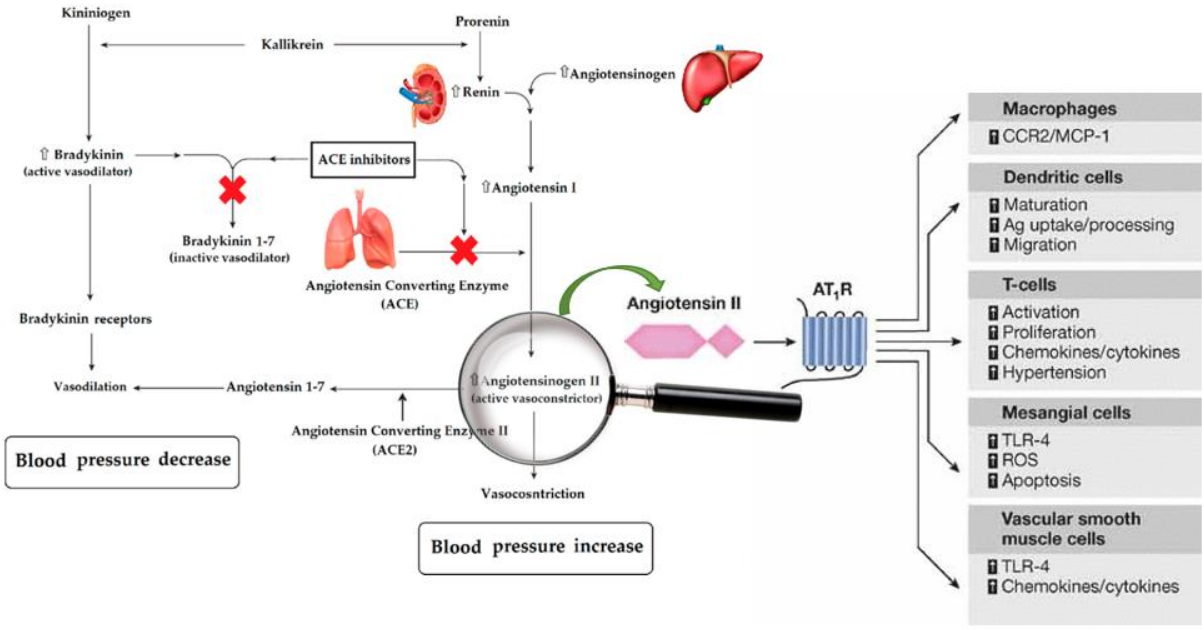

Figure 27 *- Schematics with general effects of Angiotensin-Converting Enzyme (ACE)Inhibitors and antioxidant and anti-inflammatory effects.*
Adapted from: Lizárraga-Velázquez et al.[182] and Benigni et al.[183]

ACE inhibition assay was carried out using an assay kit for ACE activity according to the method of Sentandreu et al.[184] with some modifications. This method relies on the ability of ACE to hydrolyze the fluorescent substrate, where the generation of the fluorescent product can be spectrophotometrically continuously monitored.

Prior to assay reaction, black microtiter plate and all reagents were brought to 37 ºC for 5 minutes. To each microtiter-plate well, 20 µL of 30 mU/mL of ACE (dissolved in glycerol (50%) prepared in assay buffer), 20 µL of Assay Buffer and 10 µL of each extract (diluted in Assay Buffer, in concentrations of 1, 0.5 and 0.1 mg/mL) were added. The microtiter plate was incubated at 37 º C for 10 min, separately the fluorescent substrate was incubated in the same conditions. The enzyme reaction was initiated by the addition 50 µL of fluorescent substrate. The plate was immediately mixed and incubated at 37 ºC. The generated fluorescence was monitored in kinetic mode (measurements every 5 min, during a total of 40 min) by a multiscan microplate fluorimeter (Biotek Synergy Neo2, BioTek Instruments, USA). Excitation and emission wavelengths were 350 and 420 nm, respectively.

All samples were done in sextuplicate plus duplicated of color control of each sample (reaction without addition of ACE enzyme). In total, 4 extracts were analyzed (*T. lutea-*

ethanol; *T. chui*-ethanol; *T.* CTP4-ethanol; *T.* CTP4-hexane) in 3 concentrations (1, 0.5 and 0.1 mg/mL). As positive control, enalapril was used in 5 different concentrations – 2.0 mg/L; 1.0 mg/mL; 0.5 mg/mL; 0.1 mg/mL and 0.01 mg/mL – diluted in Assay Buffer. Positive control was done in quadruplicate. As blank and negative control (sextuplicate), DMSO (1%) was used instead of sample.

The results from the microplate fluorimeter were obtained as relative fluorescence units (RFU). To convert into n mol, a standard curve was constructed (standard from kit and same procedure as described previously) and linear regression determined. The standard curve regression slope and the sample kinetic curve linear regression slope were used to transform the values of the samples from RFU/min into nmol/min.

To calculate the percentage of ACE inhibition (ACE, %) the equation 7 was used [185]:

$$\mathrm{ACE} = 100\% - \left(\frac{Act_{sample}}{Act_b} \times 100\%\right) \quad (7)$$

where Act_b is given by the slope of the blank and Act_{sample} is given by the slope of sample.

3.4.2.2. Cyclooxygenase (COX) Inhibiting Activity

Arachidonic acid (AA) is a fatty acid which is bound in the membrane phospholipids in an inactivated state. Whenever the AA is activated due to phospholipase, it becomes available as a metabolic precursor for 3 major inflammatory pathways: cytochrome P-450 monooxygenase, lipoxygenase, and cyclooxygenase pathway. Cyclooxygenase (COX, Prostaglandin H Synthase or PGHS) is a rate-limiting key enzyme involved in conversion of arachidonic acid (AA) into prostaglandins (PGs) in the cyclooxygenase pathway, which has a double function – both COX and peroxidase activity [186–188]. PGs are very important lipid mediators of various physiological and pathophysiological processes in the kidney, such as water and salt balance and renal hemodynamics. Whereas the PG's synthesis is stimulated in cases of distinct pathophysiological situations, such as inflammation, pain, and other diseases. COX is responsible for the first two steps in the synthesis of PGs. First, the COX component is responsible for the conversion of arachidonic acid into hydroperoxyl endoperoxide (PGG_2) and then, secondly, the peroxidase component turns PGG_2 into the corresponding alcohol (PGH2), the main precursor in the formation of bioactive prostaglandins—PGE_2, PGI_2, PGD_2, $PGF2_\alpha$—and thromboxane A2, which play important roles in different biological responses (figure 28) [188–190].

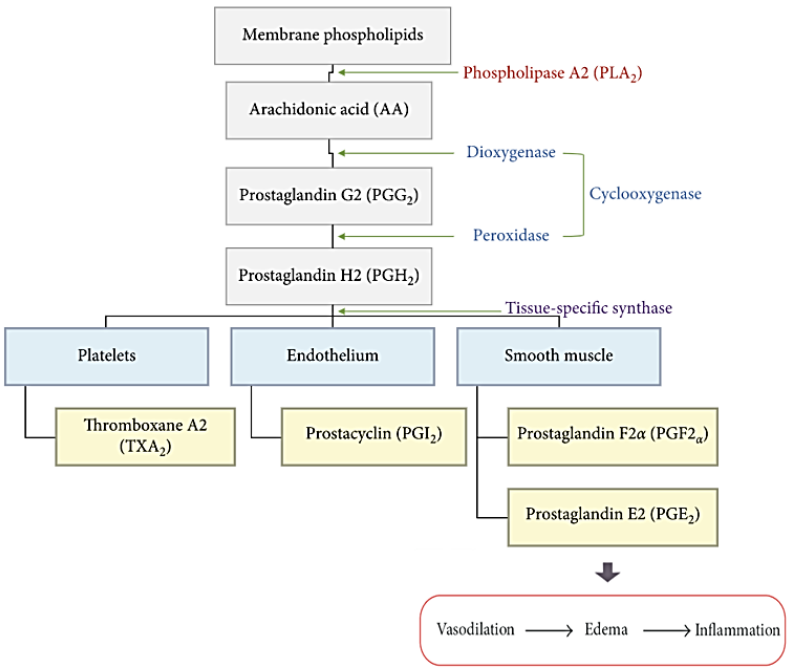

Figure 28- Mechanism of action of cyclooxygenase pathway.
Adapted from: Wong et al.[71]

The COX enzyme exists in two major isoforms: COX-1 and COX-2. COX-1 is present in various cells and tissues, being involved in normal cellular homeostasis and is associated with initiation of inflammatory acute phase. On the other hand, COX2 is an inducible enzyme is overexpressed in several pathophysiological events such as, carcinogenesis and inflammatory diseases and is responsible for maintaining the inflammatory response. To inhibit the activity of COX enzymes (specially COX-2), traditionally Non-Steroidal Anti-Inflammatory Drugs (NSAID) are used, such as Ibuprofen, Naproxen, and Ketoprofen. The NSAIDs mainly exhibit there anti-inflammatory, anti-pyretic, and analgesic activity due to the inhibition of the cyclooxygenase enzyme, responsible for the conversation of arachidonic acid to prostaglandins [186–190].

The COX inhibition assay was carried out using a COX Inhibitor Screening Assay Kit according to manufacturer's instruction.

This kit measures the changes of PGE_2 levels by $SnCl_2$ reduction of COX-derived PGH_2 produced in the COX reaction. This is a common method used because the direct COX products PGG_2 and PGH_2 are unstable. The prostanoid product is quantified via an enzyme immunoassay (ELISA) using a broadly specific antiserum that binds to all the major PG compounds, as described in figure 29. The enzymes used in the assay were ovine COX-1 and human recombinant COX-2.

The AChE competitive ELISA methodology is based on the competition between PGs and a PG-acetylcholinesterase (AChE) conjugate (from now on referred to as, PG tracer) for a limited amount of PG antiserum. As the concentration of PG tracer is held constant while the concentration of PG varies, the amount of PG tracer able to bind to the PG antiserum will be inversely proportional to the concentration of PG in the well. The rabbit antiserum PG (either free or tracer) complex binds to a mouse monoclonal anti rabbit antibody that has previously been attached to the well wall (pre-coated 96-well plate). The plate is washed to remove any unbound reagent and then the Ellman's Reagent (which contains a substrate of AChE) is added to the well. Quantification of the tracer is achieved by measuring its AChE activity with Ellman's Reagent, this reagent consists of acetylthiocholine and 5,5'-dithio-bis-(2-Nitrobenzoic Acid). The hydrolysis of acetylcholine by AChE producers thiocholine, and the non-enzymatic reaction of thiocholine with 5,5'-dithio-b*is*-(2- nitrobenzoic acid) produces 5-thio-2-nitrobenzoic

acid. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The intensity of color, determined spectrophotometrically, is proportional to the amount of PG tracer bound to the well, which is inversely proportional to the amount of free PG present in the well during the incubation. So, the higher is the PG amount in a sample, less PG tracer will be able to bind to the antibody attached to the well, and less yellow color will be seeable.

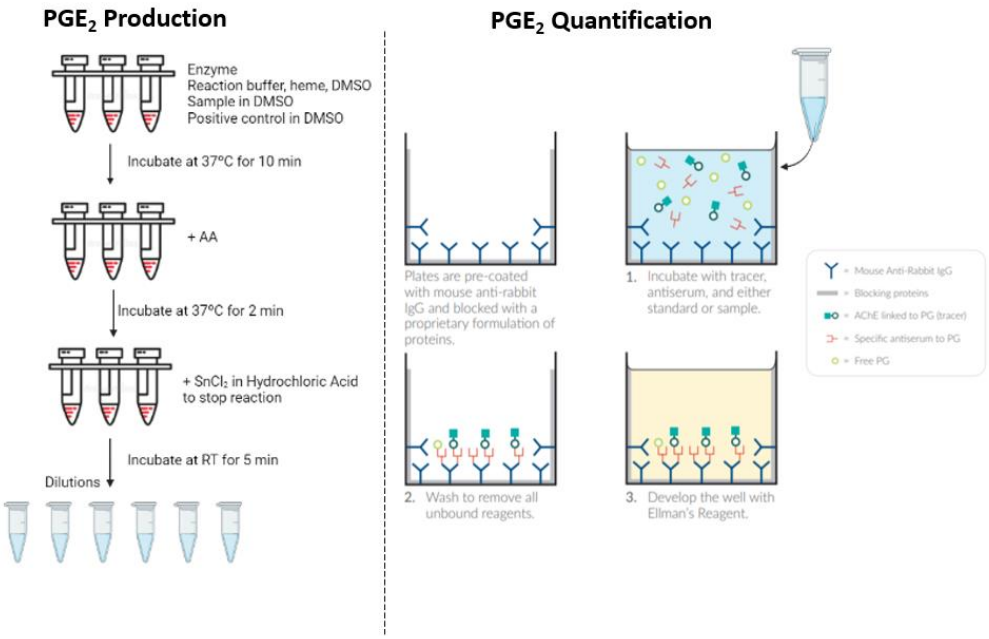

Figure 29 - Two-step determination of COX activity by measuring PGE_2 production. Adapted from: Cuendet et al.[191]

Briefly, the reaction buffer (0.1M Tris–HCl buffer, pH 8.0, containing 5 mM EDTA and 2 mM phenol) and heme (0.065 mM) were placed in reaction tubes. The initial reactions take place in reaction tubes pre-equilibrated to 37°C.

Three types of reaction tubes were made: background tubes (BCK); 100% Initial Activity tubes (100% IA) and Sample Inhibition tubes (SI). In the BCK tubes, 10 µL inactivated enzyme (enzyme put in boiling water for 3 minutes), 160 µL reaction buffer and 10 µL heme are mixed. In the 100% IA tubes, 160 µL reaction buffer, 10 µL heme, 10 µL of the enzyme in question and 10 µL DMSO are added. In the SI tubes, 160 µL reaction buffer, 10 µL heme, 10 µL inhibitor at two concentrations (1mg/mL and 0.1 mg/mL DMSO), 10 µL of DMSO and 10 µL enzyme are added. The tubes were incubated for ten minutes at 37°C. Then 10 µL of the arachidonic acid and potassium hydroxide mixture with a final

concentration of 200 µM was added to each tube, and the tubes were incubated for two minutes. To stop the reaction and to the trap the reaction product and reduce it to a more stable form, 30 µL of a HCl and stannous chloride solution (final concentration of 0.13M) was used. The tubes were incubated a final time for five minutes at room temperature.

Each BCK, 100% IA and SI reaction tube was diluted with ELISA Buffer, obtaining a 1:100 dilution of the original sample (BC tubes). BCK tubes were not anymore diluted, while both 100% IA and SI tubes were still diluted twice, obtaining 1:2.000 and 1:4000 dilutions of the original sample (IA2, IA3 and SI2 SI3, respectively).

The concentration of prostaglandin $PGF2_{\alpha}$ (final product) was measured using the enzyme immunoassay (ELISA) of the same kit and was calculated from a standard curve after the reaction liquid had been diluted to 1/4000.

In the provided ninety-six well plate coated with mouse anti-rabbit IgG, the plate setup included wells for blanks, total activity, nonspecific binding, maximum binding, background COX (1:2000 and 1:4000 dilutions), 100% Initial Activity (1:2000 and 1:4000 dilutions), standards, and the inhibitor dilutions (1:2000 and 1:4000). Different amounts of sample, ELISA buffer, tracer and antiserum were added to each well, except total activity well. The plate was covered with plastic film and incubated in the dark at room temperature with 400 rpm for eighteen hours on an orbital shaker (Eppendorf Thermomixer comfort). When ready to develop the plate, the plate was washed five times with wash buffer, developed with Ellman's Reagent, 5µL of tracer was added to the total activity well, covered with plastic film and read on a microplate reader at 410 nm and 420 nm, when maximum binding wells had absorbance 0.3-0.8 A.U. The microplate reader (Biotek Synergy Neo2, BioTek Instruments, USA) was set with orbital shaking for 40 minutes with kinetic reads every 5 minutes.

In total, 4 extracts (*T. lutea*-ethanol; *T. chui*-ethanol; *T. striata* CTP4-hexane and *T. striata* CTP4-ethanol*)* were tested in 2 concentrations (1 mg/mL and 0.1mg/mL in DMSO), all samples were done in duplicates.

The positive control used was Ibuprofen (≥98% (GC)) from Sigma–Aldrich (Munich, Germany) with different concentrations (5-100 µg/mL).

To calculate the percent inhibition of the samples, the mean values of nonspecific binding, maximum binding were subtracted from the absorbance values of samples. Calculate the % Sample or Standard Bound/Maximum Bound). The COX sample values were multiplied by the dilution factors (BC=100; IA2 = 2.000 and IA3=4000). Subtraction of the BC values from the IA2, IA3 and SI2, SI3 values. At last, subtraction of each inhibiters sample from the 100% initial activity sample, then divide by the 100% initial activity sample and multiplied by 100 to give the percent inhibition.

3.5. Phytochemical screening analysis by GC-MS

3.5.1. Gas Chromatography-Mass Spectrometry (GC-MS)

The identification of the components in each extract (*T. lutea*-ethanol and *T. chui*-ethanol) was determined by Gas Chromatography-Mass Spectrometry (GC-MS).

GC-MS encompasses two procedures: Gas Chromatography (GC) and Mass Spectrometry (MS). While GC separates the components of a mixture (based mainly on volatility, molecule size and polarity), MS characterizes each of the components individually by using an electron beam to convert the individual compounds into ions, these ions are then led through electric and magnetic fields to measure their masses. As each atom breaks into a set of characteristic ions the comparison of the MS profile of the compounds present in the extract can be compared with those in reference libraries to identify the compound. Gas chromatography (GC) is a key technique that is widely available for the screening, identification, and quantification of food components. GC-MS is the very well accepted methodology in food science, as nonpolar, volatile, and small molecular weight (such as fatty acids, amino acids, and organic acids) are most suitable for analysis by GC [192,193].

Volatility is a key factor in the effectiveness of the separation of chemical species, a strategy to increase volatility is the chemical derivatization procedure, which converts less volatile and thermally labile substances into compounds that can be analyzed in the gaseous state. A derivatization procedure is performed to extend the molecular coverage of GC-MS, in order to include smaller polar species that would be difficult to retain and separate by other methods [194,195].

3.5.2. Phytochemical screening procedure

3.5.2.1. Sample preparation

The two extracts that, based on antioxidant and anti-inflammatory assays, presented higher overall bioactivity were *T. lutea* in ethanol and *T. chui* in ethanol. Each of these extracts were prepared by the milling technique described previously and dissolved with DMSO to a concentration of 1 mg/mL. From this solution, 100 µl were transferred into a 2ml Snap Vials with integrated 0.2 ml Glass Micro-insert and then dried completely by a gentle nitrogen flow. The samples were prepared in duplicates.

3.5.2.2. Derivatization

To initiate the derivatization procedure, an intermediate stock solution (Solution A) was prepared as follows: 0.25 ml of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was mixed with 10 mg of NH_4I and 15 µl of β-mercaptoethanol. The final derivatization agent (Solution B) was prepared by mixing Solution A with pure MSTFA in a 1:10 ratio.

To the dry residue of microalgae extract, 50 µl of MSTFA/βmercaptoethanol/NH_4I reagent was added. The capped GC vials were heated in a dry bath at 90 °C for 1 hour.

3.5.2.3. GC-MS analysis

After the cooling down to room temperature of the derivatized sample, 1 µL of this sample was analyzed by GC-MS (Agilent 6890 Network GC System, 5973 Inert Mass Selective Detector). The GC conditions were as follows: temperature was ramped at 10°C/min from RT until 300 °C, where the temperature was held for 10 min. The splitless injection temperature was 300 °C and the helium flow was held constant at 1 ml/min. The injection volume was 1 µl. The mass-selective detector conditions were set at 280°C for the transfer line and 280°C for the source. The electron-impact mode with an electron voltage of 70 eV was used. The obtained spectra were measured in the full scan mode. The identification of components of the MS spectra was performed using the National Institute of Standards and Technology (NIST) library as reference.

As blank, DMSO was used.

3.6. Fiber and Moisture content and Mineral Profiling of *Tisochrysis lutea*

3.6.1. Mineral Profiling of *Tisochrysis lutea*

3.6.1.1. Instrumentation

For this analysis a microwave plasma atomic emission spectrometer, the Agilent 4200 MP-AES (Agilent Technologies, Santa Clara, CA) was used for the elemental determination of digested *T. lutea* biomass samples. An Agilent SPS 3 autosampler was used to deliver samples to the instrument. MP-AES quantifies the concentration of an element in a sample by comparing its emission to that of known concentrations of the element, plotted on a calibration curve.

Acid digestion was carried out using a Discover SP-D CEM Microwave Digester (CEM Corporation, Matthews, NC, USA). About 100 mg of *T. lutea* biomass was transferred into the microwave digestion flask with five mL of analytical grade concentrated nitric acid (HNO_3 65%) and a magnetic stirring bar. The samples were processed by microwave assisted digestion: 3 min ramp temperature to 198 °C held for five min. After the chemical digestion, the digested solution was diluted with Milli-Q water until 15 ml and was stored in the refrigerator until analysis by Microwave Plasma-Atomic Emission Spectrometer (MP-AES). Samples were analyzed in triplicate. As blank, pure nitric acid (65%) was used.

To make the standard curves, a multi element calibration standard containing Ca, Mg, Fe, K and Na (Agilent Technologies, Santa Clara, CA) was used to prepare working standards with concentrations 0.25 ppm – 20 ppm. The remaining standard curves were obtained through multi-element calibration standards, whose working standards had concentrations from 50 ppm – 2500 ppm. All calibration standards were matrix-matched with a 5% nitric acid.

Working standards of different concentrations were prepared from certified standard solutions. For analytical quality assurance results were corrected by subtracting a blank from the analyzed ion concentrations and samples were analyzed in triplicate. Method conditions for digested biomass sample analysis in the 4200 series MP-AES are listed in Table 3. Complete wavelengths and analyzed elements are in table 4.

Table 3 - MP-AES 4200 operational conditions for elemental determination in digested biomass.

Parameter	**Value**
Replicates	6
Pump rate (rpm)	15
Sample uptake time (s)	60 (with fast pump)
Rinse system (s)	65
Stabilization time (s)	20
Autosampler	Agilent SPS 3

Table 4 - Complete wavelengths and elements analyzes in MP-AES

Element	**Wavelength (nm)**		**Element**	**Wavelength (nm)**		**Element**	**Wavelength (nm)**	
Se	196.026		Pb	405,781		Al	396.152	
Zn	213.857		P	213.618		Ca	393.366	
Cd	228.802		Mo	379.825		Fe	371.993	
V	309.311		Hg	253.652		K	766.491	
Ag	328.068		Pb	405.781		Mg	383.829	
Cu	324.754		Mn	403.076		Na	588.995	
As	193.695		Cr	425.433		Li	610.365	
Sb	231.147		Sr	407.771		Co	340.512	

The Method Detection Limits (MDL) were calculated as three times the standard deviation of four consecutive blank readings divided by the slope. From the MDL, the method quantification limit (MQL) was calculated as ten times the standard deviation of four consecutive blank readings divided by the slope.

3.6.2. Proximate composition analysis of *T. lutea*

3.6.2.1. Fiber and Moisture content

Total dietary fiber (TDF) was determined according to McCleary et al.[196] by an enzymatic-gravimetric method, as described in the kit manual provided by Megazyme (Megazyme International Ireland Limited, Bray, Ireland), based on the AACC method 32–05.01 and AOAC method 985.29, and expressed as g 100 g^{-1} ww.

Total nitrogen, hydrogen and carbon content of dried biomass samples were determined using an elemental analyzer (Elementar Vario EL III, Hanau, Germany). Total protein content was estimated as N x 4.78, where N is the nitrogen content determined through the elemental analysis [197].

Moisture content in *T. lutea* biomass was determined according to the 967.03 AOAC official method – "Total Dry Matter by Oven Drying at 100 C for 24 hr" [198]. Algal biomass (100 mg) was weighed into ceramic crucibles and dried overnight in a drying oven (105 °C). The next day, the crucibles were cooled to room temperature in a desiccant chamber and weighed to determine moisture content. Moisture content (M, %) was calculated by equation 8.

$$M = \frac{(W_1 - W_2)}{W_1} \times 100\% \quad (8)$$

where W_1 is the sample weight (mg) before drying and W_2 is the sample weight (mg) after drying.

3.7. Experimental design

In figure 30 the whole methodology is summarized into a diagram.

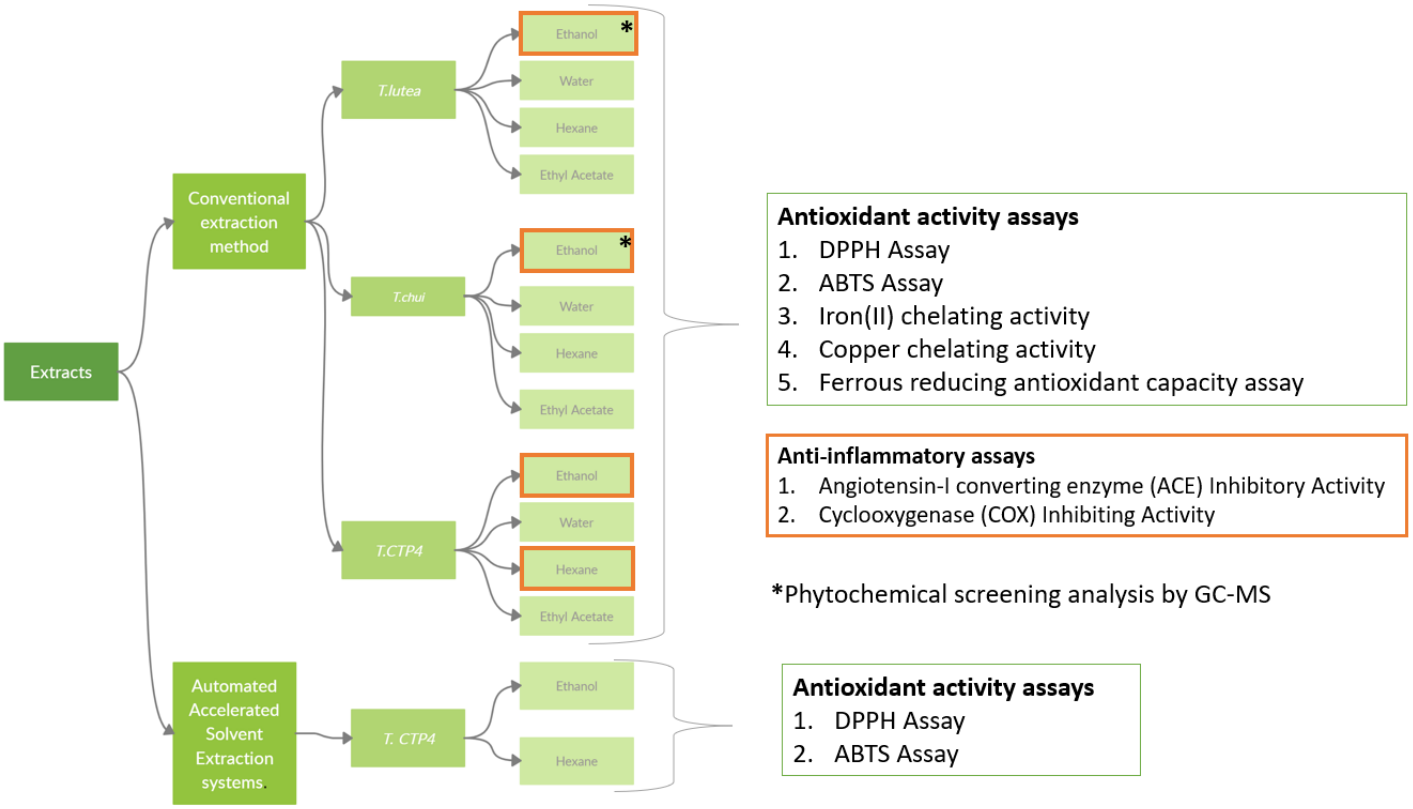

Figure 30 - Summarized methodology diagram

3.8. Data analysis

All experiments were performed in sextuplicate (n=6), if not otherwise stated and results were expressed as mean ± SD.

One way analysis of variance (ANOVA) was carried out using SPSS version 27 (IBM, USA) to determine whether significant differences exist among treatments and Tukey test was used to separate significant differences between the means at the 5% level.

IC_{50} values were determined using GraphPad Prism 9.1.2. for Windows (GraphPad Software, San Diego, CA, USA).

For all statistical tests, a significance level of 0.05 was adopted.

4. Results and Discussion

4.1. Bioactivity of microalgal extracts

Bioactivities were assessed on extracts prepared from dried biomass using four solvents of increasing polarities: hexane (PI: 0.1), ethyl acetate (PI: 4.4), ethanol (PI: 5.2) and water (PI: 10.2) in a *bead beater* with glass beads. The *bead beater* mixes the extraction components (biomass/solvent/glass beads) and facilitates the disruption of biological cells, allowing a more efficient extraction. In a *bead beater*, cell disruption occurs mainly due to two factors: the shear forces of the rotary movement of microalgae cells with glass beads (where cells also collide strongly with the beads and cell disruption also occurs) and the grinding effect of beads on cells. Milling is an attractive pre-treatment for extraction, as it can effectively break the considerable rigidity of the algal cell walls. Milling has already proven to be effective in the major disruption of microalgae cells (such as *Tisochrysis lutea*, *Tetraselmis striata* CTP4, *Chlorella vulgaris*, *Neochloris oleoabundans* and *Tetraselmis suecica)* and improve extraction yields. Due to the scalability of milling, this process could become easily industrially applicable [130,199].

Extraction made with organics solvents is a very well-known technique that allows a very efficient extraction of microalgae biomolecules, being specially effective after a cell disrupting technique was applied (for example milling), because of the easier access of the solvent into the inner cell compounds [200,201]. Different extracting solvents were used to fractionate the microalgal compounds into different extracts as solvent polarity considerably affects extract composition. Organic solvent extractions initiate by the penetration of solvent through the cell wall, weakening the cell wall and making cell content more available for extraction. Then, solvent forms a complex with the target product, and the formed complex is transferred to the outside of the cell [202]. Organic solvents are known to play an important role in the extraction of bioactive components from plant and algae materials [135,203].

Results from one-way ANOVA indicated that the means of the yields were significantly different (Tukey,p <0.05) between the three microalgal species, as shown in table 5. In organic extracts, yields from *Tisochrysis lutea* were always significantly higher than *Tetraselmis chui* and *Tetraselmis striata* CTP4. Ethanol has high recovery of membrane associated polar lipids due to its known ability to penetrate membranes and is considered

GRAS. Additionally, all three algae have a high protein content and by using polar solvents, these polar compounds can also be extracted to a higher extent, in this way increasing the total yield of extract obtained [130,199]. *Tetraselmis striata* CTP4 presented significantly higher yields in aqueous extract in comparison to the other two species (Tukey, p <0.05). The higher extraction yield of *T. striata* CTP4 with water could be explained by the higher fiber content and other polysaccharides (such as sugars) which are hydrophilic [204,205]. Other molecules that are best extracted with water are water soluble ionic components, microelements, small molecular weight compounds (such as amino acids), bioactive peptides, nuclear acids and water soluble proteins[206,207]

Table 5 - Extraction yields (%) of *Tisochrysis lutea, Tetraselmis chui* and *Tetraselmis striata* CTP4 with solvents ethanol, water, hexane, and ethyl acetate.

Solvent	Specie	*Tisochrysis lutea*	*Tetraselmis chui*	*Tetraselmis striata* CTP4
Hexane		11.4 ± 0.1^{a}	1.50 ± 0.09^{b}	2.09 ± 0.94^{b}
Ethyl Acetate		16.2 ± 0.1^{a}	4.45 ± 0.39^{b}	3.67 ± 0.01^{b}
Ethanol		33.7 ± 2.9^{a}	15.0 ± 0.80^{b}	9.29 ± 2.57^{b}
Water		14.7 ± 0.1^{a}	14.3 ± 0.10^{b}	24.4 ± 0.10^{c}

Note: n=2. Different superscript letters in a row indicate statistically significant differences bet ween means (analysis by analysis of variance and Tukey test, p <0.05)

The cell wall composition of microalgal species has a major effect on extraction yields [208]. *T. lutea* presents the highest extraction yields with organic solvents in comparison to *T. chui* and *T. striata* CTP4 . This may be due to *T. lutea* having a very thin almost non-existing cell wall, which makes it very easy for solvents to penetrate the membrane and extract the inner cell components, regardless of being polar or non-polar in nature. Due to the pre-treatment with milling, most of the cells walls probably were compromised, further exposing the intracellular content to the solvent extraction to achieve an efficient compound recovery [109,209]. On the other hand, microalgae in the genus *Tetraselmis*, such as *T. chui* and *T. striata* CTP4 , are known to have a unique cell wall formation process among green algae: the cells synthetize small non-mineralized scales that form a solid wall composed of fused scales, also called theca. The cell wall rigidity can be provided by complex sugars composition, search as arabinose, galactose, mannose, and xylose as found in *Tetraselmis suecia* and *T. striata* CTP4 is considered to have a mechanically resistant cell wall, while *T. chui* has tough and flexible walls due to being rich in a pectin-like polysaccharide. The rigid and thick cell walls are more challenging to damage, so that the intercellular content is less available for solvents to extract and lead to overall

lover extraction yields. Due to the thicker cell wall, it is essential to assure cell disruption (e.g., through bead beating) occurs to increase extraction yields [204,205]. Also, out of the three microalgal species, *T. lutea* contains the highest amounts of lipids (both soluble and insoluble fatty acids) and triglycerides, with up to 32% of total fatty acids and 15% of triglycerides per dry weight [210]. The extraction of lipids can be achieved by using nonpolar solvents such as hexane, as lipids are nonpolar compounds that dissolve in nonpolar solvents, contributing to a greater yield of *T.lutea*-hexane extract[211].

4.1.1. Antioxidant activity

The screening for the antioxidant activity was performed in the hexane (H), ethyl acetate (EA), ethanol (EtOH) and water (W) extracts obtained from *Tisochrysis lutea*, *Tetraselmis chui* and *Tetraselmis striata* CTP4. All extracts were tested at concentrations of 1, 5 and 10 mg/mL DMSO.

The antioxidant assays performed to assess antioxidant activity were DPPH assay, ABTS assay, iron (II) chelating activity assay, copper chelating activity and ferrous reducing antioxidant capacity assay. All the obtained results were presented as a percentage, and the activities compared with positive controls: BHT in case of DPPH, ABTS and ferrous reducing assay or EDTA in metal chelating activity assays (iron and copper). Statistical comparisons between solvents and microalgal species was always made at the concentration of 5 mg/mL DMSO.

4.1.1.1. DPPH assay

The DPPH radical scavenging activity of all the extracts was dose-dependent, so higher extract concentration increases DPPH scavenging activity, as can be seen in table 6.

Table 6 - Radical scavenging activity (%) and IC_{50} on the DPPH radicals of organic and water extracts *of T. lutea, T. chui* and *T. striata* CTP4.

		Concentration (mg/mL DMSO)			
Species	Solvent	**1**	**5**	**10**	**IC_{50}** (mg/mL)
T. lutea	Hexane	n.a.	26.3 ± 2.1 $^{a, A}$	60.7 ± 1.5	n.a.
	Ethyl acetate	4.04 ± 2.15	49.1 ± 2.4 $^{b, A}$	88.8 ± 2.1	4.77
	Ethanol	3.54 ± 2.63	37.3 ± 2.4 $^{c, A}$	74.4 ± 1.9	n.a.
	Water	9.15 ± 3.57	14.7 ± 3.9 $^{d, A}$	27.1 ± 3.7	n.a.
T. chui	Hexane	16.8 ± 2.7	78.8 ± 2.2 $^{a, B}$	111. ± 1.	3.09
	Ethyl acetate	8.75 ± 2.09	57.1 ± 2.6 $^{b, B}$	105. ± 2.	2.80
	Ethanol	10.1 ± 1.6	44.6 ± 2.0 $^{c, B}$	78.8 ± 1.9	3.50
	Water	9.18 ± 3.15	23.9 ± 3.5 $^{d, B}$	35.8 ± 5.3	n.a.
***T. striata* CTP4**	Hexane	14.5 ± 3.4	45.3 ± 2.8 $^{a, C}$	72.4 ± 2.3	2.55
	Ethyl acetate	15.8 ± 3.3	48.2 ± 3.2 $^{a, A}$	88.9 ± 1,9	n.a.
	Ethanol	8.52 ± 2.39	24.5 ± 3.2 $^{b, C}$	49.0 ± 2.4	n.a.
	Water	4.98 ± 3.22	9.66 ± 2.92 $^{c, A}$	17.9 ± 4.3	n.a.
Positive control (**BHT)**		n.a.	n.a.	n.a.	0.21

Note: n=6. *Different superscript letters in a column indicate statistically significant differences between means (analysis by analysis of variance and Tukey test, p <0.05). Lower case letters (a, b, c, d) refer to differences from different solvents within the same microalgal species. Upper case letters (A, B, C) refer to differences between microalgal species using the same solvent. n.a. = not analyzed*

Results from one-way ANOVA indicated that DPPH scavenging activity were significantly different (p <0.001) in between the three microalgal species. The extract with highest DPPH scavenging activity was hexane *T. chui* (78.8 ± 2.2%) and lowest was aqueous *T. striata* CTP4 (9.66 ± 2.92%). Overall, *T. chui* always presented highest antioxidant activity out of the three microalgal species regardless of the extraction solvent (IC_{50} of 3.50 mg/mL, 3.10 mg/mL, 2.80 mg/mL for ethanol, hexane, and ethyl acetate, respectively). *T. lutea* had best results with ethyl acetate (IC_{50} = 4.79 mg/mL) as solvent and lowest in aqueous extract. *T. striata* CTP4 was the microalgal species with lowest DPPH scavenging activity, whereas hexane and ethyl acetate had better results in comparison to water and ethanol. However, all the obtained values are still significantly higher than positive control BHT (IC_{50} = 0.21 mg/mL), as expected since BHT is a reference antioxidant and a pure compound.

Results from one-way ANOVA indicated that DPPH scavenging activities were significantly different (p <0.001) in between solvents. At the concentration of 5 mg/mL, ethyl acetate and hexane had highest antioxidant activity with *Tetraselmis chui.* Water extracts also had higher values with *T. chui* but found no differences between *T. lutea* and *T. striata* CTP4 . *Tetraselmis chui* is known to be rich in pigments as carotenes, and PUFAs, which are non-polar compounds with known antioxidant activity and are very effectively extracted by hexane[142,212]. The higher DPPH scavenging activity of hexane and ethyl acetate extracts suggests that non-polar compounds (such as carotenoid pigments and polyunsaturated fatty acids) may contribute to a greater extent to the antioxidant activity[213]. Non-polar solvents such as hexane are known to have higher recovery of cellular non-polar compounds such as terpenes, including neutral lipids, essential oils, and alkanes, as compared to polar solvents, with up with up to 86% yield recovery of neutral lipids recovered from *Nannochloropsis striata* [214]. Ethyl acetate possesses higher selectivity towards less polar molecules (such as alkaloids, sterols and fatty acids) and generates extracts with higher purity and is also good from economical point of view as it is inexpensive and considered as food grade [215,216]. The lower antioxidant activity of water were different of the findings of Maadane et al.[217], which found high DPPH radical scavenging activity in ethanolic and aqueous of *T. lutea.* Water is often used as solvent in food industry owing to its inexpensive, non-toxic, GRAS and environmentally friendliness. Water acts as extraction solvent by swelling and weakening the cell structure, facilitating the access to the intercellular compounds. Due to its polarity, water is effective for the extraction of polar compounds such as phenolic compounds and polysaccharides, however nonpolar molecules (such as PUFAs which have known antioxidant properties) are not extracted by water, which can decrease the antioxidant activity of these extracts in comparison to other more nonpolar solvents [218].

4.1.1.2. ABTS assay

The ABTS radical scavenging activity of all the extracts was dose-dependent, so higher extract concentration increases ABTS scavenging activity, as can be seen in table 7.

Table 7 - Radical scavenging activity (%) and IC_{50} on the ABTS radicals of organic and water extracts of *T. lutea, T. chui* and *T. striata* CTP4.

		Concentration (mg/mL DMSO)			
Species	Solvent	**1**	**5**	**10**	**IC_{50}** (mg/mL)
T. lutea	Hexane	5.24 ± 0.49	16.3 ± 1.4^{A}	31.0 ± 1.1	n.a.
	Ethyl acetate	9.58 ± 0.86	27.4 ± 1.4^{A}	45.4 ± 4.1	n.a.
	Ethanol	9.20 ± 1.35	30.1 ± 2.3^{A}	49.7 ± 3.3	n.a.
	Water	5.74 ± 2.53	14.4 ± 5.3^{A}	24.9 ± 6.5	n.a.
T. chui	Hexane	21.6 ± 1.30	68.8 ± 3.5^{B}	93.4 ± 2.6	n.a.
	Ethyl acetate	17.7 ± 2.15	51.1 ± 1.6^{B}	87.7 ± 1.4	n.a.
	Ethanol	17.2 ± 1.77	68.1 ± 3.8^{B}	91.5 ± 0.4	2.32
	Water	11.2 ± 1.40	37.9 ± 6.5^{B}	81.2 ± 12.9	n.a.
***T. striata* CTP4**	Hexane	19.7 ± 5.3	70.2 ± 5.0^{B}	91.1 ± 2.3	2.18
	Ethyl acetate	19.4 ± 5.9	52.6 ± 7.3^{B}	88.6 ± 2.1	n.a.
	Ethanol	9.72 ± 4.45	36.4 ± 4.2^{C}	62.9 ± 4.5	n.a.
	Water	7.28 ± 0.93	12.8 ± 0.7^{A}	22.6 ± 0.6	n.a.
Positive control (BHT)		n.a.	n.a.	n.a.	0.22

Note: *n*=6. Different superscript letters in a column indicate statistically significant differences between means (analysis by analysis of variance and Tukey test, p <0.05). Lower case letters (a, b, c, d) refer to differences from different solvents within the same microalgal species. Upper case letters (A, B, C) refer to differences between microalgal species using the same solvent. n.a. = not analyzed

Results from one-way ANOVA indicated that ABTS scavenging activities were significantly different (p <0.001) in between the three species. While *T. lutea* did not reach 50% scavenging activity with any solvent not even at the highest concentration of 10 mg/mL, *T. chui* extracts achieved over 50% inhibition with all organic solvents at concentration 5 mg/mL. The extract with the lowest IC_{50} value was *T. striata* CTP4 - hexane (2.17 mg/mL) closely followed by *T. chui*-ethyl acetate (2.32 mg/mL) and *T. chui*-ethanol (4.79 mg/mL), however all the extracts were far from the IC_{50} value of BHT (0.22 mg/mL), which is expected as BHT is a pure compound. This findings are in accordance with Conde et al.[219], wherein *T. chui* had one of the lowest IC_{20} values together with *Spirulina striata* and *C. vulgaris*, but is contrary to other findings where *T. lutea* showed high antioxidant activity in ABTS assay[220–222].

Results from one-way ANOVA indicated that the ABTS scavenging activities were significantly different (p <0.001) in between the four solvents. Hexane and ethyl acetate presented high ABTS scavenging activity in both *Tetraselmis* algae contrary to *T. lutea.* Ethyl acetate possesses higher selectivity towards less polar molecules (such as alkaloids, sterols and fatty acids) and hexane is known to have higher recovery of cellular non-polar compounds such as terpenes, including neutral lipids, essential oils, and alkanes[215,216]. These findings are according to Goiris et al.[203], in which ethyl acetate and hexane fractions have shown higher carotenoid content in ethyl acetate, hexane and ethanol/water extracts of various algal species, whereas water extracts presented almost no carotenoid content. Water was the solvent with less ABTS scavenging activity in all 3 algae species, which might indicate that the extracted polar compounds not greatly contribute to the antioxidant activity of the extracts but is controversially to findings of Matos et al.[221] where aqueous extracts of *T. lutea* had the highest antioxidant activity. Extracts with ethanol also had high antioxidant activity with *Tetraselmis chui* and *Tisochrysis lutea.* Various studies have demonstrated very high recovering yields of antioxidant compounds, including phenolics, from several macroalgae (such as *Haematococcus pluvialis, Tysocrysis lutea, Tetraselmis suecica, Botryococcus braunii, Neochloris oleoabundans, Chlorella vulgaris, and Phaeodactylum tricornutum* and *Dunaliella salina)* due to extraction with ethanol [135,203]. These results are in harmony with the findings of Goiris et al.[203] and Rahman et al.[223] that found high antioxidant activity in extracts *T. chui* and *Tetraselmis striata*, probably due to phenolic compounds. The effectiveness of ethanol might be related to the precipitation of proteins and leaves some of the reversibly bonded phenolic compounds into the solution [224]. These results can be attributed to high content of phenolic compounds and pigments, which various studies already correlated with antioxidant activity and these compounds are mainly extracted by organic solvents [126,203].

4.1.1.3. Iron (II) chelating activity assay

The iron chelating activity of the extracts was dose-dependent between concentrations of 1 mg/ml and 5 mg/mL, however, no substantial changes in chelating capacity exist between 5 mg/mL and 10 mg/mL, as showed in table 8.

Table 8 - Iron chelating activity (%) and IC_{50} of organic and water extracts of *T. lutea, T. chui* and *T. striata CTP4*.

		Concentration (mg/mL DMSO)			
Species	Solvent	**1**	**5**	**10**	**IC_{50}** (mg/mL)
T. lutea	Hexane	66.9 ± 3.7	67.9± 3.7 $^{a, A}$	49.0 ± 6.2	0.060
	Ethyl acetate	119 ± 7	90.1 ± 3.3$^{b, A}$	19.8 ± 6.6	0.090
	Ethanol	53.7 ± 4.3	58.1 ± 2.6$^{c, A}$	64.6 ± 1.0	0.080
	Water	47.9 ± 3.8	101 ± 3$^{d, A}$	96.6 ± 7.2	0.153
T. chui	Hexane	n.a.	n.a.	n.a.	n.a.
	Ethyl acetate	6.15 ±13.90	n.a.	n.a.	n.a.
	Ethanol	22.7 ± 1.8	n.a.	n.a.	n.a.
	Water	51.8 ± 4.3	94.1 ±4.9$^{a, A}$	99.8 ± 8.8	0.173
***T. striata* CTP4**	Hexane	15.5 ± 4.0	24.0 ± 3.0$^{a, B}$	32.4 ± 6.9	n.a.
	Ethyl acetate	14.9 ± 8.0	22.3 ± 6.7$^{b, B}$	n.a.	n.a.
	Ethanol	29.9 ± 1.5	38.8 ± 2.1$^{c, B}$	28.8 ± 5.4	n.a.
	Water	36.6 ± 10.7	97.9 ± 1.3$^{d, A}$	97.2 ± 2.8	0.372
Positive control (EDTA)		n.a.	n.a.	n.a.	0.452

Note: *n*=6. Different superscript letters in a column indicate statistically significant differences between means (analysis by analysis of variance and Tukey test, p <0.05). Lower case letters (a, b, c, d) refer to differences from different solvents within the same microalgal species. Upper case letters (A, B, C) refer to differences between microalgal species using the same solvent. n.a. = not analyzed

Results from one-way ANOVA indicated that the iron chelating activities were significantly different (p <0.05) in between the four solvents. In organic solvents (ethyl acetate, hexane, ethanol) significant differences (Tukey, p <0.05) were found among species, while whereas *Tisochrysis lutea* was the algal species with higher iron chelating activity and lowest was found for *Tetraselmis chui*. Water was the solvent which highest iron chelating activity in all three microalgal species, and no significant differences were found in between species. Various polar compounds that can be extracted with water have been reported to have metal chelating ability, such as sulphated polysaccharides (fucoidans)[225]. The presence of phenolics in extracts could also partly explain the iron chelation, since it is known that these compounds can bind to transition metals[167]. Proteins and peptides can also be co-extracted with water and have been reported to possess metal

chelating ability in seaweeds[226]. Aqueous extracts of *Sargassum hystrix* have also exhibited high iron chelating activity in the work of Budhiyanti et al.[227].

Results from one-way ANOVA indicated that the iron chelating activities were significantly different (p <0.001) in between the three species. *T. lutea* had the highest activity out of the three species regardless of solvent used (IC_{50} values of 0.06 mg/mL for hexane extract, 0.09 mg/mL for ethyl acetate, 0.08 mg/mL for the ethanolic extract, and 0.15 mg/mL for the water extract). Both algae from genus *Tetraselmis* had highest activity with water as solvent (IC_{50} of 0.17 mg/mL and 0.37 mg/mL, for *T. chui* and *T. striata* CTP4, respectively) and lowest with ethyl acetate, suggesting that polar compounds (e.g., polysaccharides, proteins) are mainly responsible for the iron chelating activity in these extracts. The ethyl acetate extracts of *T. chui* and *T. striata* CTP4 species were found to have lowest iron chelation activity, which is contradictory to the results from Khalili et al.[228] and Pereira et al.[126]. In the study of Custódio et al.[229] hexane extracts of different microalgae exhibited highest Fe^{2+}-chelating activity and were attributed to high PUFAs and sterols, which is contrary to our findings. The extracts had a higher capacity to chelate Fe^{2+} ions than Cu^{2+}, which is in accordance to the study of Custódio et al.[229].

Most of the calculated IC_{50} values are even lower than the EDTA positive control (0.14 mg/mL), which is a pure compound with known metal chelating activity. These findings are very promising and could be associated to very bioactive compounds which even in unpurified state are able to chelate iron very effectively, conferring the whole analyzed extract an extremely high iron chelating activity Oxidative damage due to reactions catalyzed by divalent metals has been implicated in the development of various diseases and by metal chelation oxidative stress is reduced, as these are unable to generate reactive species or decompose lipid peroxides [229]. Our results suggest that the water extracts are endowed with compounds with Fe^{2+}-chelating activity.

4.1.1.4. Copper chelating activity

The copper chelating capacity of the extracts was dose-dependent between concentrations of 1 mg/mL and 5 mg/mL, but no substantial changes between 5 mg/mL and 10 mg/mL were detected, as it is possible to see in table 9.

Table 9 - Copper chelating activity (%) and IC_{50} of organic and water extracts of *T. lutea, T. chui* and *T. striata* CTP4.

		Concentration (mg/mL DMSO)			
Species	Solvent	**1**	**5**	**10**	**IC_{50}** (mg/mL)
T. lutea	Hexane	73.4 ± 1.3	82.6 ± 2.1$^{a, A}$	83.3 ± 1.7	0.060
	Ethyl acetate	56.8 ± 2.1	83.3 ± 1.5$^{a, A}$	76.1 ± 1.6	0.090
	Ethanol	44.1 ± 3.3	85.9 ± 1.1$^{a, A}$	85.1 ± 2.2	0.941
	Water	30.2 ± 3.2	68.9 ± 2.5$^{b, A}$	85.6 ± 1.4	1.98
T. chui	Hexane	n.a.	15,61 ± 2.21$^{a, B}$	41.3 ± 5.7	n.a.
	Ethyl acetate	5.65 ± 2.84	27.0 ± 4.8$^{b, B}$	37.7 ± 6.5	n.a.
	Ethanol	7.19 ± 3.26	44.5 ± 3.2$^{c, B}$	86.1 ± 5.4	n.a.
	Water	18.8 ± 4.2	66.7 ± 2.8$^{d, A}$	79.2 ± 3.9	0.173
***T. striata* CTP4**	Hexane	n.a.	4.80 ± 6.20$^{a, C}$	31.2 ± 7.6	n.a.
	Ethyl acetate	n.a.	10.5 ± 3.5$^{b, C}$	9.20 ± 2.90	n.a.
	Ethanol	n.a.	19.9 ± 5.0$^{c, C}$	37.6 ± 5.6	n.a.
	Water	12.1 ± 4.6	43.0 ± 2.9$^{d, B}$	48.4 ± 8.0	0.372
Positive control (EDTA)		n.a.	n.a.	n.a.	0.136

Note: *n*=6. Different superscript letters in a column indicate statistically significant differences between means (analysis by analysis of variance and Tukey test, p <0.05). Lower case letters (a, b, c, d) refer to differences from different solvents within the same microalgal species. Upper case letters (A, B, C) refer to differences between microalgal species using the same solvent. n.a. = not analyzed

Results from one-way ANOVA indicated that the copper chelating activity was significantly different (p <0.001) in between the three species. At concentration of 5 mg/mL, the highest values of copper chelating activity were shown from *T. lutea* regardless of the extraction solvent while *T.* striata CTP4 always had lowest values, these findings are according to the works of Custódio et al.[230] and Pereira et al.[126], respectively. The work of Custódio et al.[229] confirms that the copper chelating activity is very variable among extracts and species. Copper chelating activity in microalgae can be attributed to both polar and non-polar compounds, including phenolic compounds. Higher copper chelating activity in microalgae may be related to the high abundance of polyunsaturated fatty acid and flavonoids [220,231].

Copper chelating activites were significantly different (ANOVA, p <0.001) in between the four solvents. Water had best copper chelating activity for *T. chui and T.* striata CTP4

but had low copper chelating activity for *T. lutea*. Hexane and ethyl acetate had low chelation activity for *T. chui* and *T.* striata CTP4 but presented high activity (together with the other organic solvents) with *T. lutea*. The extracts had a higher capacity to chelate Fe^{2+} ions than Cu^{2+}, which is in accordance to the study of Custódio et al.[229].

The extract with lowest IC_{50} value was *T. lutea*-hexane (0.60 mg/mL), closely followed by *T. lutea* ethyl acetate (0.65 mg/mL) and *T. lutea* ethanolic extract (0.94 mg/mL), which are quite close to the IC_{50} (0.136 mg/mL) of EDTA, which is a pure compound with known metal chelating activity. These results are according to the findings of Custódio et al.[230], where *T. lutea*-hexane extracts presented the lowest IC_{50} value at 0.90 mg/mL. Both aqueous extracts from *T. lutea* and *T. chui* also had relatively low IC_{50} values (1.98 mg/mL and 1.75 mg/mL, respectively), which is in accordance with the work of Budhiyanti et al.[227]. These findings are very promising and could be associated to very bioactive compounds which even in unpurified state are able to chelate copper very effectively, conferring the whole analyzed extract an extremely high copper chelating activity. Oxidative damage due to reactions catalyzed by divalent metals has been implicated in the development of various diseases and by metal chelation oxidative stress is reduced, as these are unable to generate reactive species or decompose lipid peroxides [229]. Our results suggest that the water extracts are endowed with compounds with Cu^{2+} chelating activity in *T. chui* and *T.* striata CTP4 while nonpolar compounds seem to provide copper chelating activity in *T. lutea*.

4.1.1.5. Ferrous reducing antioxidant capacity assay

The response of the extracts was dose-dependent in ferrous reducing antioxidant capacity assay, as shown in table 10, which is consistent with work of Rafay et al.[116].

Table 10 - Ferrous reducing antioxidant capacity (%) and IC_{50} of organic and water extracts of *T. lutea, T. chui* and *T. striata* CTP4.

		Concentration (mg/mL DMSO)			
Species	Solvent	**1**	**5**	**10**	**IC_{50}** (mg/mL)
T. lutea	Hexane	20.8 ± 1.6	27.1 ± 3.2 $^{a, A}$	n.a.	n.a.
	Ethyl acetate	21.4 ± 1.6	34.3 ± 2.5^{b,A}	53.1 ± 5.0	n.a.
	Ethanol	23.9 ± 0.7	117 ± 4^{c,A}	107 ± 6	1.23
	Water	n.a.	14.2 ± 4.0^{d,A}	33.8 ± 4.1	n.a.
T. chui	Hexane	12.1 ± 0.9	91.4 ± 7.1^{a,B}	144 ± 6	2.95
	Ethyl acetate	n.a.	70.1 ± 36.4^{b,A}	119 ± 24	n.a.
	Ethanol	49.1 ± 1.8	223 ± 7^{c,B}	276 ± 12	0.771
	Water	1.43 ± 0.92	23.9 ± 12.6^{d,A}	62.2 ± 11.9	n.a.
***T. striata* CTP4**	Hexane	20.1 ± 5.7	162 ± 10^{a,C}	220 ± 9	1.82
	Ethyl acetate	n.a.	n.a.	n.a.	n.a.
	Ethanol	22.8 ± 1.8+	65.5 ± 3.8^{b,C}	n.a.	3.83
	Water	0.29 ± 0.83	2.0 ± 2.4^{c,A}	2.96 ± 1.98	n.a.
Positive control (BHT)		n.a.	n.a.	n.a.	0.230

Note: *n*=6. Different superscript letters in a column indicate statistically significant differences between means (analysis by analysis of variance and Tukey test, p <0.05). Lower case letters (a, b, c, d) refer to differences from different solvents within the same microalgal species. Upper case letters (A, B, C) refer to differences between microalgal species using the same solvent. n.a. = not analyzed

The ferrous reducing antioxidant capacity was significantly different (ANOVA, p <0.001) among the three microalgal species. *Tetraselmis striata* CTP4 showed lowest reducing activity in ethanol and ethyl acetate extracts but highest in hexane extract. *Tetraselmis chui* had best activity in ethanol and ethyl acetate extracts and lowest in water extract. In most cases, ethanol extracts contain the highest amounts of phenolic compounds and have a wider range compared to the water extracts [224]. The extracts with higher reducing power were organic extracts from *T. chui* (IC_{50} = 0.77 mg/mL and IC_{50} = 2.95 mg/mL for ethanol and hexane, respectively), ethanolic *T. lutea* (IC_{50} = 1.23 mg/mL) and hexane extract of *T.* striata CTP4 (IC_{50} = 1.82 mg/mL). These results are according to the work from Saranya et al.[232] that showed high reducing power activity in methanolic extract of *T. lutea*, which may be related to the high phenolic and carotenoid content of this microalgae which are better extracted by methanol then by other nonpolar solvents. In most cases, ethanol extracts contain the most phenolic compounds and have a wider range compared

to the water extracts [224]. In the work of Pereira et al.[116] the extracts from *T. striata* CTP4 with higher reducing power were acetone and ethyl acetate extracts, as opposed to aqueous, ethanolic and hexane extracts, which is contrary to our findings. However, none of the extracts are particularly close to the value obtained by BHT (IC_{50} = 0.23 mg/mL).

4.1.2. Identification of extracts with highest overall antioxidant activity and performance of anti-inflammatory assays

After performing the five antioxidant assays, the IC_{50} of extracts of the individual assays were analyzed and the three extracts with highest overall antioxidant activity were selected to undergo the anti-inflammatory assays.

It should be noted that, in general, data from antioxidant assays may vary among different studies, not only according to the microalgae species studied but also due to the specific growth conditions of the biomass, as certain stresses or *stimuli* (e.g., light manipulation) influence the synthesis of valuable bio compounds. Different algal products provide diverse antioxidant activity due to many influencing factors such as algal species, geographic origin or the area of cultivation, seasonal, physiological, and environmental variations[233]. Another factor that influences assay results is the use of crude extracts, thus other molecules, other than compounds with antioxidant properties, could have contributed to the weight of the extract [234]. Also, the used antioxidant assays differ in terms of their assay principles and experimental conditions and may not always measure the same aspects of the total antioxidant potential of the extract. In free radical scavenging activity assays (DPPH and ABTS), antioxidant activity is measured by the antioxidant's ability to donate an electron to the radical while in metal reduction activity assays, the antioxidant transfers one or more electron pairs with the formation of the covalent bond by the donor-acceptor mechanism [50]. The ferrous reduction assay is based on the ability of antioxidants to reduce Fe^{3+} from ferric chloride into Fe^{2+} by accepting an electron from an antioxidant [170].

The chosen extracts were ethanolic of *T. lutea* and *T. chui* and hexane of *T. striata CTP4* due to presenting the lowest IC_{50} values in three of the five antioxidant assays. Additionally, ethanolic *T. striata CTP4* was also considered an extract of interest, as hexane extract of *T. striata CTP4* cannot be used as food ingredients, due to hexane not

being GRAS. These four extracts were used to perform ACE inhibition and COX-1/COX-2 inhibition assays.

4.1.2.1. Angiotensin-I converting enzyme (ACE) Inhibitory Activity

The inhibition of ACE activity was measured by the decrease in the production of a fluorescent caused by the presence of possible inhibitor compounds in the microalgal extracts. For this, under the assay conditions, a calibration curve of the fluorescent product was prepared by plotting the fluorescence measured at 405 nm (after excitation at 320 nm) versus the amount of standard. The calibration curve in figure 31 showed an excellent linearity over the concentration range from 0.1 to 0.6 nmol of standard (R^2=0.9988). Working standard solutions with six different concentrations were analyzed in duplicate. The linear regression of the calibration curve (y = 60.71 ± 1.06 RFU/nmol + 0.94 ± 0.39) was used to convert the relative fluorescence units (RFU) into concentration units (nmol/L). Limit of detection (LOD) in this assay was 2.29 nmol/L and limit of quantification (LOQ) was 7.66 nmol/L.

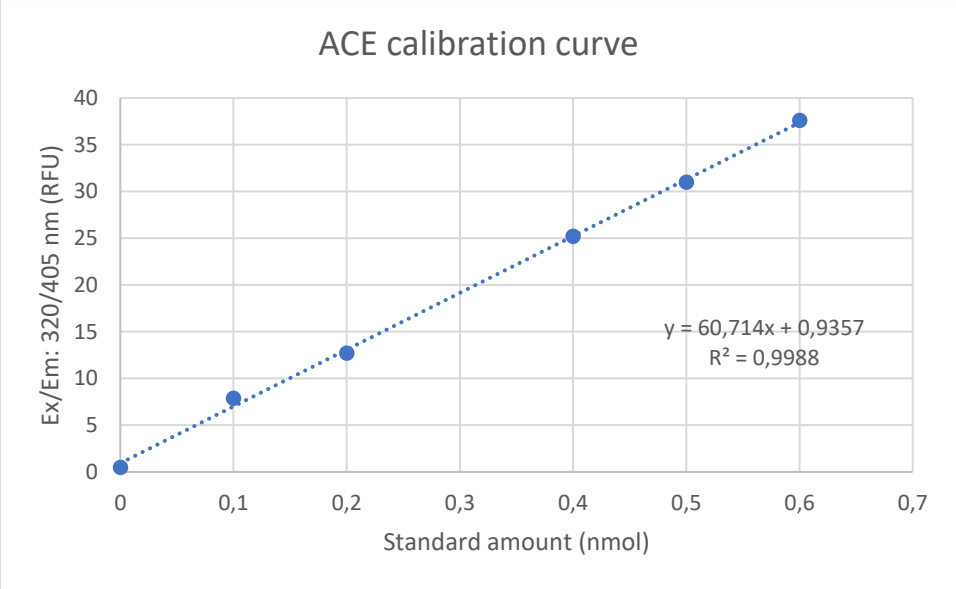

Figure 31- ACE calibration curve with six standard concentrations.

The assay was performed in the absence of inhibitor (Blank assay), containing all substances besides inhibitor/sample, and a plot of amount of fluorescent product generated (nmol/L) against time (minutes) was produced with the linear regression equation: y = 0,0179x + 0,0269. This Blank assay was performed in six replicates. Good

linearity was found over the time range from 0 to 25 min (R^2=0.9484). The slope of the Blank assay was then used to determine the percentage of ACE inhibition of the samples.

As shown in table 11, the extracts exhibited low inhibition, as at 0.5 mg/mL and 0.1 mg/mL, no ACE inhibition could be determined. However, at the concentration 1 mg/mL all extracts besides ethanolic *T. striata* CTP4.exhibited a similar inhibition percentage as the positive control (enalapril) also at 1.0 mg/mL, which indicates a promising inhibitory activity of ACE. Nonetheless, this assay needs optimization as the inhibition percentage of enalapril was below the expected value (80-100%). The low inhibition value may be due to enalapril possibly having less ACE inhibition activity comparing to other ACE inhibitors (such as captopril) or used concentrations of enalapril being too low to achieve an efficient inhibition.

Out of the three microalgae species, *T. chui* has the highest inhibitory activity, followed by *T. lutea* and *T. striata* CTP4. *Tetraselmis chui* ethanolic extract exhibited the highest ACE inhibitory activity (5.80%) and *Tetraselmis striata* CTP4 the lowest (0.79% and 2.29% in ethanolic and hexane extracts, respectively). Ethanolic and methanolic algae extracts are known to have strong ACE inhibitory activity [185,235]. Many studies have demonstrated that green algae possess anti-inflammatory properties, however few studies have been caried out regarding green algae and their ACE inhibitory properties [236,237]. In the study of Wang et al.[238], *Chlorella ellipsoidea* hydrolyzed with commercial proteases had a high ACE inhibitory. A potent ACE inhibiting tetrapeptide, Val-Glu-Gly-Tyr (IC_{50} = 128.4 μM) was isolated from this extract.

Brown algae, such as *Tisochrysis lutea,* are rich in polyphenolic compounds. In general, phlorotannins are the predominant polyphenols in brown algae, which are a known natural source of novel ACE inhibitors. Polysaccharides such as fucoidans, alginates and laminarans are also compounds which are produced by brown algae and have several biological properties, such as antitumor activity and anti-inflammatory activity [239,240]. It is possible that *T. lutea* extracts could further display higher ACE inhibitory activity after hydrolysis [241].

Table 11 - ACE inhibition (%) by blank, enalapril and microalgae extracts from *T. lutea, T. chui* and *T. striata* CTP4. n.d. – not detectable

Sample	Concentration (mg/mL)	Inhibition of ACE (%)
Blank (negative control)	-	0.00
Enalapril (Positive control)	2.0	9.89
	1.0	2.51
	0.5	n.d.
	0.1	n.d.
	0.01	n.d.
***T. lutea* EtOH**	1.0	2.9
	0.5	n.d.
	0.1	n.d.
***T. chui* EtOH**	1.0	5.80
	0.5	n.d.
	0.1	n.d.
***T. striata* CTP4 EtOH**	1.0	0.79
	0.5	n.d.
	0.1	n.d.
***T. striata* CTP4 Hx**	1.0	2.29
	0.5	n.d.
	0.1	n.d.

Note: *n*=6.

4.1.2.2. Cyclooxygenase (COX) Inhibiting Activity

COX inhibition was measured by decrease in the production of prostaglandins. Prostaglandins (PG) were quantified by ELISA by plotting $\%B/B_0$ (Standard Bound/Maximum Bound) versus PG concentrations. In this case, a calibration curve with a negative slope is obtained as the $\%B/B_0$ decreases as PG concentration increases. An excellent linearity over the concentration range (2000 to 15.6 pg/mL) of PG was obtained ($R^2 = 0.9943$). The linear regression of the calibration curve (y = -38.6 ± 0.85 + 2.09 ± 0.85) (figure 32) was used to convert sample results from %B/B0 into concentrations (pg/mL) and further into inhibition of COX percentage.

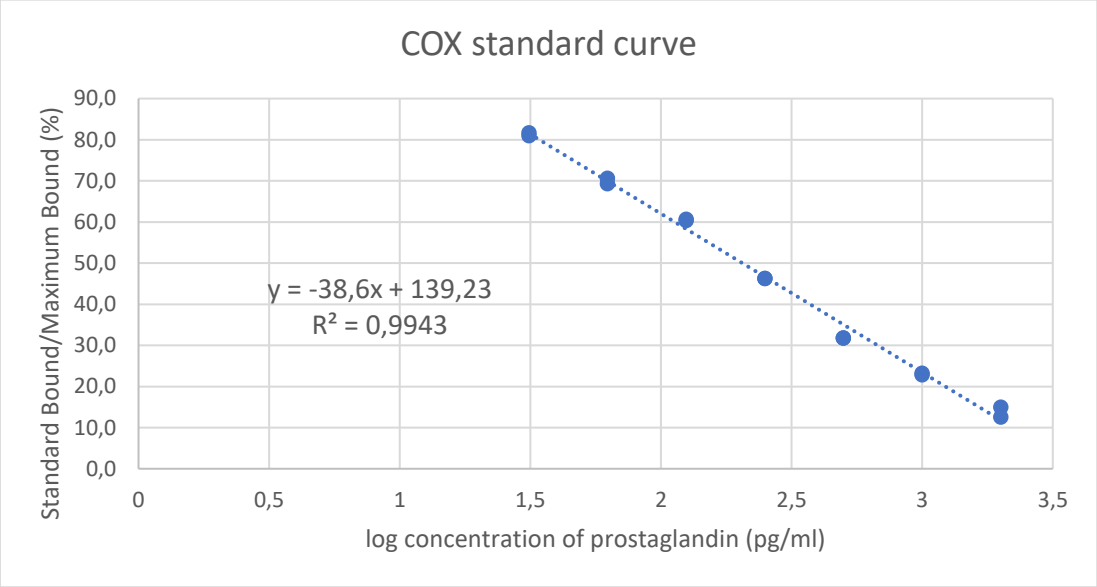

Figure 32- COX calibration curve with eight standard concentrations.

Cyclooxygenase (COX) Inhibiting Activity Assay was performed with ethanolic extracts of *T. lutea*, *T. chui* and *T. striata* CTP4 and hexane extract of *T. striata* CTP4. Each extract was tested at two concentrations, 1 mg/mL DMSO and 0.1 mg/mL DMSO. Ibuprofen was used as positive control at a concentration of 100 µg/mL. After addition of Ellman's Reagent and incubation, yellow color started to develop in the wells, as is shown in figure 33.

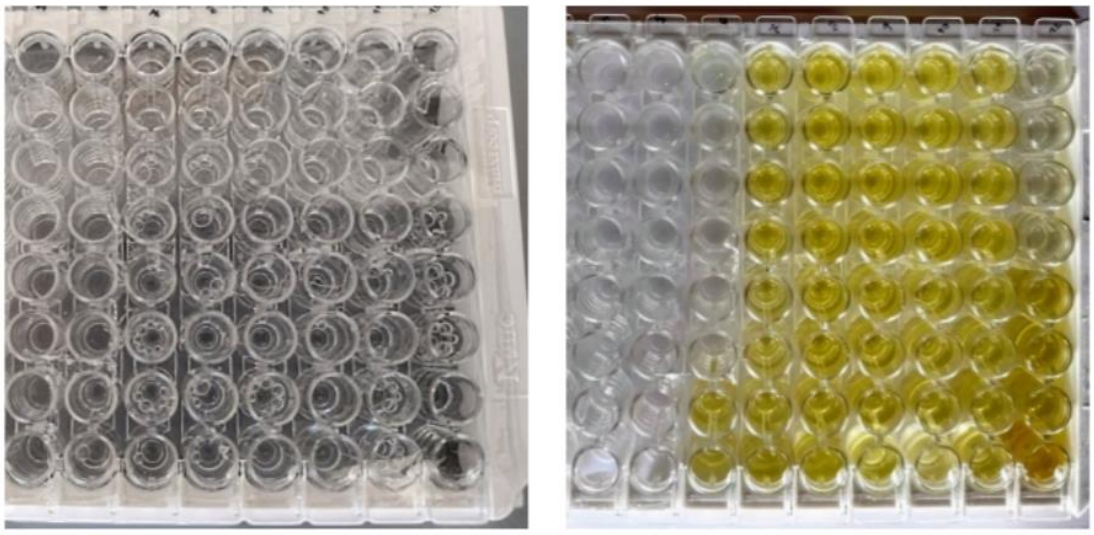

Figure 33 - Development of 96-well-plate for COX assay with Ellman's Reagent and incubation.
Before development (left) and after complete development (right)

The extract's COX inhibitory activity was assessed for COX-1. Ibuprofen at a concentration of 100 µg/mL exhibited 21.5 ± 4.1% of COX-1 inhibition. All extracts exhibited a dose dependent response, as shown in figure 34. At both concentrations (1 mg/mL and 0.1 mg/mL) all extracts had significant differences (Tukey, p <0.001) in COX inhibitory activity.

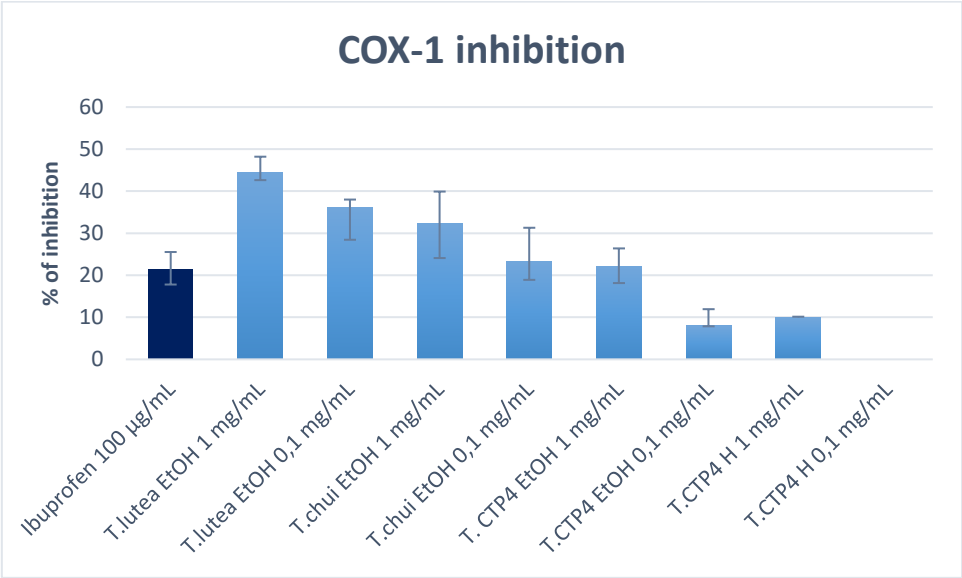

Figure 34- COX-1 inhibitory activity (%) from *T. lutea, T. chui* and *T. striata* extracts. *Note: n= 2*

T. lutea was the microalgae species with highest COX inhibition percentage (44.5 ± 3.7%; 36.1 ± 1.9%, for 1 mg/mL and 0.1 mg/mL, respectively) and consequently, showed the

highest anti-inflammatory activity. These results are expected, as shown by the recent work of Bigagli et al.[242], where a methanolic *T. lutea* extract significantly decreased the production of PGE_2 and COX-2 protein expression in a concentration dependent-manner. The work of Bigagli et al.[242] also suggests that the anti-inflammatory properties from *T. lutea* may be related not only to its fucoxanthin content but also to a high PUFA content and presence of phenolic compounds, such as gallic and hydroxybenzoic acid. Bonfanti et. al.[243] found greatest COX inhibition in oily extracts of *I. Galbana* and attributed the strong COX-inhibition (up to 79%) to various lipids and lipophilic substances (such as EPA and glycoproteins). In a recent study of Mayer et al.[110] the supplementation with *T. lutea* on rats showed an improvement of inflammatory status, among other health benefits.

T. chui had 32.2 ± 7.7% and 23.2 ± 8.1% of COX-1 inhibition at concentrations of 1 mg/mL and 0.1 mg/mL, respectively. Microalgae of the *Tetraselmis* genus, including *Tetraselmis chui,* exhibit their anti-inflammatory properties by inhibiting the release of various inflammatory mediators such as NO, TNF alpha. Usually, best anti-inflammatory activities are detected in organic solvents of lower polarity index, such as hexane and ethyl acetate. PUFAs side chains are suspected to play an important role in the inhibition of nitric oxide, therefore reducing oxidative stress and inflammation [244,245].

Tetraselmis striata CTP4 showed the lowest inhibitory effect, whereas the ethanolic extract was higher than the hexane extract at the concentration of 1 mg/mL (22.2 ± 4.3% *vs.* 10.0 ± 0.1%, respectively). These values are lower than the ones obtained by Cardoso et al.[246], where ethanolic and aqueous extracts of *Tetraselmis striata* CTP4 at concentration 1 mg/mL exhibited inhibition of 6% and 36%, respectively. The high discrepancy on inhibition maybe be endorsed by the possibility of anti-inflammatory compounds belonging to an ethanol-soluble class or that due to different polyphenols being extracted differentially by water and ethanol, the resulting extracts have different efficiency of COX inhibition [246].

4.2. Effect of extraction method on extraction yield and antioxidant activity

4.2.1. Extraction yields

Organic solvent extraction is an efficient technique to recover of microalgae biomolecules, being especially effective after a cell disrupting technique was applied, because of the easier access of the solvent into the inner cell compounds[200,201]. It has been shown that extraction efficiency depends on various factors such as temperature, pressure, time and extraction techniques [131,136]. Cell disruption plays an important role in the efficiency of extraction; therefore, several different cell disruption techniques are being studied to enhance the recovery of the intercellular target products.

Two different extracting techniques (microwave assisted extraction and automated solvent extraction) were used and compared to the previous results obtained with the *bead-beater* to evaluate the influence of extraction techniques on the yield from *Tetraselmis striata* CTP4.

Microwave assisted extraction from *Tetraselmis striata* CTP4 biomass was mainly performed at high temperatures (up to 160 ºC), as only at high extraction temperatures this extraction method involves the use of microwaves to generate thermal energy through rotation or vibration of dipoles or ionic conduction. All the extracts were produced with one extraction cycle. Microwave assisted extraction systems have been reported to be successful in the extraction of valuable bio active compounds from microalgae. Operational key factors for success of microwave assisted extractions are mainly selected microalgae species, power of the microwave, temperature, solvent volumes and solvent properties [247].

Ethanolic and hexane extracts from *Tetraselmis striata* CTP4 produced with the EDGE automated solvent extractor were performed at 3 temperatures (30 ºC, 50 ºC and 100 °C) for either 5 or 10 minutes per cycle. The number of cycles varied from 1 to 4. Some studies indicate extractions with ASE systems as a fast and easy technique to recover natural antioxidants from microalgae such as *Spirulina platensis*, whereas the main factors affecting yields and bioactivity are related to the polarity of the solvent used to perform the extractions and extraction temperature [248].

When directly comparing the three extraction techniques, as shown in table 12, the *bead-beater* showed significantly higher yields for ethanolic extracts (ANOVA, p <0.001), and no yield differences were found between EDGE and microwave-assisted extracts. Yields of hexane extracts were significantly lower with microwave assisted extraction (ANOVA, p <0.05), but no yield differences were found between extracts from EDGE and bead-beater. Highest yields were obtained at 50 ºC with EDGE (2.38% and 1.64% for 1 or 2 cycles of 10 min, respectively) and milling (6 cycles, 1.48%).

Table 12 - Comparison of yields (%) from ethanolic and hexane *Tetraselmis striata* CTP4 extracts, by milling (*Bead beater* with glass-beads, BB), EDGE, and microwave (MW) assisted extraction

					Yield (%)		
Extract	**Solvent**	**Temperature (°C)**	**Time per cycle (min)**	**Number of cycles**	**MW**	**EDGE**	**BB**
1	**Ethanol**	**<RT**	**5**	**6**			**6.71[a]**
2	Ethanol	30	5	1		2.29	
3	Ethanol	30	10	1		1.79	
4	Ethanol	30	10	1	3.34[b]	2.87[b]	
5	Ethanol	30	10	2		3.60	
6	Ethanol	50	5	2		4.35	
7	Ethanol	50	10	1	4.39[b]	3.11[b]	
8	Ethanol	50	10	2		4.50	
9	Ethanol	50	10	3		7.07	
10	Ethanol	50	10	4		6.88	
11	Ethanol	100	5	2		5.91	
12	Ethanol	100	10	1	4.27[b]	4.16[b]	
13	Ethanol	100	10	2		7.02	
14	Ethanol	120	0.5	1	3.82		
15	Ethanol	120	1	1	5.92		
16	Ethanol	160	0.5	1	5.93		
1	**Hexane**	**<RT**	**5**	**6**			**1.48[a]**
2	Hexane	50	10	1	0.40[b]	2.38[c]	
3	Hexane	50	10	2	0.66[b]	1.64[c]	
4	Hexane	50	10	3		1.20	
5	Hexane	120	0.5	1	0.93		
6	Hexane	160	0.5	1	2.29		

Note: Different superscript letters in a column indicate statistically significant differences between techniques (analysis by analysis of variance and Tukey test, p <0.05

When comparing the three techniques, *bead beater* had higher yields than EDGE and microwave assisted extraction in ethanol extracts, which may be attributed to higher cell disruption. However, amount of cycles were different between techniques, whereas *bead beater* had the highest number of cycles, which increases the time of exposure and is associated with higher extraction yields[249,250] Despite *bead-beating* being very simple, extraction technique, it has some significant downsides which limit the overall efficiency and sustainability of the extraction process, such as longer extraction times, need to evaporate big amounts of solvent (and therefore, high energy necessity) and high solvent consumption[97,250]. The higher extraction yield results would be consistent with the work of Taucher et al.[251], where microalgae extracts obtained by different methods (*bead-beater*, Ultra Turrax, freeze and thaw, freeze drying, ultra-sonication and high pressure homogenizer) were analyzed according to their disruption yields, the highest disruption yields with about 80% was reached by using mechanical methods such as *bead-beater* with glass beads and high-pressure homogenizer. Another study Schüler et al.[199], using *Tetraselmis striata* CTP4 biomass, showed that glass bead-assisted disruption significantly improved pigment recovery compared with dispersion (1.5 times fold).

With hexane as solvent, extracts obtained using EDGE exhibited the highest overall yields which may be related to the higher temperature used as in comparison with the ones used in the *bead-beater* (below room temperature), as an increase in temperature is generally related to an increase in extraction yields [248]. In the work from Tang et al.[252] the total fatty acid methyl ester content of dry biomass from ASE extraction was found to be 1.3-2.7-fold higher compared to conventional manual extraction (using mortar and pestle) from *C. vulgaris, C. sorokiniana, C. zofingiensis and Nannochloropsis gaditana*, demonstrating that ASE exhibited significant improvement for lipid and FAME recovery, due to the use of elevated temperatures and pressures. In the EDGE extractor, the reaction cell (Q-cup) is filled with biomass and transferred into a sealed reaction chamber. In the reaction, solvent is pumped into the sealed cell, and the chamber is heated. As the chamber walls are heated, the pressure increases, overcoming the pressure inside the Q-Cup, forcing the solvent to disperse into the sample. This process generates a lot of pressure on the microalgae cell wall, causing its rupture and release of active compounds from the sample [137].

Our findings suggest that, under optimized conditions, accelerated solvent extraction systems can produce similar and/or higher yields than traditional extraction methods with fewer extraction cycles, due to higher extraction temperatures.

4.2.2. Antioxidant activity

4.2.2.1. DPPH assay

DPPH assay was performed on the extracts obtained with microwave assisted extraction, EDGE and milling. Milling shows significant differences (ANOVA, p <0.05) compared to EDGE and MW. As shown in table 13, the type and conditions of extraction can dramatically affect DPPH scavenging activity results. All the extracts produced in the MW extractor presented lower antioxidant activity (max. 15.2%) than those produced in the EDGE extractor (max. 23.9%) or in the *bead-beater* with glass beads (20.3%). Considering the extraction conditions, increases in temperature strongly compromised the antioxidant activity, even if for a short period (120 °C for 30s or 1min in the MW extractor). This may be due to a structural degradation of the compounds extracted by ethanol because of high values of microwave power [135]. The time of extraction, extraction temperature or number of extraction cycles had little effect on antioxidant activity from MW and hexane EDGE extracts (ANOVA, p>0.05). The only factor that significantly affected DPPH scavenging activity of ethanolic EDGE extracts was number of extraction cycles (ANOVA, p <0.05), whereas a higher number of extraction cycles is associated with a higher antioxidant activity, suggesting a more efficient extraction of compounds due to longer exposure[249].

Table 13 - DPPH scavenging activity (%) from *Tetraselmis striata* CTP4 extracts produced with milling (*Bead beater* with glass-beads, BB), EDGE, and microwave (MW) assisted extraction

				Activity (%)		
Solvent	**Temperature (ºC)**	**Time per cycle (min)**	**Cycles**	**MW**	**EDGE**	**BB**
Ethanol	**<RT**	**5**	**5**			**17.8**
	30	5	1		11.2	
	30	5	2		14.1	
	30	10	1	15.2[a]	9.76	
	30	10	2		15.3	
	50	5	2		18.2	
	50	10	1	3.11	8.23	
	50	10	2		16.5	
	50	10	3		23.9	
	50	10	4		13.0	
	100	5	2		16.5	
	100	10	1	5.49	14.9	
	100	10	2		15.7	
	120	0.5	1	1.83		
	120	1	1	3.77		
	160	0.5	1	6.58		
Hexane	**<RT**	**5**	**6**			**20.3**
	50	10	1	6.10	13.9	
	50	10	2	9.44	16.3	
	50	10	3		16.9	
	120	0.5	1	9.28		
	160	0.5	1	15.2		

The work of Herrero et al.[135] analyzed the DPPH radical scavenging activity from *Spirulina* extracts obtained by ASE and demonstrated that non-polar compounds (such as carotenoids) mostly contributed to the antioxidant activity of the microalgae extracts, so hexane extracts generally have a slightly better antioxidant activity when compared to ethanolic extracts. The work of Zhao et al.[253] suggests that shorter extraction times (1 -2 minutes) improves the yield and antioxidant activity of astaxanthin extracted from *H. pluvialis* in comparison to other extraction methods. This is backed up by Pasquet et al.[254] who recommends a maximum extraction temperature of 60 ºC and maximum time 5 min, as well as maximum irradiation power of 50 W, to avoid degradation of compounds.

4.2.2.2. ABTS assay

The ABTS assay was performed on the extracts obtained with microwave assisted extraction, EDGE and milling. Milling shows significant differences (ANOVA, p <0.001) comparing to EDGE and MW. As shown in table 14, ABTS scavenging activity was highest with milling technique (12.5% for ethanolic extract and 26.9% for hexane).

Table 14 - ABTS scavenging activity (%) from *Tetraselmis striata* CTP4 extracts produced with milling (*Bead beater* with glass-beads, BB), EDGE, and microwave (MW) assisted extraction

				Activity(%)		
Solvent	**Temperature (ºC)**	**Time per cycle (min)**	**Cycles**	**MW**	**EDGE**	**BB**
Ethanol	**<RT**	**5**	**5**			**12.5**
	30	5	1		9.27	
	30	5	2		4.23	
	30	10	1	8.79	6.75	
	30	10	2		5.15	
	50	5	2		4.87	
	50	10	1	9.44	3.7	
	50	10	2		3.6	
	50	10	3		6.06	
	50	10	4		4.28	
	100	5	2		4.42	
	100	10	1	9.25	4.59	
	100	10	2		6.38	
	120	0.5	1	7.14		
	120	1	1	7.78		
	160	0.5	1	10.6		
Hexane	**<RT**	**5**	**6**			**26.9**
	50	10	1	14.2	9.34	
	50	10	2	15.8	10.3	
	50	10	3		3.62	
	120	0.5	1	12.2		
	160	0.5	1	18.4		

All the extracts produced in the EDGE extractor presented lower antioxidant activity (max. 9.3%) than those produced in the MW extractor (max. 18.4%) or in the *bead-beater*

with glass beads (26.9%). Microwave assisted extracts were not significantly affected by number of cycles, cycle time, temperature (ANOVA, $p>0.05$). For ethanolic extracts, microwave assisted extraction presented higher scavenging activity when compared to EDGE. Best conditions were 30 ºC, 50 ºC and 100 ºC for 10 min and 160 ºC for 30 seconds (8.79%, 9.44%, 9.25% and 10.63%, respectively). Hexane extracts also had higher ABTS scavenging activity with microwave assisted extraction when compared to EDGE. Best results were obtained at 50 ºC for 10 min with 1 and 2 cycles and at 160 ºC for 30 seconds (14.19%, 15.78% and 18.44%, respectively). ABTS scavenging activity from EDGE extracts were not significantly affected by number of cycles, cycle time, temperature nor solvent (ANOVA, $p>0.05$).

As mentioned previously, it is expected that hexane extracts have higher antioxidant activity when compared to ethanolic extracts, due to extracts obtained by ASE demonstrated that non-polar compounds (such as carotenoids) mostly contributed to the antioxidant activity of the microalgae extracts [135].

These results are in accordance with the work of Roselló-Soto et al.[131] where hexane and acetone extracts were in general the richest in β-carotene and total carotenoids, where highest antioxidant activity was obtained with microwave assisted extraction at 90 °C for 10 min.

Considering the extraction conditions, increases in temperature slightly compromised the antioxidant activity in EDGE extracts but don't seem to strongly affect MW extracts. This may be due to the bioactive compounds that have ABTS scavenging activity being less thermosensitive and being able to endure higher temperatures for a short period (120 °C for 30s or 1min in the MW extractor). In the work from Mahieddine et al.[255] the effect of microwave heat treatments on the chemical composition (polyphenols, flavonoids, and lycopene) in relation with antioxidant activities of tomato fruits was evaluated and it was reported an increase in antioxidant activity with microwave treatment, possibly due to more efficient polyphenol release from the cell matrix by heat treatment. However, these findings should be interpreted with caution as the extracts from milling (performed below room temperature) exhibited higher radical scavenging activity than both accelerated extraction systems in both antioxidant assays and most studies report that thus high microwave power is very efficient in the disruption of cells (allowing target compounds to dissolve more quickly) the structure of the antioxidant compounds is often degraded at

high values of microwave power[256]. In the study of Jaime et al. [257] where yields and antioxidant potential from *H. pluvialis* under different extraction temperatures were evaluated, extraction temperature had a positive influence on the extraction yield, while its effect on the antioxidant activity was negative, lowering the activity of the extracts with every increase of the extraction temperature, in both ethanol and hexane extracts.

In general, the same trends can be observed among the techniques despite all extracts presenting higher activity in ABTS assay when compared to DPPH assay. This is expected as, ABTS radical has a stronger tendency to donate electrons than the DPPH radical [131]. Although both scavenging activity assays exhibited higher scavenging activity for extracts from milling technique, they exhibited inverse patterns regarding MW and EDGE, the differences could be due to the present of various phenolic compounds in extract, which exert different kinetics and reaction mechanisms to different antioxidant activity [258]. Also the mechanism of both assays is slightly different, while ABTS assay is based on the generation of a blue/green ABTS•, which is applicable to both hydrophilic and lipophilic antioxidant systems, the DPPH assay is mainly applicable to hydrophobic systems [259]. Overall, higher extraction temperatures generated higher yields due to efficient cell disruption but strongly impacted antioxidant activity of the extracts possibly due to thermal degradation of compounds.

4.3. Phytochemical characterization by GC-MS

The two extracts which presented highest overall bioactivity (antioxidant and anti-inflammatory properties) were analyzed for their phytochemical composition by GC-MS: (a) *Tisochrysis lutea* ethanolic extract and (b) *Tetraselmis chui* ethanolic extract.

For compound identification, the mass spectrum (MS) of the compounds seen in the chromatogram were compared with those present in the NIST library of compounds. Only compounds with at least 70% matching were considered as identified.

4.3.1. Tisochrysis lutea ethanolic extract

Of the 36 compounds present in the chromatogram of ethanolic extract of *Tisochrysis lutea*, only 5 could be identified, representing a total of 11.8% of the total chromatogram area. The chemical constituents with their retention time (RT), molecular formula, molecular weight (Mw) relative area (%) and matching degree with NIST library (%) in ethanolic *T. lutea* extract are presented in table 15, while the chromatogram is presented in figure 35.

Table 15 - Phytoconstituents of ethanolic *T. lutea* extract.

	RT (min)	Compound name	Formula	Mw	Relative area (%)	Match with NIST library (%)
1	5.22 ± 0.01	NORLEUCINE,N,N,O	C6H13NO2	131	0.58 ± 0.05	90.9 ± 0.2
2	11.7 ± 0.1	Myristic acid	C14H28O2	228	6.06 ± 1.69	87.9 ± 0.6
3	16.9 ± 0.1	Palmitic acid	C16H32O2	256	5.02 ± 0.38	89.2 ± 1.83
4	19.7 ± 0.1	Stearic acid	C18H36O2	284	0.220 ± 0.070	77.4 ± 6.4
Total					**11.8 ± 0.1**	

Note: *n*=3.

From the identifiable compounds, l-Norleucine (2-aminohexanoic acid) is an α-amino acid, which usually is not found in the human body as it is not used to make protein. l-Norleucine is formed from the deamination of l-lysine and has a similar structure to l-methionine although it does not contain sulfur. Downshift of oxygen in hypoxic environment has been reported to trigger the synthesis of norleucine. Norleucine has been reported in dried peaches, high altitude alga *S. porticalis* and *Rhodiola imbricata* root (trans-Himalayan high altitude medicinal herb) [260–263]. Norleucine seems to have a antibacterial, antifungal and antimicrobial activity and some data indicates a protective effect of norleucine to delay encephalopathy in mice[264]. In addition, norleucine is needed

to support proper growth and bone development, maintain nitrogen balance in body, and can act as an anti-inflammatory and antioxidant compound[265].

The other three identified phytocomponents were the saturated fatty acids myristic acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0). These findings were expectable since fatty acids (FAs) are one of the primary metabolites of microalgae. Generally, microalgae are rich in palmitic-(hexadecanoic-C16:0), stearic (octadecanoic-C18:0), oleic (octadecenoic-C18:1), linoleic-(octadecadienoic-C18:2), and linolenic-(octadecatrienoic-C18:3) acids, and only few species can produce significant amounts of PUFA under certain cultivation conditions [266]. While other studies showed palmitic fatty acid (C16:0) to be the predominant fatty acid in most microalgae [266], in the *T. lutea* ethanolic extract the fatty acid with most abundance was myristic (C14:0) followed by palmitic acid. Higher myristic acid is in concordance with the work of Almutairi et al.[267] where *T. lutea* fatty acids profile was analyzed in cultures grown at different salinities and pH values. At the salinity 0.4 M, the dominant fatty acids were myristic acid (21.1%), palmitoleic (3.12%), stearic (9.12%) and arachidonic acid (8.02%). These values can have strong variations according to cultivation conditions, as fatty acid profile changes as a protective response to the stress conditions. In the study of Henry et al.[268], myristic and palmitic acids exhibited good antioxidant activities, while stearic acid was inactive. None of the three fatty acids showed anti-inflammatory properties although myristic, palmitic, oleic and eicosapentaenoic acids can exhibit antibacterial activity[269]. However, an excessive consumption of saturated fatty acids may promote a metabolic inflammatory response [270].

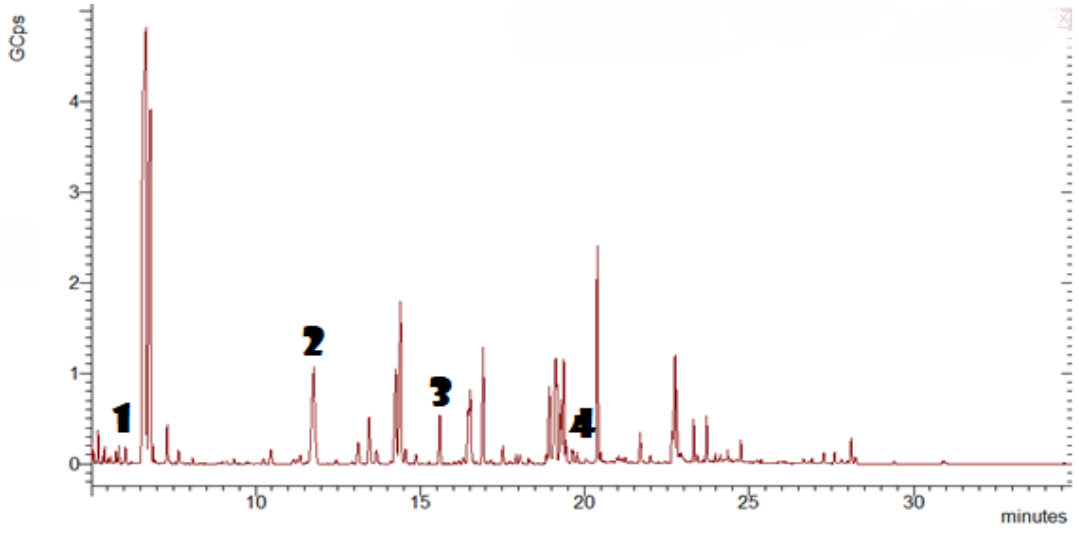

Figure 35 - GC-MS chromatogram of ethanolic extract *of T. lutea*
1- Norleucine; 2 - Myristic acid; 3 - Palmitic acid; 4- Stearic acid, n=3.

Despite the identified compounds having some antioxidant activity, it is likely that other compounds, which could not be identified in this preliminary phytochemical analysis, are also responsible for the antioxidant and anti-inflammatory activity of ethanolic *Tisochrysis lutea* extract, as many other compounds have been reported by other studies[242,271]. In the work of Bustamam et al.[272], the identification of putative metabolites from ethanolic extract of *I. galbana* by LCMS/MS was attempted and a total of 32 compounds were identified, mainly fatty acids (e.g. PUFAs, DHA), pigments (e.g. chlorophyll and fucoxanthin), sterols, sphingolipids, glycerophospholipids and glycerolipids. These compounds have reported bioactivities, such as anti-inflammatory, antioxidant, antibacterial, antiatherosclerotic, antithrombotic and anti-arrhythmic effects[110,273]. The discrepancies in our findings may be related to the type of derivatization used (silylation), as silylating reagents react with compounds containing active hydrogens (while acylating reagents react with highly polar functional groups and alkylating reagents target active hydrogens on amines and acidic hydroxyl groups), which might impact the volatilization of compounds and their identification through GC-MS. The derivatization procedure could be optimized by using multiple derivatizing reagents, which may be more indicated for extracts containing several different compounds with distinct functional groups[274].

4.3.2. *Tetraselmis chui* ethanolic extract

Of the 16 compounds present in the chromatogram of ethanolic extract of *Tetraselmis chui*, only 2 could be identified, representing a total of 21.7% of the total chromatogram area. The chemical constituents with their retention time (RT), molecular formula, molecular weight (Mw) relative area (%) and matching degree with NIST library (%) in ethanolic *T. chui* extract are presented in table 16, while the chromatogram is presented in figure 36.

Table 16 - Phytoconstituents of ethanolic *T. chui* extract.

	Retention time (min)	Compound name	Formula	Mw	Relative area (%)	Match with NIST library (%)
1	16.9 ± 0.1	Palmitic acid	C16H32O2	256	17.5 ± 2.59	84.3 ± 0.6
2	19.7 ± 0.1	Stearic acid	C18H36O2	284	5.97 ± 1.48	77.6 ± 2.0
Total					**21.7 ± 0.9**	

Note: *n*=3.

From the identifiable compounds both were saturated fatty acids: palmitic acid (C16:0) (17.5%) and stearic acid (C18:0) (5.97%). These values concur with data from Mohammadi et al.[275] and Ohse et al.[212] both showing similar amounts of palmitic acid (19%-29%, depending on cultivation conditions) but lower stearic acid content (0.3%-3.6%, depending on conditions) and high amounts of arachidonic acid (C:20). As described previously, these fatty acids have antimicrobial properties and may have antioxidant properties, however an excessive consumption may lead to increases in inflammatory response.

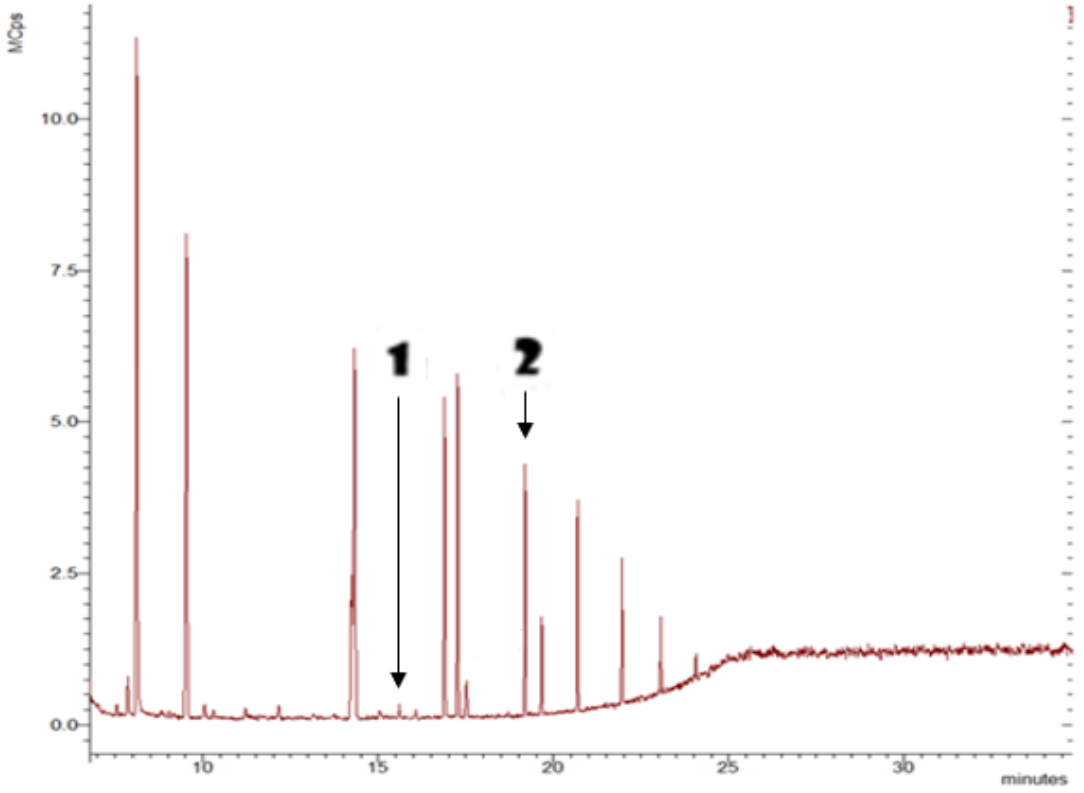

Figure 36 - GC-MS chromatogram of ethanolic extract of *T. chui*
1- Palmitic acid; 2- Stearic acid, n=3.

Despite the identified compounds having some antioxidant activity, it is likely that other compounds, which could not be identified in this preliminary phytochemical analysis, are also responsible for the antioxidant and anti-inflammatory activity of ethanolic *Tetraselmis chui* extract measured in the bioactivity assays. The study from El-Sayed et al.[276] identified many other compounds in GC-MS analysis from *Tetraselmis chui* ethanolic extracts such as benzene (1.42%), *p*-Xylene (1.67%), dodecane (0.80%), tridecane (0.78%), tetradecane (2.64%), pentadecane (1.16%), phenol (0.48%), hexadecenoic acid (5.73%), *n*-propyl hexadecanoate (1.11%), phytol (0.56%), octadecanoic acid (0.70%) and octabenzone (19.18%)[277]. Some of these compounds have known antimicrobial activity (benzene and fatty acids) hypocholesterolemic effect (sterols), antifungal activity (phenols), cytotoxic activity (phenols), and antioxidant activity (phytols and phenols. The lower compound identification in our findings may be related to the type of derivatization used (see above).

4.4. Mineral and Trace Profiling and Proximate Composition Analysis of *Tisochrysis lutea*

4.4.1. Mineral and trace analysis of *Tisochrysis lutea* biomass

Biomass mineral concentrations of *T. lutea* are listed in table 17 together with the mineral composition of other microalgae, found in the literature. Most microalgal biomasses contain considerable amounts of minerals, abundance clearly varying in between species. Generally, all algae had P, K, Na, or Ca as one of the main minerals. Values of Se and Mg were lower in our analyzed *T. lutea* biomass than reported in literature for *T. lutea* (not detectable *vs.* 10.2 µg/g and 4866 ± 94 *vs.* ~6886 µg/g of dry biomass, respectively). However, values of Fe and P in analyzed biomass were higher in comparison to literature values from *T. lutea* biomass (2912 µg/g *vs.* 2284 µg/g and 9944 µg/g *vs* 1252 µg/g, respectively). *T. lutea* had the highest zinc, copper, manganese, and iron content out of the six microalgae species.

Marine microalgae are reported to be richer in sodium than freshwater species [278], which is verified here with *Tisochrysis lutea* (~11 mg/g of dry biomass), *Tetraselmis chui* (~14 mg/g of dry biomass) and *Nannochloropsis gaditana* (25 mg/g of dry biomass). Meanwhile freshwater species *Chlorella vulgaris* and *Arthrospira striata* have much lower sodium contents (2.4 mg/g dry biomass and 2.8 mg/g dry biomass). However, amounts of minerals and trace elements are known to be strongly influenced by species, geographic area, season of the year, and environmental parameters such as growth medium composition [90].

Regarding toxic metals (Li, Hg, Pb, As, Cd), all algae species had negligible amounts, except for the analyzed *T. lutea*, which presented the highest concentrations of As (49.6 ± 3.3 µg/g of dry biomass) and Pb (6.10± 0.35 µg/g of dry biomass).

Table 17 - Mineral composition of *Tisochrysis lutea* biomass and literature values for *Tisochrysis lutea, Tetraselmis chui, Tetraselmis striata* CTP4*, Nannochloropsis gaditana, Chlorella vulgaris* and *Arthrospira sp.*

Note: n=3. n.d.- not detectable

		Element concentration (µg/g of dry biomass)																							
	Species	**Se**	**Zn**	**Cd**	**V**	**Ag**	**Cu**	**Ni**	**As**	**Pb**	**Th**	**Hg**	**Sr**	**Tl**	**Mn**	**Cr**	**Al**	**Ca**	**Fe**	**K**	**Mg**	**Na**	**Li**	**Co**	**Sb**	**Mo**	**P**
Present study	***Tisochrysis lutea***	n.d.	210 ± 3	0.39 ± 0.08	18.6 ± 0.3	n.d.	14.2 ± 0.2	n.d.	49.6 ± 3.3	6.1 ± 0.4	n.d.	n.d.	251 ± 4	n.d.	94.6 ± 2.8	9.31 ± 0.38	7.39 ± 0.69	8736 ± 32	2912 ± 48	11875 ± 150	4866 ± 94	10873 ± 37	n.d.	n.d.	140 ± 5	5.02 ± 2.26	9944 ± 315
Literature review	***Tisochrysis lutea*[273]**	10.2 ± 0.9	27.4 ± 0.5	1.3 ± 0.1			14.9 ± 1.4								56.9 ± 0.2	6.4 ± 0.3		10810 ± 0.6	2284 ± 0.4	11932 ± 0.9	6886.0 ± 1	11092 ±0.4					1252.4 ±1.3
	***Tetraselmis chui*[274]**		13 ± 1				6 ± 0		<0.03	<0.05		<0.04			80 ± 10			33800 ± 260	2010 ± 40	10400 ± 560	5060 ± 90	14330 ± 416	-	-	-	-	6270 ± 187

***Arthrospira* sp.** [272]	***Chlorella vulgaris*** [272]	***Nannochloropsis gaditana*** [275]	***Tetraselmis striata* CTP4** [126]
0.3	0.1	0.1	
19 ± 5	18 ± 8	55	
0.1	n.d.	0.1	
5 ± 0.3	6 ± 1	15	
1.3	0.1	0.68	
0.4	0.1	2.2	
n.d.	n.d.	n.d.	
38 ±1	16 ± 8	69	
7700 ± 300	4100 ± 600	5900	1190
933 ± 61	117 ± 7	627	
14000 ± 900	12200 ± 200	15000	4200
4600 ± 0.2	1100 ± 100	5300	2080
2800 ± 200	2400 ± 200	25000	1180
8300	11000	13000	710

4.4.2. Proximate composition of *Tisochrysis lutea* biomass

The macro composition of *Tisochrysis lutea* freeze dried biomass is presented in table 18. The biomass under study was compared with *Tetraselmis chui, Tetraselmis striata* CTP4, *Nannochloropsis gaditana* and to the well-known microalgae classified by EFSA as food ingredients, *Chlorella* and *Arthrospira*[93].

The protein content in microalgae is reported to range from 30% to 55% of the dry weight [279]. The determined protein content of *T. lutea* (36.7 ± 0.4%) is similar to the values found by other studies where *Isochrysis galbana* ranged between 38% and 40% [111]. Small variations in protein content can be associated with differences in cultivation conditions, as showed in the work of Mohamadnia et al.[280] where *T. lutea* control biomass had 35.2 ± 0.2% of protein but in conditions of mixotrophy this value increased to 39.8 ± 0.2%. In comparison with other microalgal species, *T. lutea* has a typical amount of protein, whereas only *Chlorella* and spirulina had higher amounts of protein. However, these results were expectable as marine species are known to exhibit lower protein content then freshwater species, such as *Chlorella* and spirulina [281].

The analyzed *T. lutea* biomass had an intermediate value of moisture (5.83 ± 0.33%), whereas *Tetraselmis striata* CTP4 had the least amount (3.88%). Ensuring a moisture content below 10% is critical for food safety. Values may vary in between species due to factors such as cell structure and drying methodology [282].

Tisochrysis lutea exhibited a high amount of dietary fibers (15.3 ± 0.1%), which is higher than the previously reported values of ~10% [283]. *Tetraselmis striata* CTP4 had the highest reported fiber content (~ 25%), while *Nannochloropsis gaditana*, *A. platensis* and *Tetraselmis chui* had lowest reported amounts (4.0%, 3.5%, 2.3%, respectively).

Table 18 - Proximate composition of *Tisochrysis lutea* biomass. Values from the literature for *Tetraselmis chui, Tetraselmis striata CTP4, Chlorella vulgaris* and *Arthrospira* sp. are also presented.

Microalgae specie	**Reference**	**Moisture (%)**	**Protein (%)**	**Dietary fibers (%)**
Tisochrysis lutea	Present study	5.83 ± 0.33	36.7 ± 0.4	15.3 ± 0.1
Tetraselmis chui	[284]	6.3	37.6-45.0	2.3
Tetraselmis striata CTP4	[126]	3.88±0.35	31.2±0.5	24.6±3.9
Nannochloropsis gaditana	[212,285–287]	4.7-5.1	31.1-54.9	4.0
Chlorella vulgaris	[288]	5.83	51.45	9.18
Arthrospira striata	[289–291]	4.7-8.0	59.0-64.8	3.5-20.0

Note: n=3.

4.5. Establishment of microalgae as functional food with antioxidant and anti-inflammatory properties

Microalgae have been reported to be rich in dietary fiber, minerals, lipids, proteins, omega-3 fatty acids, pigments, essential amino acids, polysaccharides, and vitamins, making them a well-balanced source of various nutrients[90]. Besides providing nutritional value, microalgae are also known to have compounds with several health-promoting effects, including anti-oxidative, anti-inflammatory, antimicrobial, and anti-cancer effects [90]. The use of algae as functional food, besides contributing to food sustainability, could be an important pathway to improve human health due to their rich composition in macronutrients, but also because of the presence of bioactive molecules[91]. However, the specific compounds and their amount depend on microalgae species and growing conditions [90].

Our findings suggest that *Tisochrysis lutea, Tetraselmis chui* and *Tetraselmis striata* CTP4 have potential antioxidant properties, and, despite the analyzed extracts not exhibiting much anti-hypertensive activity (evaluated through ACE inhibition assay) these extracts might have anti-inflammatory properties (evaluated through COX inhibition assay) that prompt further research. In all assays *Tisochrysis lutea* and *Tetraselmis chui* extracts demonstrated the strongest activities. Nonetheless, *Tetraselmis striata* CTP4 can still be a potential source of bioactive compounds with antioxidant and anti-inflammatory activity.

Our findings also suggest that the analyzed microalgae and their extracts may be used as bioactive ingredients for application in functional food to complement a healthy lifestyle, promoting healthy aging and mitigating diseases, acting either as preventive measure or as complement to treatment. However, the use of microalgae as a food ingredient has some limitations, which are associated with the intense green color and flavor. An excessive consumption of algae (and from unsafe sources) can induce risks (e.g., ingestion of heavy metals, allergic reactions, food poisoning), however a reasonable consumption of algae from safe sources can have several health benefits, mainly antioxidant and anti-inflammatory activity)[292].

5. Conclusion and Future Perspectives

This dissertation aimed to assess the antioxidant and anti-inflammatory potential of three microalgal species (*Tisochrysis lutea, Tetraselmis chui, Tetraselmis striata* CTP4) which might contribute for their establishment as novel functional foods.

Throughout assays it was noticeable that several factors (mainly species and extraction conditions, namely temperature, technique, and solvent influenced the chemical composition of the extracts and their bioactive properties, which indicates that an optimization of extraction process is crucial.

Our findings suggest that all three analyzed microalgal species (*Tisochrysis lutea*, *Tetraselmis chui,* and *Tetraselmis striata* CTP4) have potential antioxidant properties, and that, despite the analyzed extracts' lack of anti-hypertensive activity, they may have anti-inflammatory properties that are worth investigating. Highest bioactivities were obtained with *Tisochrysis lutea* and *Tetraselmis chui* extracts. *Tetraselmis striata* CTP4 could, however, still be a source of bioactive molecules with antioxidant and anti-inflammatory properties.

Our findings imply that the studied microalgal extracts and bioactivities may be worth researching further as bioactive ingredients for functional foods as an addition to a healthy lifestyle, aiding healthy aging and moderating diseases, either as a preventive measure or as a complement to treatment. However, more advanced research is needed to fully assess their potential.

Overall, this dissertation contributed to the current knowledge regarding microalgae potential as novel functional food with antioxidant and anti-inflammatory activities.

However, to further determine the viability of these microalgal extracts as potential functional foods, it is essential to perform extract fractionation to determine the identity of the compounds responsible for the bioactivity, or to effectively select the most promising fraction from extracts. Ideally, the fractioning technique leads to a recovery and purification of the bioactive compound, and its identification. Identified extracts/fractions/compounds should then be subject to *in vitro* bioactivity assays as well as bio-accessibility and digestibility studies, which are important factors when the

incorporation into food products is considered. Then, various studies can be carried out regarding microalgal biomass incorporation into various food products or the development of entirely new food products, optimizing sensory acceptance in food and validating biological properties specifically after digestion occurred.

Another recommendation for future studies is the optimization of extraction and recovery process, as the extraction of target compounds products from microalgae depends on many factors, mainly the class of target component. An accurate selection of the recovery techniques coupled with optimized extractions operating conditions will provide the maximum recovery of the bioactive compound/fractions. Finally, the combination of conventional and non-conventional extraction methods to improve yield recovery and bioactivities can further improve the recovery of biocompounds of interest and should, therefore, be investigated.

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