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"MARINE MICROALGAE AS SOURCES OF BIOACTIVE COMPOUNDS WITH ANTI-INFLAMMATORY ACTION"

European master's in quality in Analytical Laboratories (EMQAL)

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

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2019

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ABSTRACT

Inflammation is a defensive mechanism stimulated when the body is attacked by pathogens or irritants, or when cells are damaged. Sometimes, these defensive mechanisms can go wrong, emerging to different inflammatory diseases, such as acute inflammation and chronic inflammation. Despite the existence of several anti-inflammatory drugs on the market, new drugs with fewer side effects and higher efficacy are required for the treatment of inflammatory diseases. Attention has been given to natural bioactive compounds derived from marine organisms, since these are well known to be a source of potential bioactive compounds with different therapeutic applications in several diseases including inflammatory diseases.

The aim of this research project was therefore to find compounds that can serve as new anti-inflammatory drugs or drug leads in microalgae. For that purpose, water, ethanol and ethyl acetate extracts of different microalgae species (*Porphyridium* sp., *Nannochloropsis* sp., *Tetraselmis* sp. CTP4, *Isochrysis* sp., *Phaeodactylum tricornutum*, *Skeletonema costatum*, *Spirulina* sp., *Haematococcus pluvialis and Tetraselmis chuii*) were characterized for its antioxidant activity as a pre-screening effort to select the most bioactive species/extracts. The most antioxidant extracts (ethanol extracts of *Nannochloropsis* sp., *Tetraselmis* sp. CTP4, and *Tetraselmis chuii* and water extracts of *Nannochloropsis* sp. and *Porphyridium* sp.) were afterwards screened for their anti-inflammatory activity measuring the inhibition of TNF-α production by LPS-stimulated human macrophage-differentiated THP-1 cells (Mac-THP-1). The best results were obtained with the ethanol extract of *Tetraselmis* sp. CTP4 (87% inhibition of TNF-α at 50 µg/mL in respect to the LPS control). This extract was therefore fractionated using liquid-liquid extraction (LLE), and the fractions were re-checked for their anti-inflammatory activity, using the previous method in a bioassay-guided fractionation effort. The most active fraction (the hexane fraction) was later analyzed by GC-MS to tentatively identify some of the compounds present in the fraction that could be responsible for its anti-inflammatory properties. Most of the compounds identified were fatty acids, some of which had already been reported to have anti-inflammatory properties. Further studies are needed to identify the exact compound or compounds responsible for the anti-inflammatory effect in the active fraction. Nonetheless, these

results indicate that microalgae can be a source of compounds with the ability to minimize and reduce inflammation.

Keywords: Microalgae, Inflammation, Antioxidant activity, Bioactive compounds, Solid-liquid extraction, Liquid-liquid Fractionation, GC-MS

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LIST OF ABBREVIATIONS AND ACRONYMS

ABTS: 2.2-Azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid

BHT: Butylated hydroxytoluene

COX: Cyclo-oxygenase

DCM: Dichloromethane

DMARDs: Disease-modifying antirheumatic drugs

DPPH: 2,2-diphenyl-1-picrylhydrazyl

DXM: Dexamethasone

EA: Ethyl acetate

ELISA: Enzyme-linked immunosorbent assay

EtOH: Ethanol

GI: Gastrointestinal

HPLC: High pressure liquid chromatography

HSV-1: Herpes simplex virus type 1

IL: Interleukins

LCPUFAs: Long chain polysaccarides fatty acides

LLE: liquid-liquid extraction

LPS: Bacteria lipopolysaccharides

Mac-THP-1: Macrophage THP-1

MTS: (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)

BSA: Bovine serum albumin

NF: Nuclear factor

nitric oxide: Nitric oxide

NSAIDs:Non seroidal anti-inflammatory drugs

PBS: Phosphate-buffered saline

Phenazine methosulfate: Phenazine methosulfate

PMA: Phorbol-12-myristate-13-acetate

PPF: Pigment-protein fraction

RSA: Radical scavenging activity

RT: Room temperature

SQAG: Sulfoquinovosyldiacylglycerols

TNF-α: Tumor necrosis factor

CO_2:Carbon dioxide

CHAPTER 1: INTRODUCTION

1.1 Marine bioprospecting

Since ancient times, natural compounds have been used in disease therapy and still play a major role in modern medication. About half of the drugs existing nowadays that have been approved since 1994 are based on natural products. It is recognized that plants, microorganisms, marine organisms, vertebrates, and invertebrates are essential sources for medicines[1]-[2]. In the past 30 years, the interest in marine bioprospecting has expanded among researchers in the globe. The marine environment is different from land-based ecosystems and offers great chemical diversity and high biochemical specificity. Very low octanol-water partition coefficient, more routable bonds and stereogenic centers are some of the chemical properties of small-molecule natural products that make them favorable as lead structures for drug discovery[3]-[4]. Marine organisms are therefore known to be as treasures that remain a relatively unexplored source for novel bioactive compounds that could eventually be transferred into therapeutics.

However, as a consequence of the complex molecular structures of natural products, pharmaceutical companies have lately shifted to using synthetic chemical libraries. Hence, clinical trials for modern natural therapeutic products have decreased by 30% between 2001-2008[5]. In addition to structural complexity, drug discovery from natural products faces many other challenges, such as the difficulty of organisms' collection, limited sample quantity, and problems associated with purification and identification of the active agents. When identified, the molecules are often complex which may be too difficult and expensive to produce synthetically. Besides, crude fractions of biological materials are not as amenable to high throughput screening as libraries of pure synthetic compounds[3]-[6]. Despite this decline, the utilization of natural products as a source of novel structures in drug discovery is still in progress. Since the 1980s, 174 new compounds were approved and commercialized, from which 93 (53%) are compounds from natural sources or isolated from them[7].

So far, the Food and Drug Administration (FDA) in the United States has approved three marine-derived drugs, namely Cytarabine **1** (Cytosar-U®, discovered in sea sponges),

Vidarabine **2** (Vira-A®, discovered in sponges), and Ziconotide **3** (Prialt®, discovered in cone snails). The applications for these drugs are cancer, viral infections and pain management, respectively[8].

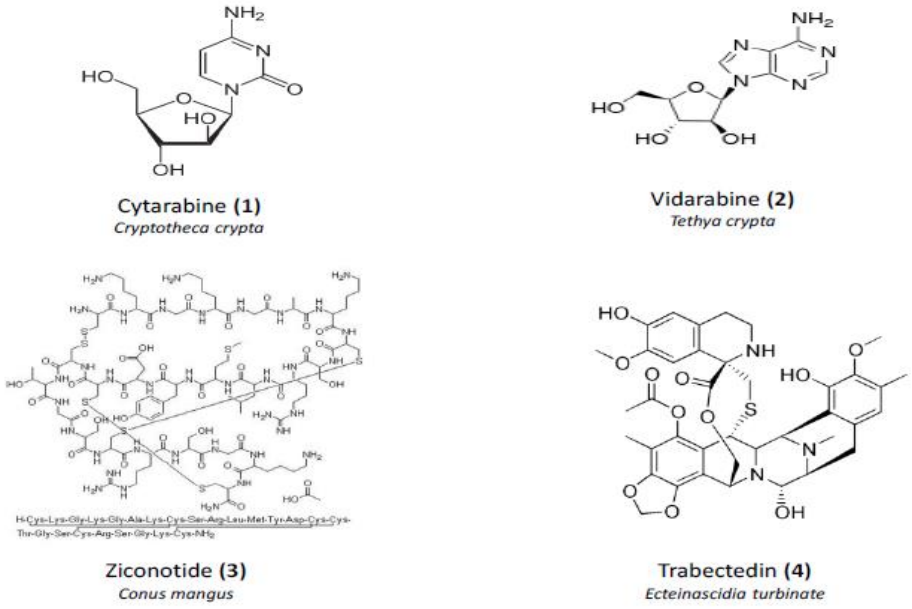

Figure 1: Marine-derived drugs available in the marked[8]**.**

In 2007, the European Union approved a marine-derived anticancer drug, Trabectedin **4** (Yondelis®). In addition, 13 marine-derived compounds are either in phase I, phase II or phase III clinical trials, the pipeline of preclinical trials is giving a hundred novel marine compounds each year those compounds are potentially variable[9]. As mentioned, marine-derived drugs have shown anticancer and antiviral activities. However, marine compounds with antibacterial, anticoagulant, antifungal, anti-inflammatory, antimalarial, antiprotozoal, and anti-tuberculosis effects, in addition to a number of other pharmacological activities are necessary for the treatment and prevention of diseases[10]. With several compounds approved as drugs, and a pipeline of agents in a clinical trial and preclinical evaluation, the marine environment has performed exceptionally well in yielding new drugs. This suggests that the value of marine natural products in new drug discovery will continue to be powerful in the years to come. There are two major methods in the research for the area of the natural products; old - and new methods[11]. The old method focuses on the chemistry of the compounds and the selection of natural sources going through ethnopharmacological information as well as traditional uses. However, the isolation and identification of compounds are being done before biological activity

testing (primarily in *vivo*). The new method, the so-called Bioactivity-guided isolation is, as the name says, highly focused on bioactivity. Biological assays (mainly in *vitro*) are being used to target the isolation and purification of the bioactive compounds. Organisms' selection is going according to ethnopharmacological information and traditional use, but it can be chosen randomly as well. In the new method, natural marine sources are particularly used because marine natural products provide a great chance in the identification of newly bioactive compounds as drugs for different types of diseases treatment.

The Marine Biodiscovery Process includes the biodiscovery pipeline for marine active bioactive compounds which is getting a biological material either via targeted sampling or through access to curated samples. This comprises analyses of the complete organism like sea squirts or sponges, or it could be sediments/cores of sediment from which microorganism can be isolated. In addition, microorganisms can be isolated from water samples to get sufficient biomass for active compound purification; the purified molecules and extracts can then be screened for the biological activity. The development process could be started if a promising result were obtained from the extracts and their purified molecules. However, that does not mean that always, results in a marketable product[12].

1.2 Inflammation

Inflammation is a defensive mechanism stimulated when the body is attacked by for example pathogens, damaged cells or irritants. These responses are important for humans in fighting infections and for starting healing and recovering to normal function in the event of injury. Sadly, these defensive mechanisms sometimes can go wrong, and develop different inflammatory diseases. Inflammation is divided into acute inflammation, which is a short-term process taking place in response to tissue injury, or chronic inflammation, which refers to a slow, long-term inflammation lasting for prolonged periods of several months to years. Acute inflammation starts rapidly, becomes severe in a short time and symptoms may last for a few days, for example, acute pneumonia. It is marked by five cardinal signs: pain, redness, loss of function, swelling and heat. The extent and effects of chronic inflammation, however, may vary with the cause of the injury and the ability

of the body to repair and overcome the damage. Chronic and low-grade inflammatory diseases include rheumatoid arthritis, atherosclerosis, Alzheimer's, asthma, psoriasis, multiple sclerosis, and inflammatory bowel diseases, and numerous of these inflammatory diseases are becoming universal[13]-[14].

As stated by the World Health Organization (WHO), about 235 million people suffer from asthma and it is a widely spread chronic disease among children. Approximately 0.3–1.0% of the general population is suffering from rheumatoid arthritis[15].The three fundamental groups of drugs used for the treatment of inflammatory diseases are corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs), and disease-modifying antirheumatic drugs (DMARDs).

These drugs are commonly used and are effective in treating many inflammatory diseases. Corticosteroids show an important role in the therapy of organ transplantation due to their anti-inflammatory and immunosuppressive effects. However, they are related to some serious side effects. Corticosteroids are recognized to cause Cushing's syndrome, in addition to other negative effects such as hyperglycemia, increased susceptibility to infection, psychiatric disturbances. On the other hand, NSAID, when used for the long term period, can lead to gastrointestinal ulceration and bleeding and platelet dysfunction[14]-[16].

Inflammation is a complicated mechanism and it involves a multifactorial network of chemical signals to mediate the action. The first anti-inflammatory targets include cyclo-oxygenase (COX)-1 and 2 enzymes, cytokines such as tumor necrosis factor (TNF)-α and interleukins (IL-1β, IL-6), and transcription factor as a nuclear factor (NF)-κB and several more. TNF-α and ILs have been recognized for their central role in the pathogenesis of numerous inflammation diseases, especially asthma and rheumatoid arthritis. TNF-α and ILs are intercellular signal proteins secreted by immune cells and have a lot of functions. The TNF-α function is responsible for a diverse range of signaling events within cells, leading to necrosis or apoptosis of the cells during acute inflammation in addition, the TNF-α protein is also important for resistance against infection and cancers. While the functions of ILs (Interleukins) is to regulate cell growth, differentiation, and motility, both are particularly important in stimulating immune responses, such as inflammation. The transcription factor NF-κB is the main regulator of the expression of several genes involved in the activation of inflammation. It is well established that the excessive

production of pro-inflammatory mediators is implicated in several inflammatory diseases. Therefore, inhibition of the overproduction of these mediators is a crucial, exciting target in the treatment of these conditions[17]-[14]-[18]. Herein, it is important to keep searching for new natural compounds possessing anti-inflammatory effects and reducing the side effects since the available drugs have a numerous side effect it's important to keep searching for new drugs with anti-inflammatory properties and less side effects. The most common side effects produced by chemically synthesized drugs are gastrointestinal (GI) and renal effects associated with NSAIDs. However, sometimes the induced gastric or intestinal ulceration can be accompanied by anemia from the resultant blood loss[19].

1. 3 Natural sources of alternative anti-inflammatory agents

It is worldwide recognized that natural products play a major role in the discovery pipeline for making and advancing of drugs for treating human diseases. Thus, natural products provide a great chance in the identification of bioactive compounds as drugs for the treatment of inflammatory diseases. Cyclosporine[20] is a natural product that has been synthesized and developed as a first-line immunosuppressive medicine for the treatment of transplant rejection. The discovery of this compound in 1972 was accidental when it was isolated from the fungus *Tolypocladium inflatum* in Hardangervidda, Norway. Cyclosporine was approved for therapeutic use in 1983 and has since been an important medication, apart from transplants, in the treatment of inflammatory diseases like rheumatoid arthritis and psoriasis. The drug possesses many actions associated with the immunosuppressive activity, but the main action is the selective inhibitory effect on IL-2 and IL-4 gene transcription. This example enlightens the powerful action of natural products as a source for drug discovery[14]-[20].

Lately, many anti-inflammatory pharmacologically active compounds from marine organisms have been identified. These compounds have been isolated and purified from numerous marine sources including sponges, mollusks, bryozoans, sea combs, algae, echinoderms, ascidians, and bacteria[21]-[22]-[23]-[24]. The Red alga *Gracilaria verrucosa* possessed anti-inflammatory activities by inhibiting lipopolysaccharide (LPS)-induced nitric oxide (NO) production, TNF-α, and IL6[25]. Fatty acids can be pro-or anti-inflammatory effects in macrophages by modulating the expression of the genes.

Specifically, n-3 long-chain polyunsaturated fatty acid (n-3 LCPUFA) have been reported to reduce the inflammatory gene expression via inhibition of nuclear factor κB signaling[26].

Astaxanthin, the major carotenoid pigment found in the marine world of algae and aquatic animals, have shown anti-inflammatory properties. Astaxanthin has exhibited anti-inflammatory activities by suppressing the NF-κB activation. It inhibited the production of pro-inflammatory mediators such as TNF-α and IL-1β via doing in vivo studies[27]. Numerous of microalgae species were studied for the purpose of searching for anti-inflammatory bioactive compounds. For instance, sulfolipids from *Porphyridium cruentum* have shown anti-inflammatory activity, the LCPUFAs existing in the crude sulfoquinovosyldiacylglycerols SQAG fraction.[28] The LCPUFAs act as anti-inflammatory compounds through the precursor compounds responsible for the amplification of the inflammation process and increasing the vascular permeability to allow self-propagating responses[29]. *Porphyridium sp*. polysaccharides derived from the red alga proved to have an anti-inflammatory effect, through interfering with the production of tumor necrosis TNF-α -induced inflammation, in human coronary artery endothelial cells[30].The unrefined polysaccharides of *Chlorella Stigmatophora* and *Phaeodactylum Tricornutum*. extracts had anti-inflammatory activity in the carrageenan-in Vivo studies in rats . Violaxanthin, isolated from *Chlorella ellipsoidea* showed an anti-inflammatory effect when tested on lipopolysaccharide (LPS)-stimulated RAW 264.7 mouse macrophage cells. Violaxanthin effectively inhibited LPS-mediated nuclear factor-κB (NF-κB) p65 subunit translocation into the nucleus, suggesting that the violaxanthin anti-inflammatory activity may be based on the inhibition of the NF-κB pathways[31]. The pigment-protein fraction (PPF) from *Nannocloropsis oculate* functioned as an anti-inflammatory extract for virus infection [32].

1.4 Microalgae

Microalgae are a very diverse assemblage of organisms and this diversity is reflected in the wide variety of chemical compounds of potential commercial interest that they produce. Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms that can grow rapidly and live in harsh conditions due to their unicellular or simple multicellular structure. Prokaryotic microalgae include Cyanobacteria (Cyanophyceae), while green algae (Chlorophyta) and diatoms (Bacillariophyta) are examples of eukaryotic microalgae. Over the past few decades, microalgae have gained great interest due to their potential for many different production purposes. They are marine photosynthetic microorganisms capable to convert carbon dioxide into biochemicals that can be further processed into high-value bioactive compounds, biofuel and food [33]. Microalgae can grow rapidly and occur in both aquatic and terrestrial environment, signifying a huge variety of species living in a wide range of condition and habitats. It is estimated that more than 50,000 species of microalgae exist, but only around 30,000, were studied and/or analyzed [34].

The main obligation for the growth of algae is sunlight, water, and nutrients. Microalgae possess the capability to fix CO_2 using solar energy with a performance 10 times better than that of terrestrial plants. The requirements of algae are very less than those of terrestrial plants for growing and they do not compete with agriculture or food for precious resources such as water, nutrient, and arable land[35]. The secondary metabolites produced by microalgae when exposed to severe conditions are being extracted from microalgae with the potential application in biomedicine and pharmacology in the modern field of microalgal biotechnology. The natural active compounds that occur in the microalgal biomass are accountable for distinct biological activities, such as cytotoxic, antibiotic, antioxidant, antifungal, anti-inflammatory and anthelminthic activity[35]-[36]. These days, there is different usage for microalgae, for example, microalgae can be utilized to improve the dietary benefit, they play a vital role in aquaculture and they can be used in cosmetic products[36]. The secondary metabolites delivered by microalgae are vitamins, proteins, lipids, pigments, and carbohydrates – for health, food, cosmetics and biofuel industries[37].

1.5 Biomedical application of Microalgae

1.5.1 Antioxidant activity

Antioxidants are compounds that provide protection for living cells against free radicals. Free radicals are molecules produced when the body breaks down food or is exposed to tobacco smoke and radiation. The anti-oxidant gives electrons to the free radical and neutralize it. The natural secondary metabolites being produced by microalgae have been reported to possess antioxidant activity. As a result, microalgae have come to be a good source for the natural antioxidants, published by recent studies[38].

1.5.2 Anticancer activity

Marine microalgae anti-cancer compounds were under investigation for a long time. The studies were done in the microalgal extracts or fractions obtained using the methods of extraction such as liquid-liquid partitioning or solid-phase extractions. It is uncommon to see dereplication methodologies, fractionations based on high throughput techniques (e.g., HPLC or gas chromatography) or a complete structural elucidation of the compounds that have been found. Despite the low data date available so far, the studies performed on *Chlorella sorokiniana* and *Chaetoceros calcitrans* show low activities compared to the commercially available marine anti-cancer drugs. Most of the pharmaceuticals on the market are active at the level of 0.6–7 ng/mL while the fractions from *C. sorokiniana* and *C. calcitrans* display significant activity at 1–3 µg/mL. Even if fractions and pure compounds cannot be directly compared in terms of activity, the anti-cancer activities of *C. sorokiniana* and *C. calcitrans* extracts seem very promising and appear preferential for further investigation and purification of the active molecules. Despite the low yield (0.0001%) of the active compound in the crude aqueous ethanol extract of the ascidian *Ecteinascidia turbinate*[39], this led to the isolation and development of the anti-cancer drug (trabectedin ET-743).

1.5.3 Antiviral activity

Most of the compounds isolated form microalgae and used as antiviral are carotenoids from *Haematococcus pluvialis* and *Dunaliella salina.* Pressurized liquid extraction (PLE), an environmentally friendly technique, was used to obtain those compounds. The evaluation of the antiviral activity was done against herpes simplex virus type 1 (HSV-1) at different stages during viral infection. Results demonstrated that the use of PLE allows obtaining antiviral compounds from microalgae used as carotenoids sources, which gives both microalgae biomass an added[40]-[41].

1.5.4 Antibiotic activity

Antimycobacterial activity was found in the hexane crude extracts of six microalgae: *Chlamydomonas mexicana, Porphyridium cruentum, Isochrysis galbana, Rhodomonas sp., Aphanocapsa marina, and Nitzschia palea*. All of the hexane extracts were able to inhibit 90% of the growth of *Mycobacterium tuberculosis* H37Rv at a concentration of 100 µg/mL-1. The hexane crude extract of *I. galbana* possessed the highest antimycobacterial activity showing a percentage inhibition that was equal to that of the anti-tuberculosis drug isoniazid and having a minimum inhibitory concentration of 50 µg µg/mL-1. These findings demonstrate that microalgae are an excellent source for the search of novel antimycobacterial compounds[42].

1.6 Microalgae with cosmeceutical uses

Numerous secondary metabolites isolated from algae are recognized for their skin benefits[43]. A worldwide tendency for products considered healthy, environmentally and ecologically sustainable, gained importance in cosmetics industries to fund the research and enhancement of new products with compounds or extracts of natural sources. Algae are natural sources of compounds against oxidative stress and have several efficient protection roles against reactive oxygen species and free radicals, producing compounds

that work in cosmetics towards the harmful effects of UV radiation, and similar acts of organic and inorganic filters that are nowadays being used in the market[43]. The production of both chlorophyll and carotenoids have been increased in *C. vulgaris*, Nostoc, and *Spirulina platensis* when cultivated in the presence of UV radiation[43]. Therefore, these compounds might assist in the protection against oil oxidation in formulations, largely in emulsions with a huge quantity of oily phase, since they possess antioxidant properties. *Fucus vesiculosus* extract is used to decrease the dark circles appear on the skin zone under the eye via stimulating the expression of heme oxygenase-l, a molecule that terminates the heme construction on the skin by removing heme catabolites. The extract with anti-inflammatory activity and antioxidant properties can make a better appearance of eye bags, and collagen production is stimulated which can assist to reduce fine lines and wrinkles. Besides, it can diminish or prevent skin aging by using make-up and sunscreens[44]. Several secondary metabolites of specific microalgae can avoid blemishes, repair damaged skin, treat seborrhea, inhibit several inflammation processes and speed up the healing process, and maintain skin moisture[45].

Furthermore, red algae extracts are being used in hair care, emollient, sun protection, skincare, refreshing or regenerate care products, anti-aging creams, and anti-irritant in peelers[45]-[46]. Algae are mainly incorporated in cosmetic formulations as thickening, water-binding and antioxidants agents. However, more than a single contribution could be attributed to each species, as reported in.[47].

1.7 Natural products drug discovery from microalgae

In this study, a bioassay-guided isolation strategy was applied to obtain molecules possessing anti-inflammatory effects isolated from marine microalgae, which could provide the market with a new natural compound to be used as an anti-inflammatory drug in the future.

The steps needed for the preparation of the material before extraction includes the collection of the microalgal biomass, species identification, and storage of the samples. There are different extraction methods available, however, the selection of the extraction method is based on the nature of the source material and the purpose of the isolation.

Extraction is a process of getting one or more compounds from a solid matrix or pull out of a solute from a liquid to another liquid (liquid-liquid extraction). Solid samples are most of the time being cut into small fragments or ground into fine particles in order to accelerate solvents penetration. Stirring or shaking can be used to advance the diffusion rate. The solvents that were used int this project for the preparation of the extracts were water, ethanol and ethyl acetate the reason behind selecting different solvents is to obtain different compounds presents in the microalgae since the extraction depends on the polarity index of solvents and compounds. The produced extracts can then be tested in the different bioassays depending on the objective. Often, a simple antioxidant test activity is done before testing with other more complex and expensive, such as the anti-inflammatory assays. Afterwards, based on the antioxidant results, the most promising extracts can be selected for further bioactivity (anti-inflammatory) assays. Anti-inflammatory assays can be performed with macrophages stimulated with a pro-inflammatory drug (i.e. LPS). A pre-incubation with the extracts is used to test for anti-inflammatory action through monitoring the inhibition of TNF-α production. Often, human THP-1 cells are used and differentiated into macrophage THP-1 cells (Mac-THP-1). The production of TNF-α is afterward measured using sandwich enzyme-linked immunosorbent assay (ELISA) which is an analytical biochemistry assay, first described by Engvall and Perlmann in 1972. The assay uses a solid-phase enzyme immunoassay to detect the presence of a ligand in a liquid sample using antibodies directed against the protein to be measured. Active extracts can undergo bioassay-guided fractionation until the purification and isolation of active compounds.

1.8 THP-1 cells

The THP-1 cell line (Figure 2: A) is a human monocytic leukemia cell line settled by Tsuchiya et al., in 1980. It was isolated from the blood of a patient suffering from acute monocytic leukemia. THP-1 cells are similar to primary monocytes and macrophages in morphology and differentiation characteristics. THP-1 cells have a large, round single-cell morphology and show distinct monocytic markers. Mostly all THP-1 cells start to adhere to culture plates and differentiate into macrophages (Figure 2: B) after being exposed to phorbol-12-myristate-13-acetate (PMA, also known as TPA,12-O-tetradecanoyl phorbol-13-acetate)

THP-1 cells have some technical advantages over human primary monocytes or macrophages. For instance, their genetic background is homogeneous which minimizes the degree of variability in the cell phenotype. Another technical advantage is that genetic modification of THP-1 cells by small interfering RNAs (siRNAs), in order to down-regulate the expression of specific proteins, is relatively simple[48]. THP-1 differentiated to macrophages have been used as a model in *vitro* at this research for the anti-inflammatory investigation.

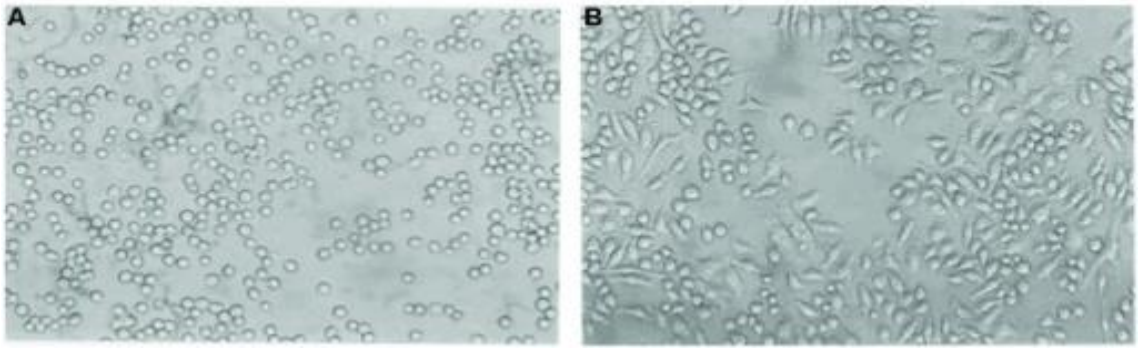

Figure 2:A: THP-cells, B: THP-1 differentiated into macrophages after treatment with PMA.

1.9. OBJECTIVE

The main objective of this master thesis is to search for a new drug lead for anti-inflammatory treatment in extracts of commercialized microalgal biomass (*Porphyridium* sp., *Nannochloropsis* sp., *Tetraselmis* sp. CTP4, *Isochrysis* sp., *Phaeodactylum tricornutum*, *Skeletonema costatum*, *Spirulina* sp., *Haematococcus pluvialis*, *and Tetraselmis chuii*). To fulfill the main objective, several specific sub-objectives were underlined:

1. To prepare ethanol, water and ethyl acetate extracts of the above-mentioned species.
2. To test the prepared extracts for antioxidant activity. A pre-screening for the antioxidant activity will help to identify and select the most promising extracts for the more expensive anti-inflammatory assays;
3. To test the most antioxidant extracts for anti-inflammatory activity, namely by measuring the production of TNF-α by LPS-stimulated THP-1 macrophages;
4. To fractionate the most active extract and produce a fraction with higher activity and less complexity than the initial extract;
5. To perform a chemical characterization of the most active fraction to identify known bioactive compounds (e.g., phenolics, vitamins, and polyunsaturated fatty acids);
6. To tentatively identify a novel drug lead in microalgae extracts.

CHAPTER 2: MATERIAL AND METHOD

2.1 Chemicals

Solvents used for extraction and fractionation: dimethyl sulfoxide (DMSO), methanol, dichloromethane (DCM), ethanol (EtOH), ethyl acetate (EA), hexane, were commercial grade with 96% purity (VWR International, Leuven, Belgium). For the GC derivatization, analytical grade solvents were purchased from the same source. Ultrapure, Type 1 water was obtained using a MilliQ® Water Purification System (Darmstadt, Germany). Roswell Park Memorial Institute medium (RPMI-1640) and fetal bovine serum (FBS), L-glutamine (200 mM), penicillin/streptomycin mixture, Phorbol 12-myristate 13-acetate (PMA), were all from Sigma-Aldrich, St. Louis, MO, USA. MTS 3-(4,5-dimethylthiazol-2-yr)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium was from the Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega Corporation, Madison USA). Butylated hydroxytoluene (BHT), phenazine methosulfate (PMS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) powder were from Aldrich, Sigma (USA), and the TNF-α Elisa kit from PeproTech.

2.2 Algal biomass

Algal biomass of the following species: *Porphyridium* sp., *Nannochloropsis* sp., *Tetraselmis* sp. CTP4, *Isochrysis* sp., *Phaeodactylum tricornutum, Skeletonema costatum, Spirulina* sp., *Haematococcus pluvialis, and Tetraselmis chuii*, were kindly provided by NECTON S.A. (Portugal). This is a company specialized in the cultivation and commercialization of microalgae in Portugal since 1996.

2.3 Preparation of extracts

The biomass of each microalga was extracted with ethyl acetate, ethanol, and water. The ratio was 1g of biomass from each algal species to 40mL of the three different solvents prepared in separated flasks. Five grams of dried biomass was mixed with 200 mL of ethanol, ethyl acetate, or distilled water. The extractions were performed overnight (16 h), at room temperature (RT) with agitation using magnetic stirring. (Figure 3: A). After extraction, the mixture was transferred to 50 mL Falcon tubes (Figure 3: B) and centrifuged (Thermo Scientific, USA) at 726.1g for 5min in order to separate the extracts from the remaining biomass. The extracts were filtered through a filter of 10-12 µm pore size (Prat Dumas, France). This process was repeated three times. Finally, the supernatants were combined and vacuum-filtered, using 0.45 µm and 0.2 µm filters sequentially (Figure 3:C). The solvents were evaporated in a rotary evaporator (IKA, RV10, IKA, Germany) to reduce the volume of the extract (Figure 3:D). The concentrated extract was transferred to a smaller pre-weighed vial and completely dried under a gentle nitrogen flow. The yield was determined, and the extracts resuspended in dimethyl sulfoxide (DMSO) at a concentration of 20 mg/mL and stored at -20ºC until analysis.

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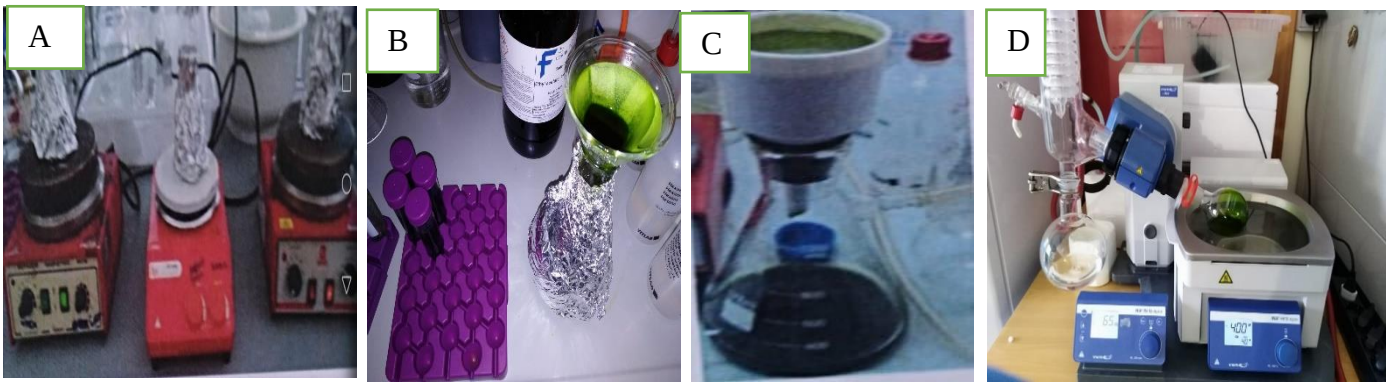

Figure 3:A: Extraction of the biomass using different solvents through stirring at room temperature. B: filtration of the extract. C: the vacuum filtration system consisting of a funnel coupled with a kitasato flask connected to water flow hose. D: evaporating the extract in a Rotary Evaporator.

2.4 Extract fractionation

The most active extract, *Tetraselmis* sp. CTP4 ethanol was selected for fractionation. The fractionation was done by liquid-liquid extraction (LLE), which is a purification technique that is used to separate compounds based on their relative solubilities in two immiscible solvents. Immiscible liquids do not dissolve in each other; they form layers when placed in the same glassware. Immiscibility is a result of two liquids having different polarity. LLE started by mixing the ethanol extract with water (20:80) and extracting sequentially with hexane (H), dichloromethane (DCM), and ethyl acetate (EA) in a separating funnel. The procedure yielded 4 fractions: H, DCM, EA and the remaining ethanol/water (E/W) fraction. For this fractionation, 3 g of the *Tetraselmis* sp. CTP4 - ethanol extract, already prepared, was mixed with 20 mL EtOH and 80 mL of Milli-Q water and placed in a separation funnel. 50 mL of hexane was added to the mixture and shook gently. After phase separation, the hexane (top) layer was collected in a glass round bottom flask (Figure 4: A). The water layer was re-extracted with another 50 mL of hexane and the process repeated until 150 mL of hexane fraction was collected (three extractions in total). After the extraction with hexane, the ethanol/water fraction was sequentially extracted with 3 x 50 mL of DCM and 3 x 50 mL of EA. The remaining fraction (a mixture of ethanol and Milli-Q water) was collected as well. All the fractions were concentrated in a rotary evaporator, transferred to a 45 mL vial size and dried under gentle nitrogen flow (organic fractions) or freeze-dried (E/W fraction). Part of each fraction was re-dissolved in DMSO at 20 mg/ mL for further anti-inflammatory analysis. However, the rest of the fractions were stored dried at – 20°C. All the fractions (hexane, dichloromethane, ethyl acetate, and water-ethanol) (Figure 4: B) were analysed for anti-inflammatory activity on macrophage differentiated THP-1 cells, as described above. The fraction with the highest activity was chemically characterized by gas chromatography-mass spectrometry **(**GC-MS) to tentatively identify the major compounds present, and possibly responsible for the observed anti-inflammatory and antioxidant activity visualized at the start point of the assay processing.

The fractions yield displayed as a percentage (%) in relative to the starting mass of the extract led for fractionation (3 g) and the calculations were done according to the following equation:

$\boxed{(m_1 - m_2)x100}$ where m_1 (g) corresponds to the weight of the extract residue after solvent removal; m_2 (g) is the weight of the dry biomass.

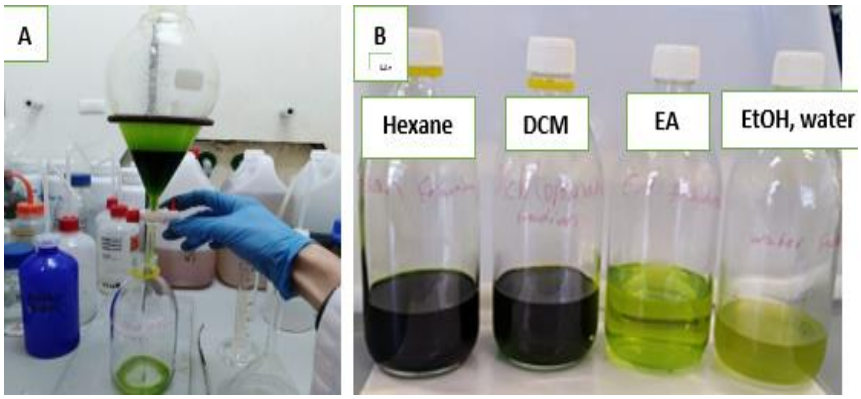

Figure 4-A: The process of collecting the fractions. B: Fractions obtained.

2.5 Free Radical Scavenging Activity

The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity is considered a basic common test for antioxidant activity assessment. DPPH is a stable free radical which has a purple color in solution (Figure 5). When scavenged by antioxidant molecules it is reduced to diphenyl picryl hydrazine, which has a yellow color in solution. This property allows the visual monitoring of the reaction, and free radical scavenging activity can be calculated by measuring the absorption changes at 517nm.

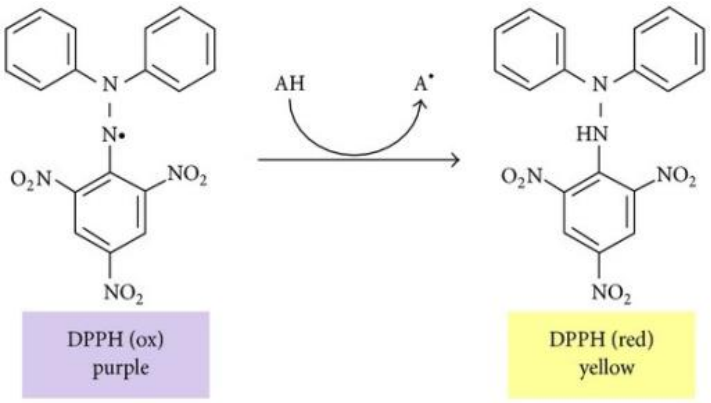

Figure 5: DPPH radical capturing a hydrogen atom from an antioxidant molecule.

The DPPH assay was determined using the method described by Moreno et al. (2006) [49]. A solution of 120 µM DPPH in methanol was prepared daily. The experiment was done on a 96 well plate (WVR, USA) (Figure 6). The extracts were tested at 1, 5 and 10 mg/mL and butylated hydroxytoluene (BHT) was used as a positive control . DMSO was used as a negative control. Extracts (22 µL) were mixed with 200 µL of DPPH solution (120 µM in methanol) in 96-well microplates. After a 30-min incubation period in the dark, the absorbance was measured at 515 nm using a microplate reader spectrophotometer (Biotech 4 Multi-Detection, USA). The antioxidant activity was calculated as the percentage inhibition relative to a blank containing DMSO in place of the samples, using the following equation:

$$\%\ inhibition = \frac{Abs_{neg\ control} - Abs_{test\ sample}}{Abs_{neg\ control}} \times 100$$

Where $\mathrm{Abs}_{\text{test sample}} = \mathrm{Abs}_{\text{test solution}} - \mathrm{Abs}_{\text{color control.}}$

IC_{50}, which is the biochemical half maximal inhibitory concentration, was determined for the extracts with radical scavenging activity (RSA)(antioxidant activity) higher than 50% when tested at 10 mg/mL.

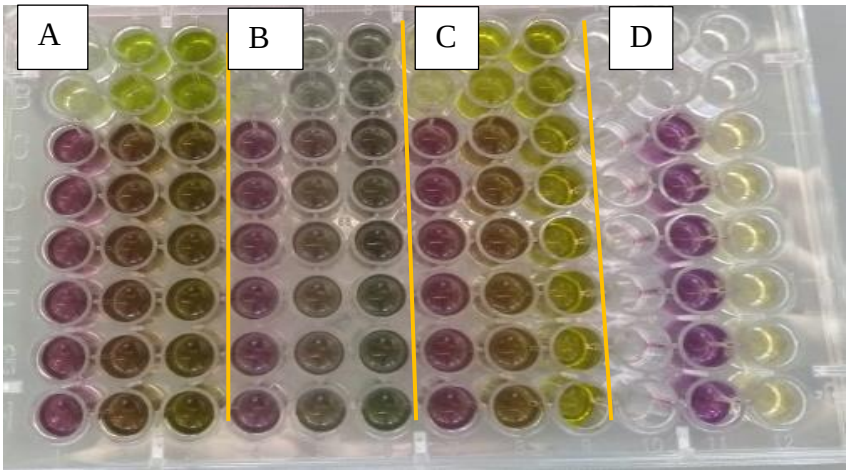

Figure 6: Antioxidant assay performed for ethyl acetate extracts at 10,5 and 1 mg/mL on the same plate. A: *Tetraselmis sp*.CTP4; B: *Skeletonema costatum*; C *Nannochloropsis* sp *oculate;* D: BHT and DMSO as positive and negative controls, respectively.

2.6 Anti-inflammatory assay

2.6.1 Cell cultivation and differentiation

THP-1 cells were kindly provided by Dr. Nuno Santos (CBME, University of Algarve, Faro, Portugal) and were cultivated in RPMI 1640 media containing 1% L-glutamine, 10% heat-inactivated FBS, 1% penicillin-streptomycin (PS), and 1% non-essential amino acids. THP-1 cells were routinely maintained in RPMI 1640 medium at 37℃ in a 5% CO_2 humidified atmosphere. To differentiate the THP-1 monocytes into macrophages (Mac THP-1), cells were plated in 96 wells plates (2.5×10^5 per well) in 200 µl/well of RPMI 1640 medium, supplemented with 25 ng/mL of phorbol 12-myristate 13-acetate (PMA), for 48h.

2.6.1.1 Cell counting

To calculate the volume of cell suspension required to plate $2.5x10^5$cells/well, cells counting was made using a Neubauer chamber (VWR International, Leuven, Belgium) (Figure 7) following the equation:

$$N = \frac{A + B + C + D}{4} \text{ x } dilution\ factor\ x\ 10^4$$

where N represents a number of cells/mL; A, B, C, D correspond to the number of cells in each quadrant of the counting chamber and 10^4 is a conversion factor.

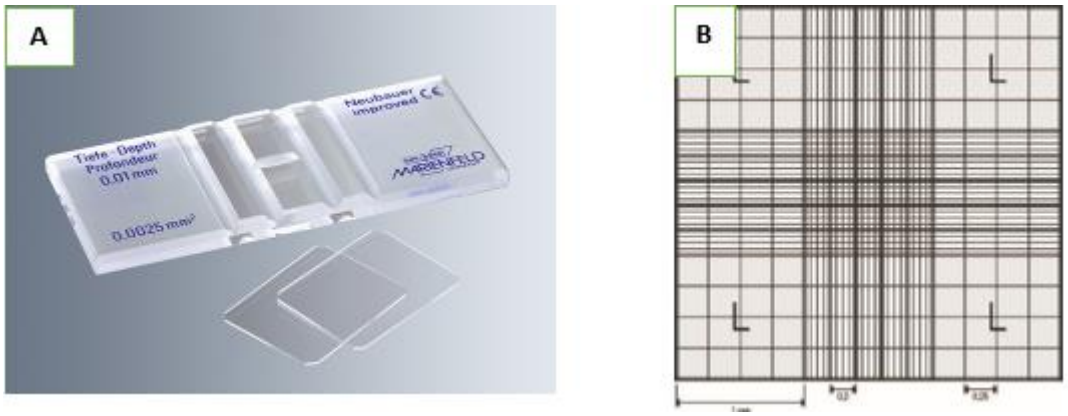

Figure 7: Neubauer counting chamber. The average count of the cells in squares multiplied by 10000 gives a number of cells per mL (A) (source: www.trade21.com; www.fishersci.co.uk)

2.6.2 Cell viability assessment (MTS assay)

Extracts were tested for cytotoxicity on Mac THP-1 cells before the anti-inflammatory assays. Cell viability was assessed using the MTS method (Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation Assay, USA). Mac THP-1 cells were differentiated as described above for 48h. T After this period, the cells were inspected under a microscope to confirm that the monocytes had differentiated to macrophages by adhering to cell culture plate, and that cells were evenly distributed in every well. Then the old media was removed, and new media, containing the five extracts: water extract of *Porphyridium* sp., and *Nannochloropsis* sp. and ethanol extract of *Nannochloropsis* sp., *Tetraselmis* sp. CTP4, and *Tetraselmis chuii* was added. The same process was done to assess the fractions (hexane, dichloromethane, ethyl acetate, and water/ethanol) resulting from the fractionation of the active extract. All were tested at a concentration of 10, 20 and 50 µg/mL. Also, DMSO vehicle at the highest concentration of 0.25% (v/v) was tested to make sure that the vehicle is not toxic. Testing conditions were added to the pre-differentiated cells, and further incubated for 48 hours. Afterwards, 11.9 µL of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-

tetrazolium) plus 0.6 µL of phenazine methosulfate (PMS) to a final volume of 120 µL RPMI media were added. The plate was incubated at 37°C, with 5 % CO_2 for 1h and the absorbance measured at 490 nm using a spectrophotometer (Biotech 4 Multi-Detection, USA). All the values were compared to the control untreated cells, considered to have 100% of cells viability. The cells viability (%) was calculated after the treatments. The average of the blank absorbance was taken first and subtracted from all the measurements on the 96-well plate, to determine the background noise. The average of the control wells (cells without treatment) was calculated representing 100% cells viability. The cell viability of the DMSO control, positive control, and cells treated with the extracts and the fractions were then calculated by the following formula:

% cell viability = $\frac{Abs_{sample} - Abs_{control}}{Abs_{control}} \times 100$

Where Abs_{sample} = absorbance of the extracts/fractions treated with the cells

$Abs_{control}$ = absorbance of untreated cells with the extracts/fractions

2.6.3 Inflammatory assay in Mac THP-1 cells

THP-1 cells were plated and differentiated to macrophages (Mac THP-1) as described in the previous section. After differentiation, cells were treated with extracts/fractions at concentrations of 10, 20 and 50 µg/mL, or not treated corresponding to the basal conditions of Mac THP-1 cells. Also, a positive control to the anti-inflammatory response was performed, by treating the cells with dexamethasone at 2 µM. This pre-treatment was performed for 24 hours. Inflammation was induced by adding 100 ng/mL of LPS to all wells, except for the negative control condition corresponding to cells without treatment, for additional 24 hours. All the extracts were tested in triplicates for each concentration. The conditioned media was collected and centrifuged for 15 minutes at 13.000 rpm and 4ºC to remove the cell debris and kept at -20°C until ELISA.

2.6.4 Evaluation of the anti-inflammatory activity through quantification of TNF-α by sandwich ELISA

The enzyme-linked immunosorbent assay (ELISA) is a commonly used analytical biochemistry assay, first described by Engvall and Perlmann in 1972. The assay uses a solid-phase enzyme immunoassay (EIA) to detect the presence of a ligand (commonly a protein) in a liquid sample using antibodies directed against the protein to be measured. ELISA has been used as a diagnostic tool in medicine, plant pathology, and biotechnology, as well as a quality control check in various industries[50]. The types of common ELISA assays include direct, indirect and sandwich ELISA. A sandwich ELISA assay (Pepro tech, USA) was used in this study. A sandwich ELISA quantify antigens detected by two antibodies (i.e. capture and detection antibody), directed against two epitopes on the target molecule. Either monoclonal or polyclonal antibodies can be used as the capture and detection antibodies in a sandwich ELISA system. Monoclonal antibodies recognize a single epitope that allows fine detection and quantification of small differences in antigen. A polyclonal is often used as the capture antibody to pull down as much of the antigen as possible. The advantage of sandwich ELISA is that the sample does not have to be purified before analysis, and the assay can be very sensitive.

2.6.4.1 Sandwich ELISA assay for the determination of TNF-α production by Mac THP-1 induced by LPS.

Sandwich ELISA was used for the determination of TNF-α production by Mac THP-1 induced by LPS. Capture antibodies Rabbit Anti-Human TNF-α (PeproTech, USA) were plated after been prepared in Phosphate-buffered saline (PBS) to a concentration of 1.0 µg/mL. 100 µL of the solution was added to each well and the plate kept at room temperature overnight. Then, aspiration of the liquid took place and the plate was washed 4 times with 300 µL wash buffer/well (wash buffer: 1xPBS with 0.05 % of Tween-20). Followed by the addition of 300 µl/well of blocking buffer (1 % of BSA diluted in 1x PBS and incubated for 1 hour at room temperature. The PBS concentrated 10x was

prepared from these compounds (15mMof Na_2PO_4, NaH_2PO_4,8.1mM $NaH_2PO_4.2H_2O$, and 1.45M NaCl) in Milli-Q water with 7.3-7.4 of pH.

Briefly, the conditioned media from the samples such as negative control (cells without treatment) were diluted 1:20 while the rest of the samples, including the inflammatory positive control (Mac THP-1 cells treated only with LPS), the anti-inflammatory positive control dexamethasone and the conditions with the tested extracts/fractions, were diluted 1:75. A serial dilution from 2000 pg/mL to zero human TNF-α standard was performed. Thus, the human TNF-α standard concentrations were 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.0156 pg/mL in Assay diluent (0.1% of BSA in 1xPBS containing 0.05%Tween-20). The plate was incubated at room temperature for 2 hours. The plates were washed with 300ul of wash buffer per well 4 times.

The detection antibodies, Biotinylated Rabbit Anti-Human TNF-α solution was prepared to a concentration 50µg/mL in a diluent, and 100 µL was added to all wells. The plates were kept at room temperature for 1 hour followed by washing and addition of 100 µL of avidin-HRP conjugate solution diluted (1:2000) to all wells. The plates were incubated at room temperature for 30 minutes before they were washed with wash buffer 300 µL wash buffer/well, 4 times. Next, 100 µL ABTS (2.2-Azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) liquid substrate solution was added to all wells, and the plate was taken to the Microplate Reader spectrophotometer. Kinetic reading of the absorbance at 405 and 650nm was determined for each well at 5 minutes interval. The concentration of TNF- α in the samples was determined using the calibration curve obtained from the Human TNF-α standard. Also, the percentage of TNF-α inhibition by the extracts and fractions was calculated in respect to the LPS -proinflammatory control, according to the following equation:

$$\% \text{ of TNF- } \alpha \; inhibition = \frac{\mathrm{LPS}_{\text{proinflammatory control}} - \text{Sample}}{\mathrm{LPS}_{\text{proinflammatory control}}} \times 100$$

Where $\mathrm{LPS}_{\text{proinflammatory control}}$ = Quantity of the TNF- α production in Mac THP-1 cells treated only with the pro-inflammatory LPS

Sample=Quantity of the TNF- α production in the cells treated pre-treated with extracts/fractions and proinflammatory LPS.

2.7 Chemical characterization by GC-MS

2.7.1 Chemical derivatization

Based on the anti-inflammatory results of the fractions, the H fractions were selected for chemical characterization by GC-MS. For this purpose, the fractions were derivatized in order to make it more volatile since GC measures the volatile compounds. The derivatization was done according to a modified protocol of Lepage and Roy[51]. This method is based on the direct transesterification with acetyl chloride/methanol, followed by direct extraction of the derivatized compounds into hexane. Briefly, 10mg of each fraction was weighed and treated with 1.5 mL of derivatization solution (methanol/acetyl chloride, 20:1, v/v), in reaction vessels. Afterward, 1 mL of hexane was added, and the mixture heated for 1 hour at 100°C. After cooling in ice baths, 1 mL of distilled water was added, and the organic phase removed and dried with anhydrous sodium sulfate. The extract was then filtered and stored at −20 °C until further analysis.

2.7.2 GC-MS analysis

Fractions were analyzed on an Agilent GC-MS (Agilent Technologies 6890 Network GC System, 5973 Inert Mass Selective Detector) equipped with a DB5-MS capillary column (25 m × 0.25 mm internal diameter, 0.25 μm film thickness, Agilent Tech) using helium as the carrier gas. Samples were injected at 300°C and the temperature profile of the GC oven was 60°C (1 min), 30°C min−1 to 120°C, 5°C min−1 to 250°C, and 20°C min−1 to 300°C (2 min). For the identification of the compounds present in the fractions, the total ion mode was used.

2.8 Statistical analysis and data interpretation

2.8.1 Statistical analysis for radical scavenging activity

The Abs color control is the Abs recorded for the (DMSO) negative blank control. The IC_{50} values were calculated for those species in which antioxidant inhibition was more than 50%. IC_{50}. The half-maximal inhibitory concentration is a measure of the potency of a substance in inhibiting a specific biological or biochemical function. IC_{50} values were estimated with GraphPad Prism v.7.0, by the sigmoidal fitting of the data.

2.8.2 Statistical analysis of sandwich ELISA assay

The results are presented as a mean ± standard deviation; all analyses were performed in triplicate in three independent experiments. Differences between species and the LPS control were assessed using analysis of variance (one-way ANOVA by Dunnett's multiple compressions tests). Significant differences were considered when $p < 0.05$ by means of the statistical program Graph Pad Prism (release 7.0).

2.8.3 Statistical analysis for the GC-MS

Bruker MSWS Software was used with integrated National Institute Standard and Technology (NIST) library to identify the compounds present in the active fraction Components' relative percentages were calculated based on GC peak areas in respect to the total area of peaks.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Screening for antioxidant activity

The screening for the antioxidant activity was performed with the ethanol (EtOH), ethyl acetate (EA) and water (W) extracts obtained from *Porphyridium* sp. (*POC*), *Phaeodacytlum tricornutum* (PHA), *Nannochloropsis* sp. (NANNO), *Tetraselmis* sp. *CTP4* (T.CTP4), *Isochrysis sp.* (ISOG), *Skeletonema costatum* (SKLT), *Spirulina sp. (SPR), Tetraselmis chuii* (T.Chuii), *Haematococcus pluvialis* (*H Pluvialis).*

Thus, extracts were tested for antioxidant activity with the DPPH assay. where the presence of compounds that are able to neutralize the free radical DPPH is determined. All the values obtained were presented as a percentage, compared to the positive control butylated hydroxytoluene (BHT)[52]. All the samples were analyzed at concentrations of 1, 5 and 10 mg/mL. The response of all the extracts was dose-dependent (Table 1), that is, DPPH scavenging activity was increased by the increasing concentration of the antioxidant compounds present in the samples[53].Since all the extracts were dissolved in DMSO prior to the experiment, DMSO was used as a negative control as well, tested at the same concentration as in the extracts. Due to its medium polarity, DMSO is a preferred solvent in extract testing assays for a wide range of compounds, as it can dissolve both polar and nonpolar compounds; in addition, it is miscible with water[54]. The results for the average antioxidant activity are summarized in Table 1. The best results were accomplished was with the EA extracts, showing the highest antioxidant activity when compared to the positive control BHT and to other water (W) and ethanol (EtOH) extracts.

3.1.1 Screening of ethyl acetate extracts for their antioxidant activity

The POC EA extract results reached to 21.4%, 89.6% and 104% for the 1,5 and 10 mg/mL respectively. These values indicate that the best results were achieved with POC EA with considerably higher antioxidant activity when compared to other extracts (Table 1). The antioxidant activity for POC EA reached 104 % and 86.6% at 10 and 5 mg/mL respectively, showed higher activity when compared to NANO EA 81%, *Tetraselmis* sp. CTP4 EA 37%, and SKLT EA with 72.3% at 10 and 5 mg/mL concentrations. However, in the case of PHA EA and ISOG EA, good results were obtained with 10 mg/mL, reaching the antioxidant activity of 94% and 97%, respectively. These values were significantly higher when compared to POC EA (89%) at 5mg/mL.

Some previous studies showed the apparent antioxidant activities of polysaccharides isolated from other *Porphyridium* species (*P.cruentum)*, however, they also revealed that the molecular weight (MW) of the polysaccharides was related to their biological activities[55]. *Phaeodactylum tricornutum.*.was also reported possessing an antioxidant activity by measuring the phenolic content and carotenoid content [56]. Also, *Isochrysis galbana* methanol extract displayed antioxidant activity. It was indicated that the total phenolic and carotenoid content play a vital role in the antioxidant activity[57]. *Skeletonema costatum* was reported to have a moderate antioxidant activity influenced by their carotenoid and phenolic continent[58]. However, *Nannochloropsis oculata*, *Tetraselmis sp. CTP4* and *Tetraselmis chuii* were not previously assessed for antioxidant activity.

3.1.2 Screening of water extracts for their antioxidant activity

Water extract of *Porphyridium* sp. (POC W) also showed good results in terms of scavenging activity with 87.5% at 10 mg/mL.

And the results obtained at 5 mg/mL showed 32.1% of antioxidant activity was higher when compared to *Tetraselmis* sp. CTP4 W (23.24%) and PHA W (24.17%), both at 10 mg/mL concentration. On the other hand, ISOG W and NANNO W antioxidant activity results were 42% and 51.9% respectively at 10 mg/mL concentration. This value showed

significantly higher antioxidant activity when compared to the other water extracts at 10 mg/mL (Table 1).

3.1.3 Screening of ethanol extracts for their antioxidant activity

The best antioxidant activity for the ethanol extracts tested was for *Haematoccoccus pluvialis* with antioxidant activity of 103% and 77% at 10 and 5 mg/mL, respectively. These values with antioxidant activity at 5 mg/mL were higher compared to *POC* antioxidant activity T. CTP4. ISOG. PHA, SKILT, *SPR. sp.* and T. *Chuii.* All at 10 mg/mL as shown in Table 1. Despite of having a high antioxidant activity *Haematoccoccus pluvialis* was not selected since it was reported that astaxanthin produced by this species possess both antioxidant and anti-inflammatory [59].

On the other hand, NANNO was an exception, presenting significantly lower antioxidant activity with 80.37%, when compared to *H. Pluvialis* at 10 mg/mL for both of them (Table 1). High antioxidant activity of *Haematoccoccus pluvialis* ethanol extract might be due to the carotenoid astaxanthin, as reported previously, and it could be a promising source to be used as a natural alternative antioxidant agents[59]. Also, *Spirulina platensis* is reported to be considered as a potential antioxidant agent[60].These results go in the same line in term of antioxidant activity possessed by most of the species screened here. However, *Tetraselmis sp. CTP* and *Tetraselmis chuii* both ethanol extracts were not reported before for antioxidant activity. Nonetheless, *Nannochloropsis sp.* was reported to be a potential source for antioxidants compounds due to the presence of a substantial amount of polyunsaturated fatty acids and carotenoids[61].

Table 1: Radical Scavenging Activity (RSA) (antioxidant activity) of DPPH free radicals of water (W), ethanol (EtOH) and ethyl acetate (EA) extracts of the different microalgal species, applied at three concentrations (1, 5 and 10 mg/mL). nd – no activity detected. Values are presented as mean ± standard deviation.

Extract	**Solvent used**	**DPPH**		
		1 mg/mL	5 mg/mL	10 mg/mL
Porphyridium sp.	Water	5.02 ± 1.75	32.1 ± 5.6	87.5 ±10.0
Porphyridium sp.	EtOH	0.49 ± 1.17	30.36 ± 1.33	59.1± 1.0
Porphyridium sp.	EA	21.4±1.9	89.6±0.8	104.5±2.6
Nannochloropsis sp.	Water	nd	46.8 ± 9.4	51.0 ± 5.2
Nannochloropsis sp.	EtOH	3.95 ± 2.97	53.7 ± 2.6	80.3±2.3
Nannochloropsis sp.	EA	4.72±0.91	36.8±1.5	81.7±2.2
Tetraselmis sp. CTP4	Water	00.2 ± 5.0	12.0±2.3	23.2 ± 3.7
Tetraselmis sp. CTP4	EtOH	8.72 ± 0.70	36.0± 1.4	58.1 ±1.5
Tetraselmis sp. CTP4	EA	0.14±2.35	17.12±1.70	37.8±3.2
Isochrysis galbana	Water	nd	21.3 ± 3.5	42.2± 3.3
Isochrysis galbana	EtOH	1.69 ± 1.31	21.8± 1.2	41.6 ± 0.2
Isochrysis galbana	EA	17.2± 2.6	51.9±2.0	97.8±2.1
Phaeodactylum tricornutum.	Water	0.50 ± 0.57	12.3± 1.2	26.8 ± 2.2
Phaeodactylum tricornutum..	EtOH	0.05 ± 1.67	32.4 ± 1.4	61.8 ±1.3
Phaeodactylum tricornutum..	EA	13.2±1.4	61.7± 2.1	94.9±3.3
Skeletonema sp.	Water	nd	16.5 ± 2.9	24.1 ± 3.1
Skeletonema sp.	EtOH	4.9 ± 0.8	36.0 ± 2.0	71.1 ± 1.2
Skeletonema sp.	EA	23.9± 1.7	60.0± 2.3	73.3± 7.25
Spirulina sp.	EtOH	15.6 ± 3.5	49.4 ± 2.8	71.5 ± 0.3
Tetraselmis chuii	EtOH	22.2± 5.1	45.3 ± 0.4	66.8 ± 1.0
Haematococcus pluvialis	EtOH	4.94 ± 7.63	77.2 ± 54.1	103.6± 58.2
BHT		94.1±2.8		

3.1.4 Determination of IC_{50} for the extracts with antioxidant activity above 50%

The IC_{50} was determined for the extract exerted antioxidant activity higher than 50%. As shown in Figure 9. The best IC_{50} achieved was with *Porphyridium* sp.(POC).ethanol extract, *Porphyridium* sp.(POC)water extract, *Nannochloropsis* sp.(NANNO) ethanol extract, *Skeletonema* sp.(SKILT) ethanol extract, *Skeletonema* sp. (SKILT) ethyl acetate extrct, *Phaeodactylum* sp.(PHA)ethanol, *Phaeodactylum* sp.(PHA) ethyl acetate,, and *Haematococcus pluvialis*,(H.pluvialis) (Figure 8).

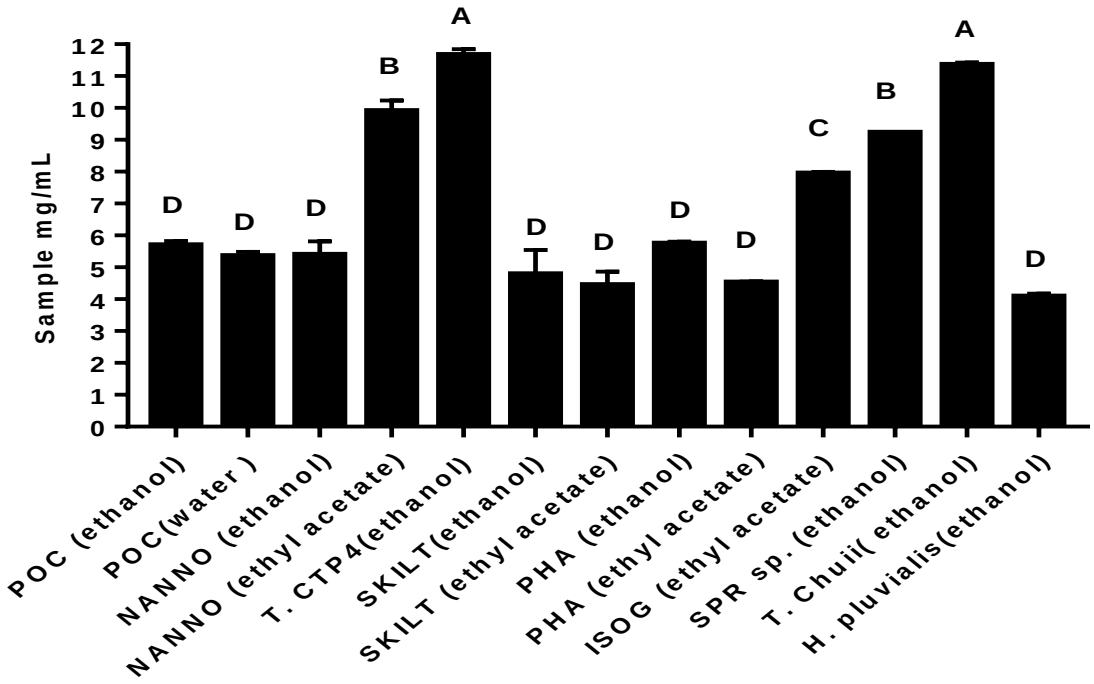

Figure 8:Radical scavenging activity (**IC_{50}**: mg/mL) of ethyl acetate, ethanol, and water extracts of the different microalgae screened values are mean with standard deviation values not sharing the same letter are significantly different from one another ($P < 0.05$).

Most of the extracts contain a lot of compounds such as phenolic, polysaccharides, carotenoids, polyunsaturated fatty acid, and vitamins, and all of these compounds were reported to be responsible for providing antioxidant activity partially or completely[62]-[55]-[63]. However, other studies showed that carotenoids are a major contributor to the antioxidant activity in some species[64].

The interest in compounds possessing high antioxidant activity from natural sources is constantly growing. The reason behind this is the role they play in the protection against oxidative stress, which has been associated with several chronic disorders with a special focus on neurodegenerative diseases[65]. *Porphyridium sp, Isochrysis galbana* and *Phaeodactylum sp* EA extracts and *Haematococcus pluvialis* ethanol extract showed similar or even higher activity to that of synthetic antioxidant BHT (showed in Table 2), used here as a positive control. BHT is a lipophilic organic compound derived from phenol, active for antioxidant activities[66].

The high antioxidant activity for the ethyl acetate extract could be due to the high efficiency of the solvent in solubilizing flavonoids, which are polyphenolic compounds present in the extract prepared[67]. However, *H. pluvialis* ethanol extract showed the highest activity compared to BHT and all extracts. This could be due to the fact that ethanol is considered a good solvent for extracting polyphenols present in the microalgae species, which might be responsible for the antioxidant property[68].

Despite the high antioxidant activity for the ethyl acetate extracts, these were excluded from further analysis of anti-inflammatory screening activities, due to the evidence that non-polar extracts are often more toxic to cells lines[69]. Therefore, the ethanol extracts of *Tetraselmis* sp. CTP4, *Tetraselmis chuii, and Nannochloropsis sp*., and the water extracts of *Porphyridium* sp., and *Nannochloropsis* sp. were chosen to be screened for the anti-inflammatory activity in the next step of this study. The microalgae extracts exerting the high antioxidant activity are a potential new source of natural antioxidants and other bioactive compounds. However, apart from ethyl acetate extracts, some other ethanol extracts were not selected. This was due to that they or their compounds were already studied for the anti-inflammatory effect.[70]-[71]-[72]-[73].

3.2 Anti-inflammatory assays

3.2.1 Cell viability assessment for the extracts

For the MTS assay, Mac THP-1 cells were used to assess potential cytotoxic effect of the microalgae extracts. In the anti-inflammatory assays, compounds tested should not exhibit any observable toxic effect on the Mac cells, as cells can release the inflammatory marker TNF-α, which is then quantified using ELISA sandwich method. Extracts and were applied for 48h in different concentrations (10, 50 and 100µg/mL).and DMSO was also applied separately at 0.25% (v/v) concentration. Then cytotoxicity was assessed using the MTS assay. The cell viability results as showed in Figure 10 indicate that most of the extracts screened for the cytotoxicity were non-toxic, as the reduction of the cell viability did not go below 80%, except for the water extracts for both *Porphyridium* sp. And *Nannochloropsis*s sp.

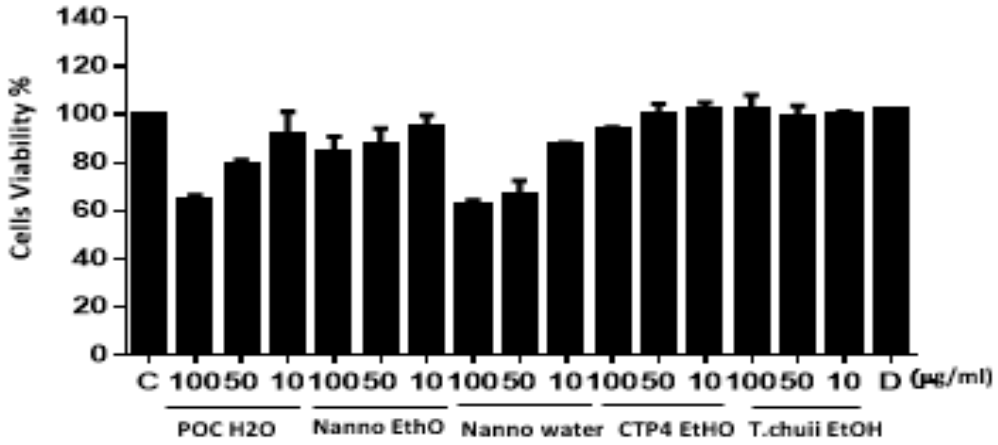

Figure 9: **MTS cytotoxicity assay screened at 100, 50 and 10 µg/mL for *Porphyridium* sp. Water (POC (Water)), *Nannochloropsis oculate* ethanol (NANNO ethanol) *Nannochloropsis* oculate water NANNO water), *Tetraselmis sp. CTP4* ethanol (T. CTP4 ethanol) *and Tetraselmis Chuii*e ethanol (T. chuii ethanol). C: negative control (cells without treatment. D: DMSO (DMSO treated with the cells only).**

Applying MTS reagent on Mac THP-1 cells, large amounts of a brown formazan were produced upon incubation with above-mentioned extracts at different concentrations. This indicates normal cell metabolism and normal mitochondrial integrity. Ethanol extracts of all species tested at the tested concentrations of 10, 50 and 100 µg/mL, did not show any toxic effect to Mac THP-1 cells, with cell viability above 80%. However, water extracts of both of *Porphyridium sp.* and *Nannochloropsis sp.* were slightly toxic at 100 µg/mL reducing cell viability of Mac THP-1 to approx. 60%. Due to this, the concentrations used for further anti-inflammatory screening were 50, 20 and 10 µg/mL for all extracts (Figure 9).

3.2.2 Cytokines targeted by anti-inflammatory drugs

Macrophages have an important role in the starting, maintaining and resolution of the inflammation caused by the stimulation with the lipopolysaccharide (LPS), which is a bacterial endotoxin. Macrophages respond to the LPS stimulation by secreting large amounts of pro-inflammatory cytokines such as TNF-α, IL-1 which in turn further increase the inflammatory response via further activation of macrophages. This is the reason behind targeting cytokines released by macrophages in therapeutic interventions, for the purpose of controlling the inflammatory response[74]. Most of the anti-inflammatory drugs used are antagonists of the inflammatory cytokines, such as IL-1-receptor antagonist (anakinra) and TNF-α antagonists (infliximab, etanercept, and adalimumab)[75]. Despite the fact that anti-inflammatory drugs from natural compounds showed remarkable success, the cost and numerous adverse effects they cause still limit their application[74]. In this study, one of our objectives was to suppress the TNF-α cytokine production by the THP-1 differentiated macrophages (Mac THP-1), for the application of extracts obtained from different microalgae species as an anti-inflammatory. ELISA assay was used to detect and quantify the production of TNF-α cytokine. In addition, dexamethasone (DXM), which is a well-known anti-inflammatory drug, was used as a positive control in the study.

3.2.3 The screening of ethanol extracts for anti-inflammatory activity against THP-1 macrophage cells (Mac THP-1)

Anti-inflammatory activity screening of ethanol extracts, previously shown to be non-toxic to Mac THP-1 cells, *Tetraselmis* sp. CTP4 (*T.*CTP4), *Tetraselmis chuii* (*T.*chuii), and *Nannochloropsis sp.* (NANO), were assessed against human THP-1 cells differentiated to macrophages (Mac THP-1) . Mac THP1- cells were pre-treated with the extracts and dexamethasone (DXM), which is being used as an anti-inflammatory [76] drug. In addition, cells treated with LPS only, without any pre-treatment was used as a positive control to the inflammatory reaction.

Three experiments were performed in triplicates for each concentration tested and the results are expressed as the concentration of TNF-α per experimental condition (Figure 11), and as the average inhibition of TNF-α (%) in respect to the LPS-induced proinflammatory control (Table 2) (Figure 10).

Both *Tetraselmis* sp. CTP4 and *T. chuii* ethanol extracts showed an interesting TNF-α downregulation in respect to the LPS control at 50 µg/mL, reaching 82% and 47% respectively. *Tetraselmis* sp. CTP4 showed a dose-dependent response with a TNF-α inhibition of 55% and 46% at 20 and 10 µg/mL, respectively. The *T. chuii* showed similar anti-inflammatory activities at 20 and 10 µg/mL, with reduction of TNF- α from 49% to 53% respectively. The reduction of the positive control (DMX) at 2µM reached 57% in respect to the LPS control. These values suggest that *Tetraselmis* sp. CTP4 at the highest concentration tested of 50 µg/mL, had the best TNFα inhibition activity, even when compared to the positive DXM control. *T.chuii* ethanol extract showed the highest inhibition of TNF-α at the lowest concentration (10 µg/mL), compared to the other two concentrations used, with suppression reaching 54% (Table 2). This might be due to several reasons since we are dealing with a complex natural extract. One of the reason, which might cause this higher inhibition at the lower concentration, is due to some compounds being present in the extract, that has the ability to mask the agents which are responsible for the anti-inflammatory effect. However, at the lowest concentration, the effect of the inhibition is similar to the positive control, which exhibited 53% of TNF- α inhibition. TNF- α inhibition might be increased by fractionation of this extract. To our

knowledge, this is the first time that *Tetraselmis* sp. CTP4 and was screened for the anti-inflammatory activity. One reason for this is the fact that *Tetraselmis* sp. CTP4 was recently isolated and identified[1]. However, both *Tetraselmis* sp. CTP4 and *T.*Chuii presented statistical significance with respect to the LPS control group.

Regarding the *Nannochloropsis sp.* (NANO) ethanol extract, the results of TNF-α suppression reached 31%, 26 and 40% (Table 2) (Figure 10) at the 50, 20 and 10 µg /mL, respectively. These results were not as promising as the previous two extracts, but it is noteworthy to mention that the highest suppression rate of TNF-α occurred at the lowest concentration. Both the highest and the lowest concentrations tested were statistically different when compared to the LPS control (Figure 10). However, no statistical difference between control and NANNO ethanol extract was observed at 20 µg/mL, with 26% of TNF-α inhibition. As mentioned previously, there are many different compounds present in natural complex extracts, and sometimes, the presence of antagonistic receptor ligand, blocking or dampening a biological response could be responsible for the observed effect.

For further investigation of this issue, fractionation needs to be performed in order to reduce the number of compounds with potential anti-inflammatory activity in each fraction, so that the identification of responsible compound for the observed effect could be facilitated.

Much of the current literature on extracts suppressing TNF-α produced by Mac THP-1 induced inflammation pays particular attention to bioactive compounds responsible for this activity. Methanol extract of *C. vulgaris* was reported to be significant different TNF-α inhibitor when compared to LPS control at concentration 125 µg/mL tested. This suggests that *C. vulgaris* has anti-inflammatory activity and could be a potential source of anti-inflammatory agents of natural origin[77]. Moreover, other species that were reported to have TNF-α inhibition were *Cylindrotheca closterium , Odontella mobiliensis, Pseudonitzschia pseudodelicatissima, C. actinocyclus, and A. minutum*. All these species showed a significant decrease of TNF-α when compared to the LPS control at 10, 25, 50 and 100 µg/mL[78]. However two tested concentrations for that studies are similar to the concetraions that we used(10 and 50 µg/mL) and they showed significance compared to the LPS control. This is a reason to believe that our results with *Tetraselmis* sp. CTP4 are very promising with possible use as an anti-inflammatory agent. Since the

most promising result was obtained from *Tetraselmis* sp. CTP4 ethanol extract, and the suppression of TNF-α was concentration-dependent, this extract was selected for fractionation.

Table 2: % of TNF-α inhibition caused by ethanol extracts at 50, 20 and 10 µg/mL in Mac THP-1 cells, relative to the LPS control, data are presented in percentage and standard deviation.

Extract	**Concentration**	**%**
Dexamethasone	2µM	57.0±8.6
Tetraselmis sp. CTP4 ethanol	50µg/mL	83.0±9.4
Tetraselmis sp. CTP4 ethanol	20µg/mL	56.0±5.1
Tetraselmis sp. CTP4 ethanol	10µg/mL	46.0±6.7
Tetraselmis chuii ethanol	50µg/mL	47.0±12.4
Tetraselmis chuii ethanol	20µg/mL	49.0±9.6
Tetraselmis chuii ethanol	10µg/mL	54.0±5.0
Nannochloropsis oculate ethanol	50µg/mL	31.0±16.1
Nannochloropsis oculate ethanol	20µg/mL	26.0±17.8
Nannochloropsis oculate ethanol	10µg/mL	40.0±2.6

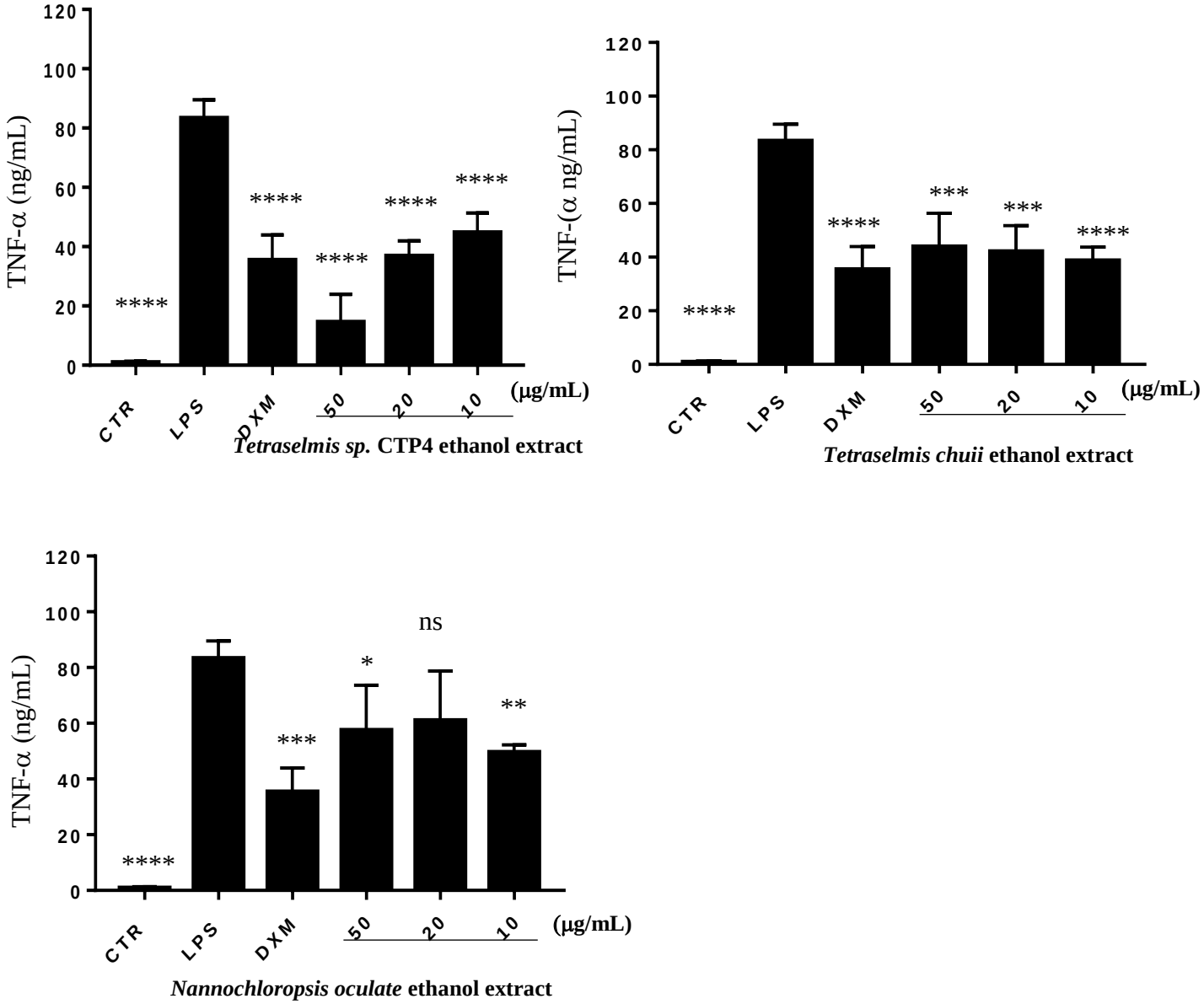

Figure 10:Anti-inflammatory activity of extract, *Nannochloropsis oculate* , *Tetraselmis sp.* CTP4 and *Tetraselmis chui* sp. all are ethanol extracts, in Mac THP-1 cells stimulated with LPS, as determined by levels of TNF-α in the cell culture media. Data are the means ± S.D. of three independent experiments. * $p < 0.05$, ** $p < 0.005$, ***$p < 0.0005$, ****$p < 0.0001$ vs LPS control. CTR, negative control (cells without treatment); LPS, LPS pro-inflammatory control; DXM, Dexamethasone positive anti-inflammatory controls: ns, no significance, (50, 20 and 10µg/mL): concentrations tested of each extract.

3.2.4 The screening of water extracts for anti-inflammatory activity against THP-1 macrophage cells (Mac THP-1)

During the next step of this study, water extracts obtained from *Porphyridium* sp. (POC) and *Nannochloropsis* sp. (NANNO) were screened for anti-inflammatory activity in Mac THP-1 cells. However, obtained results were not as promising as the ethanol extracts described in the previous section. The results achieved for the TNFα inhibition by POC were 32%, 4% and 20 % of inhibition for the 50, 20 and 10 µg /mL, respectively (Table 2). Yet, these results were not significantly different in comparison to the LPS control except for the highest concentration (50 µg /mL) (Table 3)(Figure 11). A previous study has described a potent anti-inflammatory activity of isolated polysaccharides from this microalga on Angiotensin II-stimulated human coronary artery endothelial cells[79]. However, the water extract used in this study is not expected to contain a high amount of polysaccharides, since their solubility in the water is low due to the long chains.

For the *Nannochloropsis* sp. (NANO), the results for all the concentrations tested were below 25% of TNFα inhibition (Table 3), and they were not significantly different from the LPS control group (Figure 11).

Our results suggest that ethanol extracts might display higher anti-inflammatory activity than water extract. This might be due to ionic and polar substances present in water, that is not cell membrane permeable, and therefore unable to reach a potential cellular target. At this step, both water extracts were excluded from further studies, and ethanol extract *Tetraselmis* sp. CTP4 was subjected to the fractionation process.

Table 3: % of TNF-α inhibition caused by water extracts at 50, 20 and 10 µg/mL in Mac THP-1 cells, relative to the LPS control, data are presented in percentage and standard deviation.

Dexamethasone	2µM	57.0±8.6
Porphyridium sp.water	50µg/mL	33.0±2.4
Porphyridium sp.water	20µg/mL	05.0±17.9
Porphyridium sp.water	10µg/mL	21.0±3.6
Nannochloropsis oculate water	50µg/mL	0
Nannochloropsis oculate water	20µg/mL	26.0±11.6
Nannochloropsis oculate water	10µg/mL	18.0±17.6

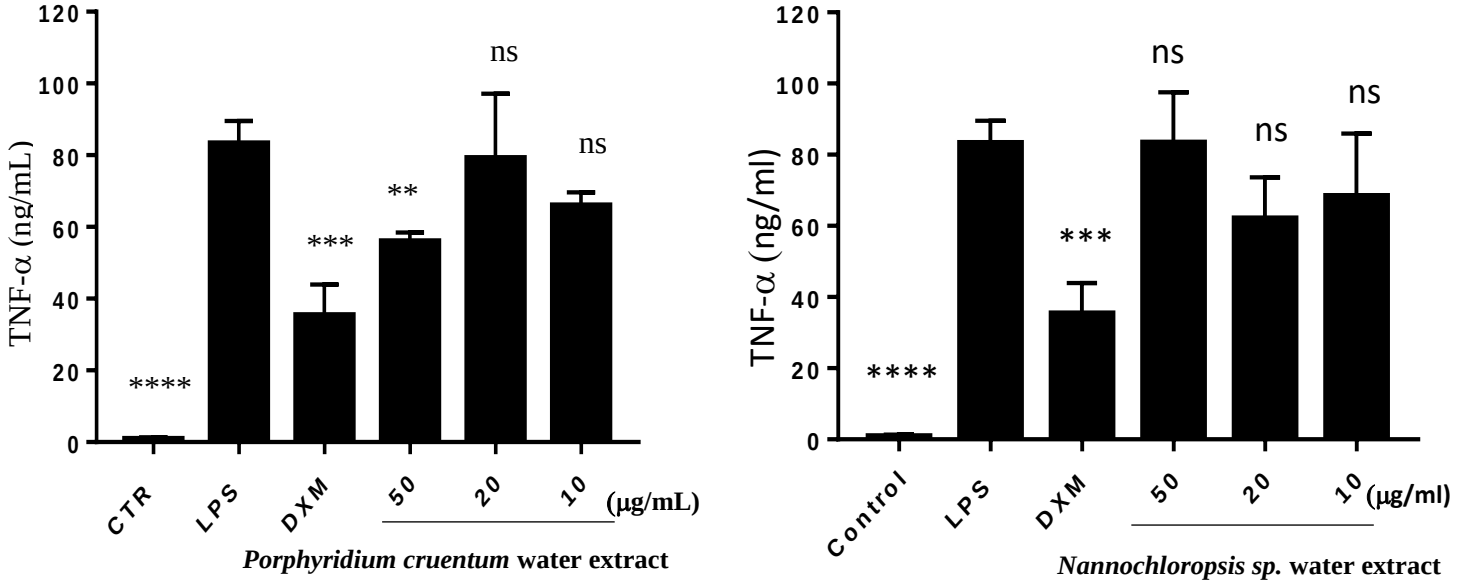

Figure 11: Anti-inflammatory activity of , *Nannochloropsis oculate* , *Porphyridium cruentum* both are water extracts in Mac THP-1 cells stimulated with LPS, as determined by levels of TNFα in the cell culture media. Data are the means ± S.D. of three independent experiments. * $p < 0.05$, ** $p < 0.005$, ***$p < 0.0005$, ****$p < 0.0001$ vs LPS control. CTR, negative control (cells without treatment); LPS, LPS pro-inflammatory control; DXM, Dexamethasone positive anti-inflammatory control, ns, no significance , (50, 20 and 10µg/mL): concentrations tested of each extract.

During the last few years, a remarkable proportion of compounds with an anti-inflammatory effect were discovered in the marine environments. Due to the issues regarding the serious side effects of the traditional anti-inflammatory drugs, the need for novel anti-inflammatory drugs is increasing. The aim of this research was to find and investigate the anti-inflammatory activities of extracts from microalgae. Thus, all foreign substances present in the extracts that are not being recognized by the cells might trigger the inflammatory response. These non-specific interactions may also occur between extracts and Mac THP-1 cells and trigger the production of IL-1β and TNF-α, or maybe the concentrations of the substances present in the extract are too high so that they interact non-specifically with a receptor in the cells. Since the screening for compounds exerting anti-inflammatory activities was not always easy owing to the complexity of cellular interactions involved during an inflammatory response, the same extracts were tested in 3 experiments in triplicates for each one. The data generated from 3 experiments made it possible to compare whether the results were consistent with each other. This concluded that the most promising result obtained was with *Tetraselmis* sp. CTP4 ethanol extract.

3.3 Fractionation of *Tetraselmis* sp.CTP4 ethanol extract

Our previous results showed that *Tetraselmis sp.* CTP4 (T.CTP4)ethanol extract was the most promising species in regard to the anti-inflammatory activity in Mac THP-1 cells. Therefore, it was subjected to a four-step fractionation process using solvents of different polarity indexes. The polarity and the chemical nature of the solvent used in the extraction method are responsible for the extraction yield, the composition, and thus biological activity of a given extract since they will have different compounds both qualitatively and quantitively [80]. In this way, in order to obtain the knowledge on the phytochemical composition and bioactivity profile of the microalgal species selected for fractionation, a sequential extraction process using funnel separation with extremely non-polar hexane(H), medium polar dichloromethane (DCM) and ethyl acetate (EA), and polar water was performed. Water was added to ethanol to increase the polarity of the ethanol extract and favour the extraction of the non-polar and intermediate polarity compounds to the other solvents. With this method, more diverse fractions are obtained, and the bioactive compounds might be more concentrated in some of the fractions. This way,

false-negative results are minimized as well. The algal neutral/nonpolar lipids such as the mono-, di- and tri-acyl glycerides and carotenoids are hydrophobic[81]-[82]. Thus, these non-polar compounds are extractable by the hexane solvent since it is non-polar with a polarity index of 0.1.

The DCM solvent is a moderately polar solvent with a polarity index of (3.1). This solvent will probably extract both polar and non-polar compounds such as pigments and the hydrophilic polar lipids (phospholipids and glycolipids) present in the microalgal extract[82]. Thus, polar compounds could also be extractable by the ethyl acetate solvent since its more polar with a polarity index of 4.4. In addition, most intermediate polarity compounds would be extracted with the DCM since its polarity is much closer to the polarity of the ethyl acetate, hence the lowest yield was obtained for the EA fraction.

However, the solvent with the highest polarity used here was water with a polarity index of 10.2 and ethanol with a polarity index of 4.8, was added in the begging of the fractionation. This combined fraction W/EtOH was the last fraction to be collected and was expected that all the polar compounds would remain in this fraction. The yield of this fraction was the largest (Table 4) comparing the other fractions yield obtained. This is due to the high polarity of both combined solvents (water and ethanol). It was assumed that this fraction would contain most of the polar compounds in the extract. The yield of the hexane fraction was the second largest (Table 4) after the combined ethanol-water fraction, and this is due to the previous explanation that all non-polar compounds present in the extract would be extracted by hexane.

Table 4: The yield quantity for both extract and fractions obtained.

Species	Solvent	Yield (g)	Fraction	Yield (g)
***Tetraselmis* sp. CTP4**	Ethanol	3g	Hexane	0.599
			Dichloromethane	0.0475
			EA	0.0105
			Water/ EtHO	1.6532

3.3.1 Cell viability of the fractions of *Tetraselmis* sp. CTP4 extract

MTS assay was performed to test potential cytotoxicity of fractions prior to the anti-inflammatory assay. Mac THP-1 cells were treated with different doses (10, 20 and 50 μg/mL) of hexane (H), dichloromethane (DCM), ethyl acetate (EA), and ethanol-water (E-W) fractions and DMSO was added as well separately at 0.25% (v/v) concentration all of them for 48 h. The results showed almost no cell toxicity in all the fractions tested since none of them decreased the cell viability below 90% (Figure 12). Instead, cell proliferation was observed at the highest concentrations for H and EA fractions. In this regard, observed increased metabolic activity induced by high concentrations of the different fractions can only mean cell proliferation. This means that cells were metabolically active, viable and were also able to grow in the presence of the fractions at all concentrations (10, 20 and 50 μg /mL).

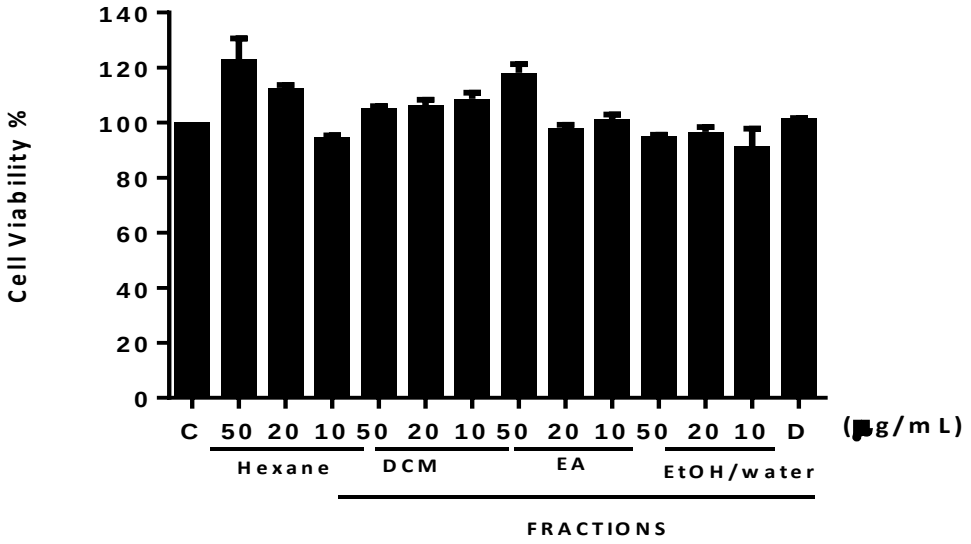

Figure 12: MTS cytotoxicity assay screened at 50, 20 and 10 μg/mL for hexane, dichloromethane (DCM), ethyl acetate (EA) and ethanol/water (EtOH/water) fractions. C: negative control cells without treatment. D: DMSO (DMSO treated with the cells only).

3.3.2 Anti-inflammatory activity of fractions obtained from *Tetraselmis* sp. CTP4 ethanol extract against THP-1 macrophage cells (Mac THP-1)

The same assay used to check the anti-inflammatory effect of the extracts explained above was applied for the fractions as well.

In the secondary screening for the anti-inflammatory activity, the best results were obtained with hexane (H) and dichloromethane (DCM) fractions. Both H and DCM fractions showed very promising TNFα inhibition in Mac THP-1 cells, with respect to the LPS control (Figure 13). At 50 µg/mL they hit 99% and 100% of TNF- α inhibition, respectively (Table 5). The hexane fraction induced inhibition of 98% and 97%, at 20 and 10 µg/mL, respectively.

Nonetheless, the DCM showed a slight decrease in the TNF-α inhibition with 89% and 87% at concentrations of 20 and 10 µg/mL, respectively. Both fractions showed higher activity at all concentrations when compared to the positive control DXM that reached 70%.

In addition, both of the fractions presented levels of TNF-α similar to the non-stimulated control cells, at the highest concentration tested (Figure 13). This indicates that those fractions display strong anti-inflammatory activity, even at the lower concentration tested, with a higher effect than DXM. This might suggest that a complex mixture with a lot of compounds can have a higher anti-inflammatory effect that a purified compound used as an anti-inflammatory drug, which in our case is DXM. If the compound responsible for this anti-inflammatory effect was isolated and purified, it may have the same effect as the DXM, but with considerably lower concentration administered. Since the drug potency is a measure of the drug activity, expressed in terms of the amount required to produce an effect of given intensity, highly potent drugs (e.g., fentanyl, alprazolam, risperidone) evoke a given response at low concentrations, while a drug of lower potency (meperidine, diazepam, ziprasidone) evokes the same response only at higher concentrations[83]**.** And that means that our fraction contains a compound or compounds with high potency, could be used as a potential anti-inflammatory drug. In fact, both H and DCM showed significantly highest downregulation of the TNF-α in respect to the LPS control at all concentrations (Figure 13).

Ethyl acetate (EA) and Water/ethanol (W/E) fractions at 50 µg/mL inhibited TNF-α production by 90% and 5 %, respectively (Table 5). Ethyl acetate fraction showed a really interesting result at the highest concentration, in which the suppression of TNF-α in Mac THP-1 cells, reached 90%. This result at this concentration was close to the exhibited activity of DCM at 20 µg/mL, that reached 89% (Table 5). Yet, the result of EA at 20 and 10 µg/was not that interesting, with no effect of inhibition at 20 µg/mL, and 35% of TNF-α inhibition at 10 µg/mL (Table 5). However, this might be due to several impacts like freezing and defrosting the extract each time prior use may cause some degradation and the extract may lose the activity. This part might need to be repeated for better confirmation of the observed effect. Yet, this EA fraction showed that it might contain a compound which would have an interesting anti-inflammatory effect if purified. Hence the effect showed at 50 µg/mL was really interesting compared to DXM positive control. Ethanol/water (W/E) fraction TNF-α downregulation results at 20 and 10 µg/mL were 32% and 1% respectively (Table 5). However, W/E was not significantly different compared to LPS pro-inflammatory control (Figure 13). These results could be due to that most of the bioactive compounds were extracted by H, DC, and EA fractions. W/E fraction was the last fraction to be collected during the fractionation for the the *Tetraselmis* sp. CTP4 ethanol extract. Based on these results, the hexane fraction was the most promising and interesting fraction among all the others, which was the reason behind further characterization by GC-MS**.**

Table 5: % of TNF-α inhibition caused by different fractions at 50, 20 and 10 µg/mL in Mac THP-1 cells, relative to the LPS control, data are presented in percentage and standard deviation

Fractions	**Concentrations**	**%**
Dexamethasone	2µM	70.0±5.6
Hexane fraction	50µg/mL	99.0±0.8
Hexane fraction	20µg/mL	98.0±2.0
Hexane fraction	10µg/mL	97.0±1.7
Dichloromethane fraction	50µg/mL	100±1
Dichloromethane fraction	20µg/mL	89.0±3.4
Dichloromethane fraction	10µg/mL	87.0±11.4
Ethyl acetate fraction	50µg/mL	90.0±9.1
Ethyl acetate fraction	20µg/mL	0
Ethyl acetate fraction	10µg/mL	35.0±32.0
Water/Ethanol fraction	50µg/mL	05.0±46.1
Water/Ethanol fraction	20µg/mL	32.0±38.0
Water/Ethanol fraction	10µg/mL	1.0±55.4

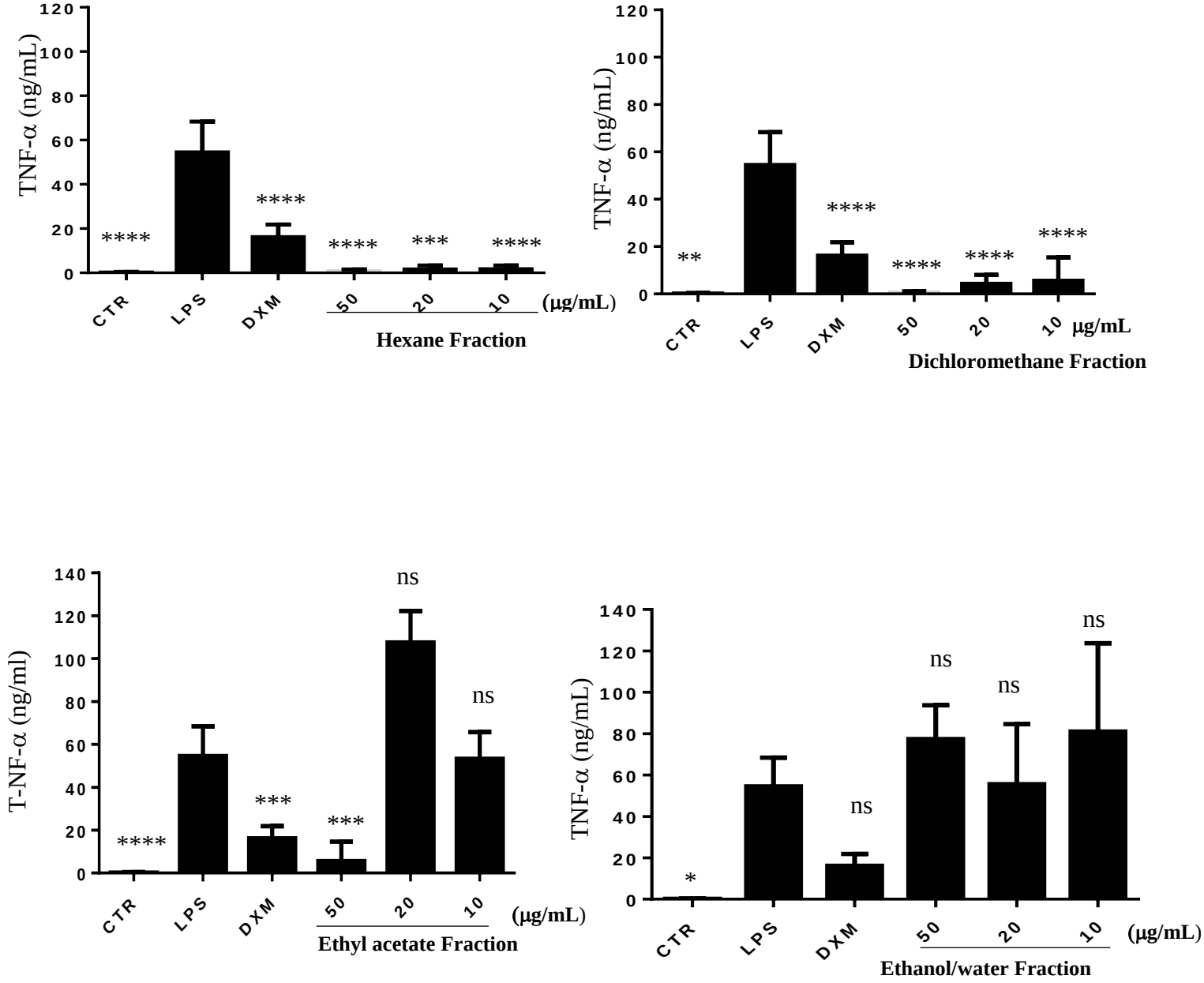

Figure 13: Anti-inflammatory activity for (hexane, dichloromethane, ethyl acetate and ethanol/water) fractions of *Tetraselims sp CTP4* extracts in Mac THP-1 cells stimulated with LPS, as determined by levels of TNF-α in the cell culture media. Data are the means ± S.D. of three independent experiments. * $p < 0.05$, ** $p < 0.005$, ***$p < 0.0005$, ****$p < 0.0001$ vs LPS control. CTR, negative control (cells without treatment); LPS, LPS pro-inflammatory control; DXM, Dexamethasone positive anti-inflammatory control, ns, no significance , (50, 20 and 10µg/mL): concentrations tested of each extract.

3.4 Chemical composition of the hexane fraction obtained from *Tetraselmis* sp. CTP4 by GC–MS

In the above sub-chapter, our results demonstrated that the hexane fraction was the most active fraction with promising anti-inflammatory activity in terms of TNF-α suppression by 98%, 97% and 96% at concentrations of 50, 20 and 10 μg/mL, respectively. In order to identify the compound possibly responsible for the anti-inflammatory effect, this fraction was analysed by GC/MS.

In the GC-MS analysis, 42 compounds were successfully identified (Table 6). The compounds were identified by comparing the mass spectra of each peak with the spectral data of different compounds obtained from the NIST library. Twelve of the identified compounds were already reported by other authors, to possess anti-inflammatory activity. These 12 compounds could be responsible for the observed effect of our extract.

These 12 compounds were: octadecenoic acid, hexadecatrienoic acid, octadecadienoic acid stearidonic acid, heptadecanoic acid, hexadecenoic acid, hexadecene-1-ol, valeric acid 1, docosahexaenoic acid, linolenic acid, eicosapentaenoic acid, and vitamin E. Furthermore, from these 12 compounds, only 8 were reported to act as an antioxidant.

Fatty acids derived from marine organisms have varies chemical structures which serve as a biological marker. They possess biological activity due to the characteristic of the living environment. Numeral studies have reported the activity of essential fatty acids from seaweed. For example, ω-3 and ω-6 obtained from *Undaria pinnatifida* could act as anti-inflammatory[84] agents. Most of the compounds identified in our sample were fatty acids which have been reported in different studies to possess anti-inflammatory effect[85]. Most of the microalgae produce fatty acids in high quantities and *Tetraselmis* sp. CTP4 is one of them[1]. In addition, the dietary intake of fatty acids is important to the human body. In particular, eicosapentaenoic (EPA) and docosahexaenoic acids (DHA), were reported to lower the incidence of cardiovascular diseases[86].

From the table 4 below, it can be seen that the most abundant compounds detected by GC-MS corresponded to Octadecenoic acid, hexadecatrienoic acid, and Octadecadienoic acid with 28.71%, 12.63%, and 6.12% for the total peak area, respectively.

In published literature octadecenoic acid, from the macroalga *Sargassum wightii* was shown to possess anti-inflammatory activity against the inflammatory marker Cycloxigenase-2 [87]. Furthermore, this compound is reported to improve the anti-oxidant activity associated with decreased expression of maturation markers in LPS-matured DCs ,and in murine enterocyte cells (MODE-K)[88]. In the line of previous studies, hexadecatetraenoic acid isolated from the algae *U. pinnatifida* and *Ulva pertusa* was reported to have an anti-inflammatory effect using MC/9 Mouse Mast Cell model[89].

In addition, other published study reported that octadecadienoic acid from the marine red alga *Gracilaria verrucosa* acted as an anti-inflammatory agent against nitric oxide (NO), IL-6, and TNF-α production, in LPS stimulated RAW 264.7 cells[90]. The same compound derived from *Peperomia pellucida* leaf extract was reported to possess antioxidant activity as well as [91]. Sardonic acid was relatively abundant in our fraction, with 4.49% of the peak area. It was shown by other researchers that this compound extracted from microalgae present in the marine environment, exhibits an anti-inflammatory effect by inhibition of mouse ear inflammation induced by phorbol myristate acetate[90].

The results of heptadecanoic acid with of 2.79% of total peak area ties well with a previous study in which this compound isolated from Ziziphus jujube Mill, a Chinese date acted as an anti-inflammatory agent against the irritant action of Euphorbiaplants[92]. This compound was not reported to act as an antioxidant in other studies. Overall the finding of hexadecanoic acid with an abundance of 1.61% are in accordance with previous findings reported to have an anti-inflammatory effect[93]. in addition, this compound is used in cosmetics, in make-up products, to hide blemishes[94]. A similar effect was achieved by another study in which 9-Hexadecen-1-ol isolated from the chlorophyll showed an anti-inflammatory effect[95]. Both valeric acid and docosahexaenoic acid with their abundant peaks of 1.15% and 0.67% in our results, were earlier reported to act as an anti-inflammatory agent. Yet both of them were isolated from the plant, not from microalgae species [96]. On the other hand, docosahexaenoic acid was reported to enhance the antioxidant activity[97].

Similar anti-inflammatory and antioxidant effects were found for linolenic acid with an abundance of 0.61% obtained from plant[98]. In addition to that, eicosapentaenoic acid (EPA) with an abundance of 0.37% have been shown to have an anti-inflammatory effect and antioxidant properties [99-100]. The last compound reported to act as both anti-

inflammatory and powerful antioxidant and a scavenger of hydroxyl radicals was vitamin E with a low abundance of 0.33% in our fraction [101].

However, the rest of the compounds with the low abundances presented int Table 6 have not been previously described for their anti-inflammatory effect.

Since there were a variety of promising compounds with potential anti-inflammatory activities obtained from *Tetraselmis* sp. CTP4 ethanol extract by GC-MS injection, it was not an easy task to speculate and point out the compound causing the anti-inflammatory effect observed before. But we do acknowledge that the most abundant compounds in the fraction (octadecenoic acid, hexadecatetraenoic acid, and octadecadienoic acid) could have strongly contributed. This means that the anti-inflammatory effect could be due to the most abundant compounds, or due to a combination of all or some fatty acids with low abundancy present in this fraction. In addition, most of the fatty acids are considered to be novel anti-anti-inflammatory and antioxidant compounds.

Nonetheless, we believe that a synergic effect of all the compounds in the fractions cannot be ignored. For the purpose of inspecting this, more research is required. The fraction of hexane must be further fractionated into multiple other sub-fractions in order to reduce the number of compounds present in each subfraction and the activity would be increased in some of those, as a number of active compounds would be limited in each sub-fraction. However, if one compound is responsible and is purified, and re-tested again, giving promising results, it could be considered as a new potential anti-inflammatory agent. However, because of the lack of the time and the fractions quantity prepared we decided not to investigate the antioxidant properties for the fractions obtained *Tetraselmis* sp. CTP4 Also, both dichloromethane and ethyl acetate fractions were not characterized by GC/MS in spite of their anti-inflammatory activity. T.chuii extract was not fractionated as well. To our knowledge, there are no publications reporting anti-inflammatory nor anti-oxidant effect for both of hexane fraction and extract of *Tetraselmis* sp. CTP4 microalga. Furthermore, there are no studies which describe the association between the compounds detected in this study and the anti-inflammatory effect of *Tetraselmis* sp. CTP4 against Mac THP-1 cells.

Table 6: Chemical composition of the hexane fraction determined by GC-MS. The percentage of each compound represents its relative abundance in the whole chromatogram.

RT	Tentatively identified compound	% of the total peak area
25.62	Octadecenoic acid	28.71
22.36	hexadecatrienoic acid	12.63
25.62	Octadecadienoic acid	6.12
22.13	trans-octadecadienoic acid	6.12
24.00	stearidonic acid	4.49
20.91	Tetrahydropyranyl ether of citronellol	2.96
31.08	2-Norpinanol, 3,6,6-trimethyl-	2.96
24.00	Tetrahydrofuran, 2-methyl-5-pentyl-	2.96
25.91	Heptadecanoic acid	2.79
26.02	Octadecatrienoic acid	2.79
24.11	eicosatetraenoic acid	2.01
22.44	9,12-Hexadecadienoic acid	1.95
26.31	Hexadecenoic acid	1.61
26.59	cis-9-hexadecenoic acid	1.61
20.91	Phytol, acetate	1.52
20.91	9-Hexadecen-1-ol	1.51
21.85	9-Eicosyne	1.51
18.08	eicosenoic acid	1.18
30.67	10-Undecenoic acid	1.15
31.08	Valeric acid	1.15
23.54	Heneicosapentaenoic Acid	0.84
26.72	Myristoleic acid	0.82
31.52	Stearic acid	0.68
31.52	Docosahexaenoic acid	0.67
22.01	linolenic acid	0.61
22.24	Hexadecatrienoic acid	0.61
21.85	Tetrahydropyran 12-tetradecyn-1-ol ether	0.60
27.01	Lup-20(29)-en-21-ol, 3,28-bis[(tetrahydro)	0.60
27.40	(1-Methoxy-pentyl)-cyclopropane	0.60
27.40	7-Heptadecene, 17-chloro-	0.59
30.53	7-hexadecenic acid	0.59

26.31	Heptadecenoic acid	0.58
22.36	5.alpha.-Androstan-3-one, 17.beta.-hydroxy-4.alpha.-methyl-,	0.48
27.77	Cholestan-3-one, 4,4-dimethyl-, cyclic 3	0.48
30.53	Tetrahydrofuran-2-one, 3-[2-pentenyl]-4-methyl-	0.48
23.15	Eicosapentaenoic acid	0.37
18.08	Tetradecanoic acid	0.37
41.58	Arachidonic acid	0.35
22.96	Pregna-5,9(11)-dien-20-ol-3-one ethylene	0.34
22.44	Vitamin E	0.34
19.69	pentadecanoic acid	0.27
19.13	cis-10-Heptadecenoic acid	0.26
28.74	unknown compound	0.74

4. CONCLUSION

Tetraselmis sp. CTP4 was isolated and characterized recently as a robust, euryhaline, lipid-rich microalga. This genus belongs to the Chlorophyta, family Chlorodendraceae. This species is unicellular flagellate with elliptical or almost spherical, slightly flattened cells.

In addition *Tetraselmis* sp. CTP4 be used as human food, it can also be used in the production of fish feed, biofuels and even to treat sewage[102]. Microalgae are not yet considered as traditional food, and this microalga needs to undergo an EU approval process, in order to be actually marketed for human consumption, which still takes some time. However, this species is a rich source of lipids since it has been reported recently has a high potential for biotechnological applications[1]

Herein, we are presenting for the first time, that *Tetraselmis* sp. CTP4 ethanol extract has a strong anti-inflammatory activity against human macrophages differentiated from monocytic leukemia (THP-1) cell line. More specifically, the hexane fraction obtained from this extract was found to have a % of TNFα inhibition higher than dexamethasone, frequently used as an anti-inflammatory drug for inflammation treatment. However, the antioxidant activity for this extract was not that high.

The GC-MS analysis of hexane fraction revealed 12 compounds potentially (according to the literature) responsible for the anti-inflammatory activity, and 8 of them might be responsible for the antioxidant activity. More studies are needed in order to purify those compounds and test each compound separately in order to identify the compound responsible for the effect observed.

Recommendations for the future work include additional fractionation to obtain more potentially active fractions, with a more limited number of compounds, and to perform characterization with NMR. Nevertheless, our results suggest that *Tetraselmis* sp. CTP4 is a source of promising anti-inflammatory agents. *T. chuii* ethanol extract should be further investigated hence it possesses higher anti-inflammatory effect at the lowest concentration. Furthermore, dichloromethane and ethyl acetate fractions from *Tetraselmis* sp. CTP4 Ethanol extract, should be further characterized hence they displayed a high TNF-α inhibition (anti-inflammatory effect) results. In addition to the identification of compounds from the hexane fraction by GC-MS, those compounds should be purified and tested for their anti-inflammatory effect, in order to obtain pure drug leads with proved anti-inflammatory effect. Last but not least, HPLC analysis should be done in order to detect non-volatile compounds, such as some carotenoids, which might be present in the fraction but are not volatile and cannot be detected by GC-MS.

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