„Searching for innovative anti-tumoral drugs in marine microalgae”

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DECLARATION ABOUT THE AUTORSHIP

“Searching for innovative antitumoral drugs in marine microalgae”

Declaration of Authorship

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

(Farah Kamberović)

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ABSTRACT

Cancer is one of the leading causes of death globally. Current available chemotherapeutics are aggressive and not specific to cancer cells, causing damage and death of healthy cells as well. As a consequence, the number of side-effects in patients arise. Another important therapeutic issue is the development of resistance and/or development of secondary malignancies. In some types of cancer, such as hepatocellular carcinoma (HCC) and acute monocytic leukaemia (AML), chemotherapy is associated with high mortality rate. This points out to the need to search and identify new sources of anti-cancer drugs with high selectivity and toxicity only for malignant cells, while conserving healthy cells.

Marine microalgae are a rich source of different bioactive metabolites (e.g. poly-unsaturated fatty acids, carotenoids, polysaccharides, phenols, sterols, vitamins) with anti-inflammatory, anti-bacterial, anti-diabetic and anti-hypertensive properties, among others. During the past few years, marine microalgae have been featured in cancer research. In this research, we studied the cytotoxic effect of six selected microalgae species against adherent (HepG2) and suspended (THP-1) human cancer cell lines.

The ethanolic extract of *Phaeodactylum tricornutum* was the most bioactive with an IC_{50} of 19.4±2.2 μg/mL for HepG2 cells. In addition, this extract was highly selective for HepG2 cells (SI=4.40) in comparison with a non-tumoural derived cell line (S17). The active extract was further subjected to bio-guided fractionation process to obtain four fractions: hexane, dichloromethane, ethyl-acetate and water with ethanol. Among these fractions, the dichloromethane fraction displayed high cytotoxicity towards both HepG2 and THP-1 cell lines with IC_{50} of 27.5±1.6 and 22.3±1.8 μg/mL, and selectivity of SI>4.54 and SI>5.60, respectively.

In order to tentatively identify compounds responsible for the observable cytotoxic effect, the dichloromethane fraction was analysed by gas chromatography – mass spectrometry (GC/MS). Thirteen molecules with potential anti-cancer properties were identified, belonging to six different classes of metabolites: saturated fatty acids (SFA), polyunsaturated fatty acids (PUFAs), sterols, vitamins (dl-α-Tocopherol), phenols and terpenoid alcohols. The most abundant compounds detected were hexadecanoic acid, 9-hexadecenoic acid and 5,8,11,14,17-Eicosapentaenoic acid (EPA).

ABBREVIATIONS

GS	Growth signals
G0	Quiescent (resting) phase in the cell cycle
G1	First growth period of the cell cycle
pRB	Retinoblastoma protein
Apaf-1	Apoptotic peptidase activating factor 1
VEGF	Vascular endothelial growth factor
TSP-1	Thrombospondin 1
RSV	Rous sarcoma virus
v-SRC	Rous sarcoma virus oncogene
HPV	Human papillomavirus
CIN	Cervical intraepithelial neoplasia
SSBs	Single-strand breaks in the DNA
DSBs	Double-strand breaks in the DNA
HCC	Hepatocellular carcinoma
AML	Acute monocytic leukaemia
HepG2	Hepatic carcinoma cell line
THP-1	Human monocytic leukaemia cell line
S-17	Murine bone marrow stromal cell line
SV-40	Simian virus 40
ATCC	American type culture collection
CYP	Liver cytochrome P_{450} enzymes
PMA	Phorbol 12-myristate 13-acetate
Fas	Surface death receptors involved in apoptosis
FADD	Death receptors ligand molecules involved in apoptosis
Topo I	Topoisomerase enzyme I
Topo II	Topoisomerase enzyme II
DEPBG	4′-demethylepipodophyllotoxin benzylidene*glucoside*
NP	Natural products
FDA	Food and drug administration
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid

PUFAs	Polyunsaturated fatty acids
EA	Ethyl acetate
ETH	Ethanol
DCM	Dichloromethane
Hex	Hexane
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's modified eagle medium
RPMI	Roswell park memorial institute medium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide
RT	Room temperature
FBS	Foetal bovine serum
PBS	Phosphate buffer saline
IC_{50}	Half maximum inhibitory concentration
SI	Selectivity index
LLE	Liquid-liquid extraction
GC/MS	Gas chromatography mass spectrometry
PHA	Phaeodactylum tricornutum
ISO	*Isochrysis* sp.
POC	*Porphyridium* sp.
SKLT	*Skeletonema costatum*
T CTP4	*Tetraselmis* sp. *CTP4*
NANNO	*Nannochloropsis* sp.

INDEX

1. INTRODUCTION………………………………………………………… **1**

1.1. Cancer biology………………………………………………….. **1**

1.1.1. Hallmarks of cancer cells………………………………………. **1**

1.1.2. Causes of cancer……………………………………………….. **4**

1.1.3. Types of cancer………………………………………………… **5**

1.1.4. Hepatocellular arcinoma……………………………………… **6**

1.1.5. Monocytic leukaemia…………………………………………….. **7**

1.2. Cancer cell lines …………………………………………………. **8**

1.2.1. HepG2 cells……………………………………………………. **10**

1.2.2. THP-1 cells……………………………………………………. **12**

1.3. Cancer therapies……………………………………………….. **14**

1.3.1. Chemotherapeutic agents……………………………………… **14**

1.3.2. Etoposide……………………………………………………….. **15**

1.4. Marine environment as promising source of new drug leads…….. **17**

1.4.1. Marine microalgae as a novel source of anticancer……………………… **19**

1.4.2. Marine microalgae used in this study.. **23**

1.5. Objectives... **24**

2. MATERIALS AND METHODS………………………………………… **25**

2.1. Chemicals and reagents ……………………………………….. **25**

2.1.1. Algal biomass…………………………………………………….. **25**

2.2. Preparation of extracts…………………………………………… **25**

2.3. Selection of extracts for testing……………………………………... **26**

2.4. Bioassays………………………………………………………….. **27**

2.4.1. Cell cultures………………………………………………………. **27**

2.4.2. In vitro cytotoxicity……………………………………………….. **28**

2.4.2.1. MTT assay………………………………………………………… **28**

2.4.2.2. Calculations and data interpretation………………………............. **30**

2.5. Liquid-liquid extraction (LLE)……………………………………….. **32**

2.5.1. Determination of extract yield…………………………………………. **33**

2.6. Characterization with gas chromatography – mass spectrometry (GC/MS)………………………………………………………………… **34**

2.6.1. Derivatization of samples………………………………………………… **34**

2.6.2. GC/MS analysis……………………………………………………….... **35**

2.6.2.1. Expression of results………………………………………………….. **35**

2.7. Statistical analysis……………………………………………………....... **35**

3. RESULTS AND DISCUSSION……………….. **36**

3.1. Initial screening for cytotoxicity against HepG2 and S17 cell lines – ethyl acetate (EA) extracts…………………………………………………….. **36**

3.2 Screening for cytotoxicity of *P. tricornutum* (PHA) ethanol extract against HepG2 and S17 cell lines.. **40**

3.3. Screening for cytotoxicity of *P. tricornutum* (PHA) ethanol extract against THP-1 cell line.. **43**

3.4. Evalution of cytotoxic activity of fractions obtained from *P. tricornutum* ethanol extract against HepG2, THP-1 and S17 cell lines...................................... **46**

3.5. Microscopic examination of morphological changes using inverted microscope.. **49**

3.6. Identification of compounds present in the extract using gas chromatography-mass spectrometry (GC/MS).. **51**

4. CONCLUSIONS……………………………………………………....... **57**

5. ANNEX………………………………………………………………... **59**

6. BIBLIOGRAPHY………………………………………………………… **61**

INDEX TABLES AND FIGURES

Table 1:	Summary of most commonly used cancer cell lines with origin in different cell types……………………………………………….	**10**
Table 2:	Main characteristics of HepG2 cell line (ATCC® HB-8065™)…	**11**
Table 3:	Main characteristics of THP-1 cell line (ATCC® TB-202™)……	**13**
Table 4:	Characterization of chemotherapeutic agents with some examples.	**15**
Table 5:	Bioactive compounds obtained from microalgae and their effect on human health…………………………………………………………	**20**
Table 6:	*In vitro* cytotoxicity of microalgae on cancer cell lines……………	**21**
Table 7:	Properties of solvents used in liquid-liquid extraction (LLE) and operating conditions for rotary evaporator…………………………	**33**
Table 8:	*In vitro* cytotoxic activity (IC_{50}; µg/mL) of the ethyl acetate (EA) extracts of the species *P. tricornutum, Isochrysis* sp., *Porphyridium* sp*., Skeletonema costatum, Nannochloropsis* sp*., Tetraselmis* sp. CTP4 and etoposide, on HepG2 and S17 cell lines..	**40**
Table 9:	*In vitro* cytotoxic activity (IC_{50}; µg/mL) of ethanol (ETH) extracts of *P. tricornutum*, *Isochrysis* sp. and etoposide on HepG2 and S17 cell lines...	**42**
Table 10:	Cytotoxicity (IC_{50}; µg/mL) of *P. tricornutum* ethanol extract and etoposide against HepG2, THP1 and S17 cell lines.........................	**44**
Table 11:	Cytotoxicity (IC_{50}; µg/mL) of fractions (hexane, dichloromethane, ethyl acetate, water+ethanol) from *P. tricornutum* ethanol extract and etoposide against HepG2, THP1 and S17 cell lines..................	**49**
Table 12:	Characterisation of compounds present in dichloromethane fraction by (GC/MS)...	**56**

Figure 1:	HepG2 cells in culture…………………………………………………..	**12**
Figure 2:	THP-1 cells in culture…………………………………………………	**13**
Figure 3:	Extract preparation from dried algal biomass..................................	**26**
Figure 4:	Microalgae species used to test cytotoxicity on HepG2 and THP-1 cell lines ……………………………………………..................	**27**
Figure 5:	MTT assay performed on 96-well plate (HepG2 cell line)…………	**30**
Figure 6:	Neubauer chamber: counting cells	**31**
Figure 7:	Liquid-liquid extraction (LLE) for *Phaeodactylumtricornutum* ethanol extract……………………………………………........	**34**
Figure 8:	Cell viability (%) after 48 h exposure to different concentrations (3.9 – 125 µg/mL) of the ethyl acetate (EA) extracts of the species *P. tricornutum, Isochrysis* sp., *Porphyridium* sp*., Skeletonema costatum, Nannochloropsis* sp*., and* Tetraselmis sp. CTP4) and etoposide, on HepG2 and S17 cell lines..	**39**
Figure 9:	Cell viability (%) after 48 h exposure to different concentrations (3.9 – 125 µg/mL) of the ethanol (ETH) extracts of the species *P. tricornutum* and *Isochrysis* sp., on HepG2 and S17 cells.................	**42**
Figure 10:	Cell viability (%) after 48 h exposure to different concentrations (0.24– 125 µg/mL) of the ethanol (ETH) extract of *P. tricornutum* and etoposide on HepG2 and S17 cells..	**44**
Figure 11:	Cell viability (%) after 48 h exposure to different concentrations (3.9– 125 µg/mL) of the fractions (hexane, dichloromethane, ethyl-acetate, water+ethanol) from *P. tricornutum* ethanol (ETH) crude extract and etoposide, on HepG2, THP-1 and S17 cells........	**50**
Figure 12:	Examination of morphological changes on cells with light inverted microscopy..	**51**

1. INTRODUCTION

1.1. Cancer biology

Cancer is a global health issue and the second leading cause of death in high-income countries and the third leading cause of death in low- and middle-income countries worldwide[1]. It is estimated that cancer was responsible for 9.6 million deaths in 2018[2]. Lung, prostate, colorectal, stomach and liver cancer are the most common types of cancer in men, while breast, colorectal, lung, cervix and thyroid cancer are the most common among women[2]. By 2030, it is estimated that the incidence of cancer in the world will grow to over 21.7 million new cases of disease and lead to 13 million deaths[1].

According to current evidence, between 30% and 50% of cancer deaths could be prevented simply by avoiding tobacco products, reducing alcohol consumption, maintaining a healthy body weight, exercising regularly, and addressing infection-related risk factors[2].

The term cancer derives from the Greek word "karkinos", which means crab. Cancer encompasses over 100 distinct diseases having in common the abnormal growth of cells that invade adjoining parts of the body and/or spread to other organs. Other common terms used for cancer are malignant tumours and neoplasms. Cancer can affect almost any organ in the human body and has many anatomic and molecular subtypes, that require specific management strategies. The oldest written description of cancer known to exist dates back to about 1600 BC, but it is believed to be based on a much earlier document, from ca. 3000 BC. Hippocrates first used the words *carcinos* and *carcinoma to* describe cancer, referring to the thick blood vessels that surround and feed the tumours and that resemble the claws of a crab[3,4].

1.1.1. Hallmarks of cancer cells

Cancer cells differ from normal cells in many ways due to the fact these cells do not respond properly to the signals that regulate normal cell behaviour, and consequently cancer cells are able to proliferate continuously avoiding cell death[1,3,4].

Hanahan and Weinberg (2000) established six hallmarks that are common for all human tumours and are responsible for their malignant properties: self-sufficiency in growth signals (GS), insensitivity to antigrowth signals, ability to avoid apoptosis, limitless replication potential, angiogenesis and capacity to produce metastasis.

Self-sufficiency in growth signals. Normal cells need mitogenic growth signals (GS) in order to proliferate. Most soluble GSs are produced by one cell type in order to stimulate the proliferation of another cell type in a process called heterotypic signalling. Cancer cells, on the other hand, have acquired the ability to synthesize their own GSs to which they respond by proliferating and creating a positive feedback signalling loop called autocrine stimulation[5].

Insensitivity to antigrowth signals. In order to maintain normal tissue homeostasis, many antigrowth signals are synthesized and recognized by transmembrane cell surface receptors to prevent excessive proliferation. These antigrowth signals can block proliferation either by forcing cells from proliferative cycle (G1) back to quiescent state (G0) or by inducing permanent loss of proliferative potential of the cell[5,6]. One of the most studied mechanisms of negative cell cycle control is the retinoblastoma protein (pRb) pathway. It has been shown that pRb is responsible for a major G1 checkpoint, blocking proliferation and progression from G1 to S-phase of cell cycle. Cancer cells can disrupt the pRb pathway by releasing signalling molecules like TGFβ that prevents phosphorylation, which in turn inactivates pRb. In this way tumour cells turn themselves insensitive to antigrowth factors and are able to keep proliferating[5,7].

Avoiding apoptosis. There are various strategies through which cancer cells avoid apoptosis. One of the most common mechanisms is through a mutation of the p53 tumour suppressor gene[5,6]. The p53 is a transcription factor that binds to damaged DNA and promotes tumour suppression by two distinct mechanisms. One mechanism includes inducing cell cycle arrest in G1 phase and preventing S-phase entry; the other one is promoting apoptosis dependent on the Apoptotic Peptidase Activating Factor 1 (Apaf-1)/caspase-9 pathway and involves mitochondrial cytochrome *c* release. However, it remains unclear why certain cells undergo apoptosis in response to p53 activation while other cells undergo p53-dependent cell cycle arrest. More than half of all types of human cancers have a mutation or missing gene for p53 resulting in a damaged or missing p53 protein[5,6,8]. As an alternative, cancer cells can compromise the activity of p53 by increasing its inhibitors or by silencing the activators of p53. One typical example of this is E6 protein produced by human papilloma virus, which inactivates p53 and enables cancer cells to evade apoptosis and spread[8].

Limitless replication potential. The three acquired capabilities of cancer cells described above (self-sufficiency in growth signals, insensitivity to antitumor growth factors and avoiding apoptosis) are major responsible factors for tumour malignancies, but still not enough to cause macroscopic tumours. Research performed with cultured cell lines over the years revealed that after a certain number of cell divisions (60-70) cells stop growing and enter a

phase called *senescence*[9]. After this they slowly start to die. The reason behind this limited number of replications is, as it was discovered later, progressive telomeres shortening which leads to an inability of DNA polymerases to completely replicate the 3' ends of chromosomal DNA. Cancer cells have acquired a mechanism to avoid senescence by maintaining telomere length. This is achieved by increased expression of the telomerase enzyme, which adds hexanucleotide repeats on the ends of telomeric DNA, keeping the length of telomeres always above a certain threshold and assuring constant cell replication.[5,6,9,10].

Angiogenesis. The process through which new blood and lymphatic vessels are formed from pre-existing vessels is called angiogenesis. This process is essential during foetal development, the female reproductive cycle, and for tissue repair. One of the most specific and critical regulators of angiogenesis is the vascular endothelial growth factor (VEGF), which regulates endothelial proliferation, permeability, and survival; and thrombospondin-1 (TSP-1), which is an endothelial growth inhibitor. During normal physiological processes these two factors are always at equilibrium assuring tissue homeostasis. When tumours are present, as they grow, their need for oxygen, nutrients and waste disposal is increasing. Because of this, an “angiogenic switch“ is activated in order to sustain the expanding neoplastic growth. This is achieved by changing the balance between VEGF and TSP-1, mainly through altered gene transcription[6,11].

Metastasis. Almost all types of solid tumours during their development enter a process called metastasis. Metastasis involves the spread of cancer cells from the primary tumour to the surrounding tissues and to distant organs, and it is the primary cause of cancer morbidity and mortality. The complete metastatic process consists of the following steps: first, cancer cells detach from the primary tumour, and intravasate into the circulatory and lymphatic system where they evade immune responses, then extravasation of cells at distant capillary beds occurs while invading and proliferating in distant organs. Several hypotheses ,which explain the origin of metastasis, exist: epithelial mesenchymal transition, an accumulation of mutations in stem cells, a macrophage facilitation process, and a macrophage origin involving either transformation or fusion hybridization with neoplastic cells[6,12].

1.1.2. Causes of cancer

Cancer is a heterogeneous group of diseases with diverse aetiology and pathogenesis. Each type of cancer has different mechanism of arising and progressing. Because of that, one general cause of cancer cannot be established as a rule.

There are different hypothesis and explanations for cancer evolution[4]:

- Genetic changes
- Epigenetic changes
- Viruses infection
- DNA lesions

Genetic changes. For the past 30 years cancer has been thought to arise from a single cell which goes through a series of different genetic alterations causing tumour proliferation, invasion, metastasis and drug resistance. This theory is called clonal genetic model. On the other hand, the multiple-mutation model, which was proposed later, was based on a belief that cancer arises as a result of many different mutations accumulating in different cells over time. According to this model, it should take decades for a tumour to arise, and then it should also take decades for tumour progression, which is not the case. The genetic instability model has been suggested as an alternative model. This model proposes that cancer arises due to an accumulation of genetic changes over time, but in specific genes that were found to be altered in many cancers – these genes are called oncogenes and tumour-suppressor genes[4]. Proto-oncogenes stimulate cell growth, division, and survival. When a proto-oncogene mutates, it becomes permanently activated, which enables cells to grow without control and become oncogenic. Oncogenes are activated by chromosome rearrangements or gene duplication. Tumour suppressor genes normally help to prevent unrestrained cellular growth and promote DNA repair and cell cycle checkpoint activation. When they lose their function, cells tend to acquire abnormal behaviour[13].

Epigenetic changes . Epigenetics is the study of non-sequence-based alterations that are inherited through cell division and affect gene expression. Epigenetic alterations do not involve changes in the underlying DNA sequence but only phenotype change. Epigenetic change is a regular and natural occurrence, but it can also be influenced by several factors including age, the environment/lifestyle, and disease state. The most studied epigenetic modifications include: DNA hypomethylation, hypermethylation and hypomethylation of specific genes, chromatin alterations and loss of imprinting. All these lead to aberrant activation of growth-promoting genes and aberrant silencing of tumour-suppressor genes[4,13].

Viral infections. There are some viruses that possess oncogenes and are able to cause cancer. The first discovered oncovirus was Rous sarcoma virus (RSV), which causes avian sarcoma and was named by its discoverer, Peyton Rous. Thanks to this discovery, many years of research led to another important discovery – the first oncogene, *v-SRC* as well as its cellular precursor, *c-SRC*[3]. Another example of an oncogene virus is the human papillomavirus (HPV) that causes a series of transformations on squamous epithelial cells in woman's cervix. These transformations, which are called cervical intraepithelial neoplasia (CIN), are reversible in the beginning, and the affected part of tissue can be successfully removed. However, if CIN is not detected on time, it can progress to irreversible cervical cancer[14].

DNA lesions. One of the most frequent causes amongst all types of cancers are DNA lesions which cannot be controlled and arise from human genome instability. It is estimated that there are tens of thousands of DNA lesions per cell per day that can corrupt human genetic information[3].

Factors responsible for DNA lesions occurrence are:

- chemical reactions (hydrolysis of nucleotide bases and non-enzymatic methylations);
- occasional mismatches introduced by DNA polymerases during replication and by DNA strand breaks generated as a consequence of abortive activities of topoisomerases I and II;
- endogenous agents (reactive oxygen species – ROS, generated through aerobic cellular respiration);
- exogenous agents (UV light, tobacco-derived chemicals and ionizing radiation).

DNA lesions include adducts, oxidized bases, abasic sites, DNA crosslinks, single-strand breaks (SSBs) and, less frequently, double-strand breaks (DSBs)[3].

1.1.3. Types of cancer

Cancers are named after the area in which they begin and the type of cells they are made of, even if they spread to other parts of the body.

Carcinoma refers to a cancer that starts in the skin or the tissues that line internal organs (liver, lungs, kidneys, intestines, reproductive organs, etc.).

Sarcoma refers to a cancer of connective tissues such as bones, muscles, cartilage, and blood vessels.

Leukaemia is a cancer of bone marrow, affecting the production of white blood cells (monocytes, platelets, granulocytes).

Lymphoma is a cancer of the immune system cells (T lymphocytes and B lymphocytes)[15]. For the purpose of this Master thesis, the focus was on two different types of cancer: **hepatocellular carcinoma (HCC),** which is fast-growing solid tumour primary in liver, and **acute monocytic leukaemia (AML), a** haematological malignant disorder or “suspended” tumour. The major difference between HCC and AML is obviously in their phenotype and area where they begin (liver and bone marrow, respectively). Apart from that, it is interesting to mention that the prevalence of haematological malignancies is much lower comparing to solid tumours, and accounts for only 6,2% of all deaths caused by cancer. However, the number of publications in the field of haematological malignancies is much higher than those on solid tumours as specimens of malignant cells can be obtained easily from peripheral blood of patients[16].

1.1.4. Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the most common type of primary liver carcinoma. It is usually found in people with chronic liver disease and it is a leading cause of cancer-related deaths in the world. Despite the fact that huge progress has been made in terms of prevention, screening and treatment of HCC, incidence and mortality of this disease continue to rise. This carcinoma occurs more often in males than in females (2.4:1), with a higher incidence in Eastern and Southern Asia, Middle and Western Africa, Melanesia, and Micronesia/Polynesia[17].

The main important risk factor for developing HCC is cirrhosis. This is a condition where liver is severely damaged and scarring tissue is more abundant than healthy one, compromising liver function. Cirrhosis develops over time due to liver damage caused by infection with hepatitis B and C viruses and alcohol consumption. Approximately 80% of patients infected with hepatitis C develop chronic hepatitis, and ~20% end up developing cirrhosis. The development of HCC occurs almost exclusively in livers with already established cirrhosis[17].

The HCC diagnosis is confirmed without pathologic confirmation. Screening includes radiologic tests (ultrasound, computerized tomography, and magnetic resonance imaging), and the presence of a serological marker (fetoprotein at 6-month intervals)[17].

In spite of all available screening techniques, the majority of patients are not properly screened and HCC is many times diagnosed at an already advanced stage when patients are symptomatic and liver is severely damaged. A problem at this late stage is that there is no effective treatment to improve the survival rate of patients. The treatment option with higher

successful rate is surgical resection or liver transplant, which is not always possible. Nonsurgical treatments in the form of chemotherapy are usually not successful for HCC patients. Moreover, higher mortality rates in HCC are associated with chemotherapy. Currently, scientists worldwide are working on finding a new potential therapeutic agent which could improve life of HCC patients[17].

1.1.5. Acute monocytic leukaemia

Acute myeloblastic leukaemia (AML) is a group of bone marrow neoplasms of myeloid precursors of white blood cells. One of the most common types of AML is acute monocytic leukaemia (AML-M5) that usually occurs in young children (< 2 years). However, the condition is rare and represents approximately 2.5% of all leukaemias during childhood and it has an incidence of 0.8 – 1.1 per million per year[18].

The AML-M5 is defined by the presence of more than 20% (WHO classification) or more than 30% (French-American-British, FAB classification) of myeloblasts in the bone marrow aspirate while normal bone marrow myeloblast count is less than 5%. Malignancy can develop as a result of congenital (Down syndrome, Fanconi's anaemia, congenital neutropenia) and / or acquired (prenatal exposure to tobacco, alcohol, radiation) factors[18].

The symptoms are not specific and include asthenia, pallor, fever, dizziness and respiratory failure. More specific symptoms are the appearance of unexplained bruises and/or (excessive) bleeding, coagulation disorders (DIC), neurological disorders and gingival hyperplasia.

Diagnostic methods include blood analysis, bone marrow aspirate for cytochemical, immunological and cytogenetical analysis, and cerebrospinal fluid (CSF) investigations[18].

Treatment options for AML consist of aggressive multidrug chemotherapy regimens, which are associated with high mortality and morbidity. The main drugs used for the treatment of AML are: arecytarabine, anthracyclins (daunorubicin, idarubicin and mitoxantrone) and etoposide. Therapy protocols include 3-7 courses of drug administration with intervals of 3-4 weeks. Chemotherapy can have serious consequences as severe bone marrow suppression, leading to leukocytopenia, neutropenia and thrombocytopenia. The overall 5-year survival rate for AML patients is 30-60% and long-term event free survival rate is only 20-50%. Therefore, new therapeutics are needed to increase the probability of cure in this disorder[19].

1.2. Cancer cell lines as *in vitro* models for cancer research

Different experiment*al model* systems, both *in vivo* and *in vitro*, exist for studying pathobiology of cancer. In vivo models include primary tumours, patient-derived xenografts and mice. The most used in vitro models are: paraffin-embedded samples, cancer cell lines and tumour primary cell cultures. Each of these models is used for different studies due to limitations associated with possible genetic manipulation[20].

For example, primary tumours (samples obtained by surgery from patients) represent the state of the tumour *in vivo* with its heterogeneity, but only at a specific evolutionary moment of the tumour. This sample is difficult to obtain, its amount is limited and genetic manipulation is almost impossible[21,22].

Patient-derived xenografts in nude mice models are often used for drug testing, providing the *in vivo* microenvironment for human tumour original cells but, on the other hand, they have an important limitation – these mice are immune-compromised which contributes to an overall inflammation process in cancer.

Animal models with spontaneous or induced tumours are used in the pathobiology research of cancer and for testing new therapeutics *in vivo*. However, using animal models in research means following strict ethical rules and the number of animals is always limited. Apart from that, it is difficult to extrapolate obtained results to humans[20].

As for *in vitro* systems, primary cell cultures are good models since they are derived directly from the tumours of the patient, which means they maintain some of the heterogeneity of the original tumour. However, the tissue environment is lost while culturing and samples are scarce and difficult to obtain[20].

This brings us to the conclusion that every experimental model for cancer research has certain advantages and disadvantages and none of them is completely representative of the phenotype of the tumour.

Nevertheless, cancer cell lines are the most adequate *in vitro* model for cancer research for many reasons. Cell lines are permanently established cell cultures that differ from cell strains in that they are immortalize. E6/E7 gene of human papillomavirus16 (HPV-16), or small and large T-antigen of the simian virus 40 (SV-40) are some examples of strategies used for cell transformation and immortalization . This means that cell lines will proliferate indefinitely given appropriate fresh medium and space, which makes them easy to handle and manipulate. Due to the progress in immortalization and cell culture techniques, almost every tissue can be

cultured nowadays and there is a large number and variety of cell lines immediately available for studying different types of cancer[21].

The most important characteristic of cancer cell lines is their high genomic similarity with the original tumour. Cancer cell lines maintain the tumour-specific chromosome abnormalities and oncogene mutations during the first passages and show the same morphologic and molecular characteristics of the primary tumour. In general, cell lines conserve well all "hallmarks of cancer" (described in Section 1.1.1), with an exception of angiogenesis due to the fact it requires the presence of stromal tissues[20,21].

Cancer cell lines also contain cancer stem cells, which makes them an excellent model for development and testing of anticancer drugs, which was proven by many scientists over two decades ago. Finlay and Bagulay (1984) demonstrated that cancer cell lines display a similar response to anticancer drugs when compared to the original tumour.

However, cancer lines do have some limitations. As the number of cell passages tends to increase, cells start to lose their phenotypic properties and become genomically instable. This leads to the rise of molecular changes including modifications in cell signalling pathways. Because of that, cell culturing for long time periods should be avoided[21,23].

To summarize, chosing the appropriate *in vitro* model in cancer research is the most important step for the screening of new potential cancer therapies. The results from the research done on cancer cell lines can be extrapolated to *in vivo* human tumours and this has a great importance for drug testing and translational studies. This characteristic has been recognized by many biomedical and pharmaceutical companies[20].Nevertheless, proper molecular characterization (genetic, epigenetic) of cell lines is fundamental before their use in order to determine the polygenetic aetiology of the type of cancer studied and the molecular mechanisms involved. This enables assessment of different types and subtypes of cancer as well as suitability of cell line established for studying that particular cancer type. Characterization is especially important for the development of new anticancer drugs as it can aid in revealing new targets and it also helps to understand the mechanisms involved in the resistance patterns of cells to certain chemotherapeutics[22].

As mentioned earlier in this section, there are a large number of cancer cell lines available in different culture collections. However, some of them are poorly characterized and may not bare the same molecular characteristics as the tumours they represent. In Table 1, the most commonly used cancer cell lines are summarized[20].

To conduct this Master Thesis, two cell lines from this list (Table 1) were studied in detail – HepG2 and THP-1 cells, representing hepatocellular carcinoma and acute monocytic leukaemia, respectively.

Table 1. Summary of most commonly used cancer cell lines with origin in different cell types[20]

Cancer cell line	Species of origin	Type of cancer	Morphology
HeLa	*Homo sapiens*	Cervix adenocarcinoma	Epithelial
MCF-7	*Homo sapiens*	Brest adenocarcinoma	Epithelial
U87MG	*Homo sapiens*	Glioblastoma-astrocytoma	Epithelial
HT-29	*Homo sapiens*	Colon adenocarcinoma	Epithelial
A549	*Homo sapiens*	Lung carcinoma	Epithelial
HepG2	*Homo sapiens*	Hepatocellular carcinoma	Epithelial
K-562	*Homo sapiens*	Chronic myeloid leukaemia	Lymphoblast
THP-1	*Homo sapiens*	Acute monocytic leukaemia	Lymphoblast
PC3	*Homo sapiens*	Prostate adenocarcinoma	Epithelial
A375	*Homo sapiens*	Malignant melanoma	Epithelial

1.2.1. HepG2 cell line

HepG2 is an immortalized cell line consisting of human liver carcinoma cells. It is the most widely used human hepatoma cell line and it was derived from a liver hepatocellular carcinoma of a 15 year old Caucasian male[23–25].

Hepatocellular carcinoma (HCC), the major type of liver cancer, has emerged as the second most common cause of cancer-related death. The only approved drug for treating advanced HCC, developed so far, is Sorafenib. As previously described in Section 1.1.4, chemotherapy used for treating HCC is not successful and is associated with high mortality in patients; moreover, liver transplant is the only effective treatment option. Therefore, it is highly desirable to develop new drugs for this disease[24].

The HepG2 cell line used in this study was obtained from the American Type Culture Collection (ATCC® HB-8056™). The morphology of HepG2 cells is epithelial and contains 55 chromosome pairs. Cells are adherent and grow as monolayers and in small aggregates (Figure 1). HepG2 cells can be grown successfully at large scale, and secrete many plasma proteins, such as transferrin, fibrinogen, plasminogen and albumin[26].

A detailed summary of HepG2 characteristics provided by ATTC is given in Table 2.

Table 2. Main characteristics of HepG2 cell line (ATCC® HB-8065™)[26]

Characteristics	Description
Karyotype	modal number = 55 (range = 50 to 60); has a rearranged chromosome 1
Receptor expression	insulin; insulin-like growth factor II (IGF II)
Genes expressed	alpha-fetoprotein (alpha fetoprotein); albumin; alpha2 macroglobulin (alpha-2-macroglobulin); alpha1 antitrypsin (alpha-1-antitrypsin); transferrin; alpha1 antichymotrypsin; (alpha-1-antichymotrypsin); haptoglobin; ceruloplasmin; plasminogen;,complement (C4); C3 activator; fibrinogen; alpha1 acid glycoprotein (alpha-1 acid glycoprotein); alpha2 HS glycoprotein (alpha-2-HS-glycoprotein); beta lipoprotein (beta-lipoprotein); retinol binding protein (retinol-binding protein
Cellular products	alpha-fetoprotein (alpha fetoprotein); albumin; alpha2 macroglobulin (alpha-2-macroglobulin); alpha1 antitrypsin (alpha-1-antitrypsin); transferrin; alpha1 antichymotrypsin; (alpha-1-antichymotrypsin); haptoglobin; ceruloplasmin; plasminogen; complement (C4); C3 activator; fibrinogen; alpha1 acid glycoprotein (alpha-1 acid glycoprotein); alpha2 HS glycoprotein (alpha-2-HS-glycoprotein); beta lipoprotein (beta-lipoprotein); retinol binding protein (retinol-binding protein)

The HepG2 cell line has several advantages comparing to primary cultured human hepatocytes: it grows continuously, has almost an unlimited life span and a stable phenotype. It is easily available; culture conditions are simpler than primary hepatocytes, and are easily standardized among laboratories. These features make the HepG2 cell line appropriate for drug screening purposes[24,25].

Despite the fact they express many liver-specific functions, the major drawback of this cell line is the lack of functional expression of almost all relevant human liver cytochrome P450 enzymes (CYP) that are involved in phase I of the oxidative metabolism of xenobiotics (drugs) system. There are about 50 CYP genes in the human genome encoding for catalytically active monooxygenase haemic proteins; however, only a few of them are capable to metabolize drugs and xenobiotics, which takes place in the liver. As a consequence, HepG2 cell lines show very limited drug metabolism activities and they are resistant to most anticancer drugs[23–25].

Primary human hepatocytes are still considered the best *in vitro* model for studying new therapeutic agents for hepatocellular carcinoma because they can provide information on how

the potential drug will be metabolized *in vivo*. Despite this, HepG2 cell lines are usually the first option and they are the most commonly used for screening and selection of new drugs as they provide the fastest way to identify drug leads with favourable pharmacokinetic and metabolic properties; sometimes, they are also the only option[25].

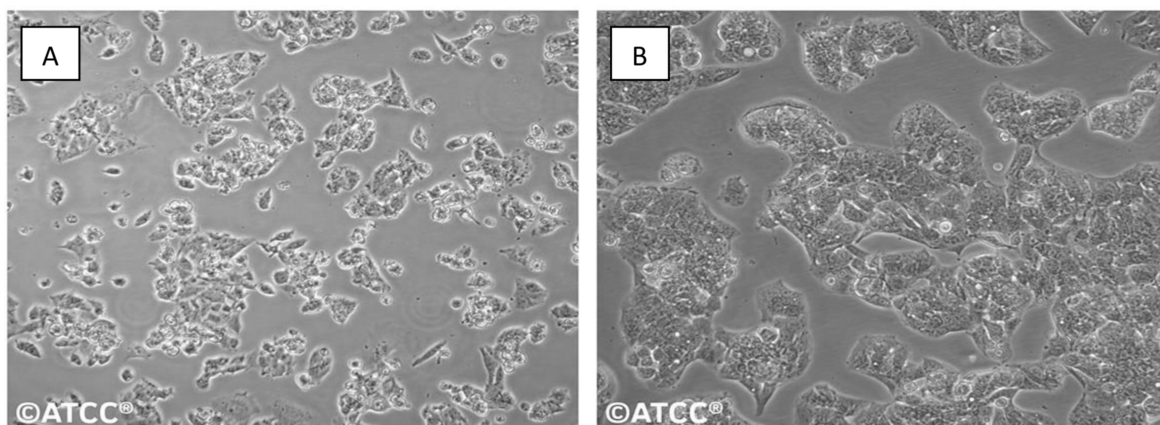


Figure 1.HepG2 (ATCC® HB-8056™)in culture. Cells grow as monolayer forming aggregates: A) Low cell density; B) High cell density. Source: www.atcc.org.

1.2.2. THP-1 Cell line

THP-1 is a human monocytic leukaemia cell line that was firstly cultured from the peripheral blood of a 1-year-old male with acute monocytic leukaemia[27,28]. Acute monocytic leukaemia (AML) is a malignant disorder that affects hematopoietic cells (bone marrow). Usually occurs in young children (< 2 years) and it is associated with high mortality due to aggressive chemotherapeutic treatment. New therapies are needed to improve the probability of cure for this disease[18,19].

The THP-1 cell line is a non-adherent, suspension cell line (Figure 2)[29]. The cells used in this study were obtained from the American Type Culture Collection (ATCC® TIB-202™). A summary of the main characteristics of THP-1 cell lines is given in Table 3.

The THP-1 cell line is one of the few leukaemia cell lines available and it is a good model for studying acute monocytic leukaemia since it retains monocytic properties (phagocytosis, activation of T-lymphocytes, lysozyme production, presence of alpha naphthyl butirate esterase) for prolonged period of time in culture conditions (>14 months)[28].

Table 3.Main characteristics of THP-1 cell line (ATCC® TB-202™)[29]

Characteristics	Description
Karyotype	diploid (46, XY);
Receptor expression	complement (C3); Fc
Genes expressed	lysozyme,HLA A2, A9, B5, DRw1, DRw2
Cellular products	lysozyme
Antigen expression	HLA A2, A9, B5, DRw1, DRw2

In addition, upon stimulation with phorbol 12-myristate 13-acetate (PMA), monocyte (THP-1) cells start to differentiate into macrophage-like cells,becoming adherent. They resemble native monocyte-derived macrophages with respect to numerous criteria which makes THP-1 a valuable model for studying the role of macrophages in human immune response[27,28]. Moreover, both monocytes and macrophages play an important part in cancer progression. In tumour settings, monocytes are recruited from peripheral blood to the tumour tissue, where they differentiate into tumour-associated macrophages (TAM). This subclass of macrophages is a heterogeneous cell population regarding their phenotype and pro-tumour function. They have abilities to support tumour initiation, local progression and distant metastasis. Therefore, targeting monocytes and macrophages in cancer is a promising therapeutic approach[30].

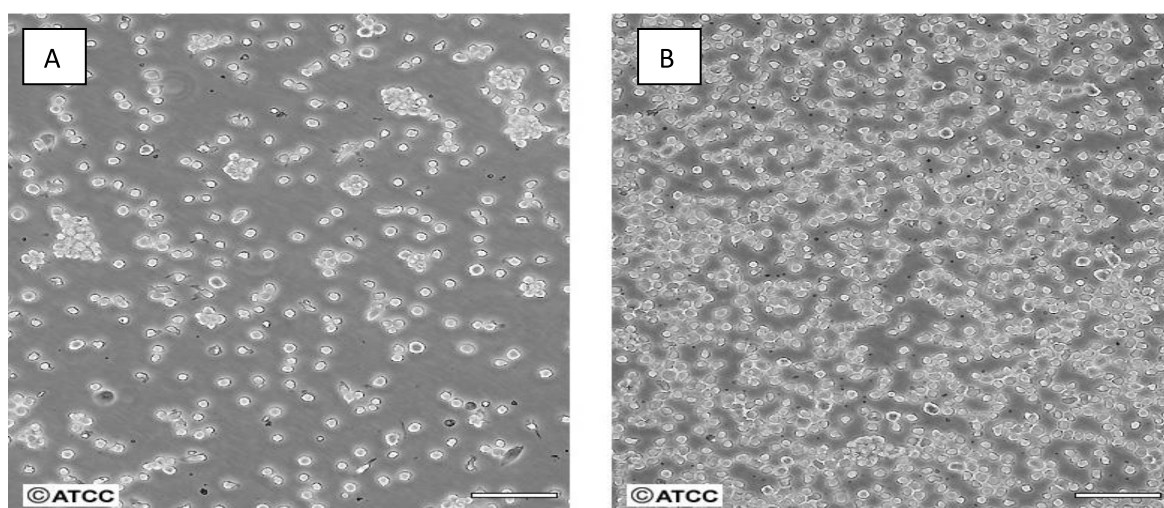


Figure 2. THP-1 (ATCC® TB-202™) in culture. Cells grow in suspension: A) Low cell density; B) High cell density. Source: www.atcc.org.

1.3. Cancer therapy

There are many types of cancer treatment. The types of treatment that patients receive will depend on the type of cancer and its stage of progression[31].

According to the National Cancer Institute[31] available cancer therapies are:

- Surgery (procedure in which the malignant tissue is removed from the body);
- Radiation therapy (using high-energy particles or waves, such as X-*rays*, γ-*rays*, electron beams or protons, in order to destroy or shrink cancer cells);
- Chemotherapy (special drugs are administered orally or intravenously to stop the proliferation of malignant cancer cells);
- Hormone therapy (slows or stops the growth of cancer cells; used only for prostate and breast cancer which need hormones to grow);
- Immunotherapy (aims to boost human immune system in order to increase production of T cytotoxic cells, natural killer (NK) cells, or specific antibodies which target cancer cells);
- Stem cell transplant (bone marrow transplant to induce production of new hematopoietic cells, which can increase ability to fight off cancer cells).

Some patients with cancer will have only one treatment while some will receive a combination of treatments, such as surgery with chemotherapy and/or radiation therapy[31]. Among all currently used cancer treatments, chemotherapy continues to play an extremely important role. However, its effectiveness is limited in some cases by the existence of drug resistance, making it necessary to define optimal combinations for therapeutic strategies in order to ensure an efficient elimination of the tumour[1].

1.3.1. Chemotherapeutic agents

In general, chemotherapeutic agents kill malignant cells causing apoptosis. This was demonstrated *in vitro* with tumour cell lines, as well as *in vivo*, in patients receiving chemotherapy. Apoptosis is a normal cell response to irreparable DNA damage, which leads to cell death. There are two major cell-intrinsic pathways for inducing apoptosis. One involves inducing the expression of cell surface death receptors (Fas) that bind to ligands (FasL), which in turn activate adaptor molecules (FADD) and finally procaspase-8. Another pathway involves the release of cytochrome *c* from mitochondria, binding Apaf-1, and activatingprocaspase-9. Once activated, caspase-8 or caspase-9 can in turn activate caspases-3, 6, and 7[32]. Caspases are a unique family of cysteine-dependent proteases that are

responsible for proteolytic cleavages during apoptosis. These cleavages include internucleosomal DNA degradation and overall cytoskeleton break up leading to the formation of apoptotic bodies. Caspases are present in the cell as inactive zymogens and go through a cascade of catalytic activations at the onset of apoptosis[33].

Chemotherapeutics are generally classified in five groups: alkylating agents, antimetabolites, mitotic inhibitors, topoisomerase inhibitors and tumour antibiotics. Each group has a different mechanism of action on cancer cells (Table 4).

The most widely used antineoplastic drug is etoposide, from the topoisomerase inhibitors group[34].

Table 4.Classification of chemotherapeutic agents with some examples. Source: www.chemocare.com[35]

Drug class	Drug	Mechanism
Alkylating agents	Cyclophosphamide, Cisplatin	Alkylation of DNA/RNA leading to cross-link between DNA strands
Mitotic inhibitors	Vincristine, Docetaxel	Destruction of tubulin causing mitotic arrest in metaphase
Topoisomerase inhibitors	Topotecan, Etoposide	Inhibition of topoisomerase I and II causing DNA degradation
Antimetabolites	Methotrexate,Hydroxyurea	Inhibit ion of DNA replication and prevention of cell growth
Cancer antibiotics	Mitomycin, Actinomycin D	Inhibition of RNA synthesis which prevents cell mitosis

1.3.2. Etoposide as a topoisomerase II inhibitor

DNA topoisomerases are essential enzymes that regulate the topological state of genetic material by introducing transient breaks in the DNA molecule. They are involved in fundamental biological processes such as DNA replication, transcription, DNA repair and chromatin remodelling. Two major topoisomerase forms are present in all cells: type I (Topo I) which makes single-strand cuts in DNA, and type II (Topo II) which modulates DNA topology by passing an intact helix through a transient double-stranded break created in the DNA backbone. As a result, Topo II is able to regulate DNA catenation/decatenation, relaxation/supercoiling and knotting/unknotting. The covalent topoisomerase-cleaved DNA complex is a short-live intermediate and the breaks created are quickly repaired after passing the helix. Etoposide does not directly inhibit the action of Topo II but rather stabilizes this covalent cleavage complex introducing more breaks in DNA, which become permanent and irreparable. When these permanent DNA breaks are present at sufficient concentration, they trigger a series of events that culminate in cell death by apoptosis[32,36,37].

Etoposide is an analogue of 4-demethylepipodophyllin benzylideneglucoside (DEPBG), which is a plant toxin obtained from *Podophyllum* (American Mayapple or Mandrake). Podophyllotoxins have been used as medication by various cultures for over 1,000 years and were found to be topically effective for skin cancers, although, when ingested, they displayed high toxicity[37].

Etoposide was first synthesized by Sandoz Pharmaceuticals in 1966 and approved for cancer therapy in 1983 by the U.S. Food and Drug Administration. During clinical trials, etoposide demonstrated antineoplastic activity in various types of leukaemias, lymphomas and solid tumours. Namely, acute monocytic leukaemia (AML), Hodgkin's disease, non-Hodgkin's lymphoma, lung cancer (both small cell and non-small cell), gastric cancer, breast cancer and ovarian cancer, sarcoma and neuroblastoma. It could be administered both orally and intravenously to patients[36,37].

However, etoposide, as all chemotherapy drugs, has certain drawbacks. The first issue addresses drug specificity. All mammals have two Topo II isoenzymes, Topo II α and β that are differently regulated during cell growth. Topo II α is a proliferation marker, greatly elevated in tumour cells, whereas the TopoII β is presented in proliferating, as well as post-mitotic cells. Both isoenzymes are the target of etoposide which leads to off-target toxicity destroying not only malignant cells but also healthy cells including white blood cells needed for immunity[36,37]. As a consequence, the number of side-effects arise in patients: hair loss, low white blood cell count, vomiting, nausea, mouth sores, diarrhoea, fatigue, low blood pressure, etc.[38]. Another important therapeutic issue is the development of resistance and/or development of secondary malignancies, such as AML[37].

These side effects point out to the need to search, find and identify new sources of anticancer drugs with high selectivity and toxicity only for malignant cells, while conserving healthy immune cells.

Moreover, in the last decades, with the continuous growth of cancer cases and concerns over resistance to certain drugs, development of secondary cancers and the unwanted side effects observed, scientists are turning to alternative sources, trying to find potential drug leads in natural products (NP)[1].

1.4. The marine environment as promising source of new drug leads

Natural products (NPs) have been used as a source of therapeutic agents for the treatment of a wide spectrum of illnesses for thousands of years. Plants, in particular, have formed the basis of traditional medicine with the earliest records from Mesopotamia, around 2600 BCE, where usage of approximately 1000 plant-derived substances for curing different disorders was documented.[39,40]Even nowadays, many drugs present on the market are plant-derived NPs or their derivatives.

However, since natural product-based drug discovery is sometimes difficult due to their complexity, pharmaceutical industry has shifted its focus toward synthetic compound libraries for discovery of new drug leads. Unfortunately, obtained results did not meet expectations, especially for autoimmune diseases or cancer. This resulted in a declining number of new drugs on the market and, at the same time, revitalizing interests for NP. Bioactive compounds obtained from NPs are interesting due to their complex chemical structures. This complexity is a result of a phenomenon called biodiversity, where the interactions between organisms and their environment happen all the time, leading to the production of chemical compounds within the organisms, that enhance their survival and competitiveness in the environment[39,41,42].

Apart from terrestrial organisms, marine organisms have emerged as potential new sources of different NPs over the past two decades.

The systematic investigation of marine environments in order to identify novel biologically active compounds started only in the mid-1970s with the development of new technologies in scuba diving techniques and engineering of manned submersibles and remotely operated vehicles (ROVs) which facilitated exploring and sampling of deep waters[1,40,43].

During the period 1977–1987, about 2,500 new metabolites were reported from a variety of marine organisms. In 2015, a review performed by Blunt et al. revealed that the number of compounds of marine origin reached 30,000 with more than 300 patents waiting for approval. Moreover, in their last review from 2018, they reported additional 1277 new compounds isolated and described from marine microorganisms and phytoplankton, green, brown and red algae, sponges, cnidarians, bryozoans, molluscs, tunicates, echinoderms, mangroves and other intertidal plants and microorganisms, only for the year 2016. These numbers suggest that the marine environment indeed is a rich source of NPs with uncommon and unique chemical features that are not found in terrestrial species. The reason for this is a large biodiversity of marine organisms harboured by the oceans. Among 33 animal phyla listed, 32

are represented in aquatic environments, with 15 exclusively marine. 17 are found in marine and non-marine environments (with 5 of these having more than 95% of their species only in marine environments), and only one is exclusively non-marine (*Onychophora*). It has been predicted that approximately 8.9 million of eukaryotic species exist, of which approximately 2.2 million are marine organisms. This means that around 86% of the species on the earth, and 91% in the ocean, have not yet been described[1,41].

The coexistence of so many species in marine habitats and constant competition between them, as well as demanding environmental conditions (UV light, salinity, temperature, high pressure), resulted in the development of different mechanisms by marine organisms to defend themselves against predation or overgrowth of competing species. These mechanisms are actually chemical adaptions, defined as secondary metabolites that involve different classes of chemical compounds. They are generally specific for a particular taxonomic family, genus, species or even organism, and constitute a very small fraction of the total biomass of the organism. Predominantly, production of these NPs with excellent pharmacological potential occurs in sessile or slow-moving organisms (e.g. algae, sponges, cnidarians, tunicates and bryozoans) that, without effective escape mechanisms or structural protection, ensure their protection through chemical defence. Moreover, high pharmacological activity of NPs obtained from marine organisms comes from the fact that these compounds are released to the water and are rapidly diluted, therefore, they have to be highly potent to retain their efficacy[1,40,41,43].

To date, different types of secondary metabolites were isolated from marine organisms: terpenoids, alkaloids, polyketides, peptides, shikimic acid derivatives, sugars, steroids, and a large mixture of biogenesis metabolites. These compounds were found to exhibit many biological activities (antimicrobial, anti-tumoural, anti-diabetic, anticoagulant, antioxidant, anti-inflammatory, antiviral, antimalarial, anti-tubercular, anti-aging antifouling, and antiprotozoal) with huge industrial and therapeutic potentials[1,41,43].

The current pipeline for new compounds, from the initial demonstration that a molecule may have therapeutic potential to the production and approval, a drug needs to pass pre-clinical testing, complex clinical trials in humans, and post-trial approval by the regulatory organisms - Food and Drug Administration (FDA) (in US) and European Medicines Agency (EMA) (EU). This process can take 10 to 15 years with less than 12% of the potential drugs receiving final approval[44].

To date, seven approved marine-derived pharmaceuticals are in clinical use, four of which are anticancer drugs. The first marine derived anticancer agent developed for clinical use,

cytarabine or Ara-C, is a synthetic analogue of a C-nucleoside from the Caribbean sponge, *Cryptothethya crypta*, approved in 1969 and still in use for the treatment of acute myelocytic leukaemia and non-Hodgkin's lymphoma. Another example is Aplidine (dehydrodidemnin B) developed by PharmaMar©. This depsipeptide hydrodidemnin was isolated from the Mediterranean tunicate *Aplidiumalbicans*, and used in the treatment of multiple myeloma (phase III of clinical trials), and for solid and haematological malignant neoplasias, like T-cell lymphoma (phase II of clinical trials)[45].

1.4.1. Marine microalgae as a novel source of anti-tumoural drugs

Among marine organisms, algae and microalgae are one of the most important resources of the ocean, economically and ecologically. Microalgae are microscopic, photosynthetic organisms found in both marine and freshwater environments and represent the major component of phytoplankton. They are primary producers, responsible for up to 50% of global carbon fixation. Their photosynthetic mechanism is similar to that of land-based plants, converting solar energy into biomass, mainly because of their simple cellular structure and being submerged in an aqueous environment with access to water, CO_2, and other nutrients[46,47].

Microalgae are a polyphyletic and highly diverse group of prokaryotic and eukaryotic organisms[48]. The classification into divisions is based on various properties: pigmentation, chemical nature of photosynthetic storage products, the organization of photosynthetic membranes, and other morphological features. The most abundant microalgal classes are: *Cyanophyceae* (blue-green and red algae), *Chlorophyceae* (green algae), *Bacillariophyceae* (diatoms), *Chrysophyceae* (including golden algae) and *Phaeophyceae* (brown algae)[46,49].

Microalgae are considered as one of the most promising sources for new products and applications due to their chemical composition. These organisms are rich in important biomolecules such as polyunsaturated fatty acids (PUFAs), pigments (chlorophylls and carotenoids), polyphenolic compounds, phycobilins, vitamins, sterols and polysaccharides. It has been found that these biomolecules have not only nutritional value but also display bioactivities and have a potential use as therapeutic agents in the biomedical area. Many of these algae-derived compounds have been associated with numerous health promoting effects including anti-obesity, anti-diabetic, antihypertensive, anti-hyperlipidaemia, antioxidant, anticoagulant, anti-inflammatory, immune-modulatory, anti-estrogenic, thyroid-stimulating, neuroprotective, anti-osteoarthritic, anti-osteoporosis, antiviral, antimicrobial and anti-tumoural (Table 5)[44,50,51].

Table 5.Bioactive compounds obtained from microalgae ant their effect on human health[49–51]

Bioactive compound isolated	Health-promoting effect	Microalgae
Carotenoids		
β-carotene	antioxidant activity	*Dunaliellasalina; Haematococcus pluvialis; Tetraselmisspp.*
astaxanthin	antioxidant, immunomodulation, and cancer prevention	*H.pluvialis; Chlorella vulgaris*
fucoxanthin	antioxidant, immunomodulation, and cancer prevention	*Isochrysisgalbana; Phaeodactylum tricornutum*
lutein	antioxidant activity	*Chlorella pyrenoidosa; Tetraselmis spp.*
violaxanthin	antioxidant activity	*Chlorella ellipsoidea; Nannochloropsisoculata; Tetraselmis spp.*
PUFAs		
eicosapentaenoic acid (EPA)	reduce risk of certain heart diseases, anticoagulation,anti-inflammatory, neuroprotective	*P.tricornutum;Porphyridiumcruentum; I. galbana*
oleic acid	antioxidant activity	*C. vulgaris; H.pluvialis; Spirulinaplatensis*
linoleic acid	antimicrobial activity	*D.salina; S.platensis*
palmitic acid	antimicrobial activity	*D.salina*
palmitoleic acid	Antihypertensive	*S.platensis*
Docosahexaenoic acid (DHA)	reduce risk of certain heart diseases, anticoagulation,anti-inflammatory, neuroprotective	*S.platensis*
Proteins		
Phycobiliproteins	immunomodulation activity, anticancer activity, hepatoprotective, anti-inflammatory, and antioxidant properties	*S.platensis; Porphyridium spp.*
Polysaccharides		
sulfated polysaccharide	antiviral, antitumor, antihyperlipidemia, and anticoagulant	*C. pyrenoidosa; Porphyridium spp.*
Insoluble fiber	reduce total and LDL cholesterol	*C. vulgaris*
Vitamins		
tocopherols (vitamin E)	antioxidant activity	*S.platensis; Porphyridium spp., Tetraselmisspp*
Phenolic compounds		
benzoic acid derivatives, hydroxybenzaldehydes, and cinnamic acid derivatives	antioxidant activity	*S.platensis*

Along the last five decades it is estimated that more than 3,000 NPs have been discovered from algae and among all of the mentioned biological activities, anti-tumoural activity seems to be one of the most promising[44]. Many studies demonstrated the *in vitro* cytotoxicity of different extracts (water, ethanol, ethyl acetate, hexane, acetone) obtained from microalgal biomass on tumoural cell lines (Table 6).

Table 6. ***In vitro*** **cytotoxicity of microalgae on cancer cell lines**

Microalgae	**Extract tested**	**Cell line**	**IC_{50} (µM or µg/mL)**	**Compound responsible (if applicable)**	**Observed effects on cells (if applicable**
Spirulina platensis[52]	water	Colon carcinoma(HCT116), hepatocellular carcinoma (HepG2)	18.8 µg/mL for HCT116; 22.3 µg/mL for HepG2		
Porphyridium purpureum[53]	isolated and purified carotenoid	Human melanoma (A2058)	40 µM	zeaxanthin	Chromatin condensation, nuclear blebbing, DNA nucleosomal fragmentation, activation of caspase-3 (apoptosis)
Nitzschia sp[54]	isolated and purified carotenoid	Human glioma (U251)		fucoxanthin	Induction of apoptosis with PARP cleavage and caspase activation
Isocrysis galbana[55]	acetone	HepG2	81.3 µg/mL		Showed high selectivity (3.1) comparing to non-tumoural, murine stromal S17 cell line
Chaetoceros calcitrans[56]	hexane, dichloromethane, ethyl acetate and methanol	Breast adenocarcinoma (MDA-MB-231) , mouse breast carcinoma (4T1), HepG2, cervix epithelial carcinoma (HeLa), human prostate carcinoma (PC-3), human lung adenocarcinoma (A549), human colon adenocarcinoma (HT-29)	60 µg/mL, ethyl acetate extract on the MDA-MB-231 cancer cell line		Extract did not show cytotoxicity on non-tumorigenic cells (mouse embryo fibroblast (3T3))
Porphyridium cruentum[64]	sulfoglyco-lipidic fraction (SF)	Colon adenocarcinoma (DLD-1), human breast adenocarcinoma (MCF-7), human prostate adenocarcinoma (PC-3), and human malignant melanoma (M4)	20-46 µg/mL (range applies to all tested cell cultures)	sulfolipids	Inhibition of DNA α polymerase
Skeletonema costatum[67]	organic extract	human non-small-cell bronchoplumanory carcinoma line (NSCLC-N6)			G1 cell cycle arrest
Phaeodactylum tricornutum	ethanol	HepG2	250 µg/mL	Sulphated polysaccharide	
Nannochloropsis oculata	methanol	human chronic myeloid leukemia cell line (K562)	50 µg/mL		Induction of apoptosis with PARP cleavage and caspase activation

Despite the number of compounds isolated from microalgae and biological activities attributed to these, they still have not entered clinical trials or been marketed.[51,57].
There are several reasons for this, including the time and cost it takes to reach the market, difficulties in harvesting the organism, low titres of natural product in producing organisms, difficulties in isolation and purification procedures, problems in obtaining a sustainable supply of the compound, high toxicity of the active compound, ecological impact on natural populations, and insufficient investment by pharmaceutical companies[45]. In spite of the high number of microalgae species identified, only a few of them have been successfully commercialized for biotechnological applications.
Examples are *Chlorella spp.* (production of lutein)[57], *Spirulina platensis* (production of phycobiliproteins)[51], *Dunaliella salina* (natural source of β-carotene)[46,57], *Haematococcus pluvialis* (production of astaxanthin)[46,57], *Porphyridium cruentum* (production of sulphated polysaccharides, phycocyanin, and phycoerithrin)[49,51], *Phaeodactylum tricornutum* (production of eicosapentaenoic acid)[58], etc.
A reason behind this small number of commercialized microalgae species is a complex process of growth optimization of these organisms and in obtaining the compound of interest in large quantities. The chemical composition of microalgae is not an intrinsically constant factor, it varies among strains and batch cultures, seasons, and it is affected by environmental and culture conditions (temperature, pH, mineral content of water, light exposure, and agitation). Therefore, it is very important to select adequate culturing methods (e.g. open or closed photobioreactors, indoors or outdoors) with carefully controlled conditions, so that the metabolism of the microalgae favours a high production of the particular compound of commercial interest (e.g. PUFAs antioxidants, polysaccharides)[46,50,51]. Once the biomass is enriched in the target compound, the next step is to optimize the conditions to extract the valuable component with high yields while maintaining its activity. An efficient separation process of the desired compound should be able to process a large volume of biomass, yield a product with a high dry weight percentage, and require modest investment, low consumption of energy, and low maintenance cost[46,50,51].
Another difficulty in the production of microalgae is their recovery and preservation. The low productivity of biomass means that recovery systems need to efficiently manage very large volumes of medium with a very low concentration of biomass. This alone constitutes a great challenge to engineers who need to combine various recovery operations such as sedimentation, flotation, filtration, or centrifugation to recover and then preserve (freeze-

drying or spray-drying) the microalgae cells produced, so as to maintain the quality of the biomass and the activity of compounds of interest[49–51].

1.4.2. Microalgae used in this study to assess cytotoxicty

In this study, ethyl acetate and ethanol extracts of six microalgal species were selected to be screened for cytotoxicity in cancer cell lines: *Nannochloropsis* sp., *Isochrysis* sp., *Skeletonema costatum, Porphyridium* sp., *Tetraselmis* sp. *CTP4*, and *Phaeodactylum tricornutum.* In literature, up to date, there are no reported cytotoxicity assays that involve testing ethyl acetate extracts of these species on both HepG2 and THP-1 cells. For example, previous studies from MarBiotech research group reported toxicity of acetone, hexane, water and diethyl ether extracts of *Isochrysis galbana* on HepG2 cells, but research did not involve ethyl acetate extract and THP-1 cells[55]. In another study, a sulfoglycolipidic fraction (SF) isolated from *Porphyridium cruentum* was screened for anti-proliferative activity on human breast adenocarcinoma (MCF-7), human prostate adenocarcinoma (PC-3), and human malignant melanoma (M4) Beu cell-lines[59]. No studies are available reporting anti-proliferative activity of this microalgae on HepG2 and THP1. Sulphated polysaccharides extracted in ethanol from *Phaeodactylum tricornutum*, showed strong anticancer activity on HepG2 cells, but not on THP-1 cells[60]. Also, *Phaeodactylum tricornutum* methanol extract was toxic to human promyelocytic leukemia cell line (HL60), while *Nannochloropsis oculata* methanol extracts were toxic to human chronic myeloid leukemia cell line (K562)[61]. As for *Skeletonema costatum*, organic extract of this diatom was studied *in vitro* for its effect on asynchronous cells of a human non-small-cell bronchopulmonary carcinoma line (NSCLC-N6) and showed inhibition of growth in G1 stage of cell cycle[62](Table 6). To the extent of our knowledge, *Tetraselmis* sp. CTP4 was not assessed for cytotoxic activity so far. Based on all stated above, we decided to screen for cytotoxic effects of ethyl acetate and ethanol extracts of already explained microalgal species on HepG2 and THP-1 cancer cells.

1.5. Objectives

The objective of this thesis is to search for innovative drug leads for hepatocellular carcinoma and acute monocytic leukaemia treatment. For this purpose, different extracts (ethanol, ethyl acetate) from already commercialized, available microalgal biomass (*Porphyridium* sp., *Skeletonema costatum, Nannochloropsis* sp., *Phaeodactylum tricornutum, Tetraselmis* sp. CTP4 *and Isochrysis* sp.) were screened for cytotoxicity on HepG2 and THP-1 tumoural cell lines. In addition, a cell line (S-17) derived from a murine bone marrow (non-tumoural) was used to assess selectivity. Results were compared with etoposide, a widely used agent in chemotherapy.

In order to study the anti-tumoural effect of microalgae extracts on HepG2 and THP-1 cells and propose a new anticancer drug lead, we have established the following specific objectives:

1. To test different concentrations of ethanol and ethyl acetate extracts from microalgae (*Porphyridium* sp., *Skeletonema costatum, Nannochloropsis* sp., *Phaeodactylum tricornutum, Tetraselmis* sp. CTP4 *and Isochrysis* sp.) on HepG2 and THP-1 cell lines in order to determine the effects on cell viability.
2. To test different concentrations of ethanol and ethyl acetate extracts on S-17 non-tumoural cell line to assess selectivity.
3. To fractionate the most active extracts and produce active fractions with a more limited number of compounds.
4. To chemically characterize the most active fraction and tentatively identify a potential drug lead in microalgae extracts/fractions.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Solvents needed for extraction, fractionation and derivatization: hexane, ethyl acetate (EA), ethanol (ETH), dichloromethane (DCM), methanol (metOH), acetyl chloride and dimethyl sulfoxide (DMSO), were all analytical grade and purchased from VWR International (Leuven, Belgium). Ultrapure, Type 1 water was obtained using a MilliQ® Water Purification System (Darmstadt, Germany). For characterization by gas chromatography – mass spectrometry (GC/MS), n-hexane GC analysis grade was used (VWR International (Leuven, Belgium). Dulbecco's Modified Eagle's medium (DMEM), foetal bovine serum (FBS), L-glutamine (200 mM), penicillin/streptomycin mixture (10,000 U), trypsin/EDTA solution (10x), phosphate buffer saline (PBS) (10X) were obtained from Lonza Ibérica (Barcelona, Spain).
Roswell Park Memorial Institute medium (RPMI-1640) was from Thermo Fischer Scientific. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)(ultrapure grade) was obtained from VWR International (Leuven, Belgium). Etoposide (synthetic, 98-105%, powder) was purchased from Sigma-Aldrich (Steinheim, Germany).

2.1.1. Algal biomass

Algal biomass was kindly provided by NECTON S.A. (Olhão, Portugal), a company specialized in the cultivation and commercialization of microalgae in Portugal since 1996. Biomass was dried, in the form of a powder, stored at room temperature and protected from light and humidity before conducting experiments.

2.2. Preparation of the extracts

Powdered (dry) biomass was mixed with the selected solvent (water, ethanol or ethyl acetate) (1:40, w/v) and left overnight (12-15 h) under continuous stirring, at room temperature (RT, 20°C). Next morning, samples were centrifuged (2500 rpm, 5 min, RT), supernatants recovered and filtered with qualitative, medium flow rate filter paper (10-12 µm pore size; Prat Dumas, France). The process was repeated three times. Finally, supernatants were combined and vacuum-filtered, using 0.45 µm and 0.2 µm filters (Prat Dumas, France) sequentially. Extracts were then dried using a rotary evaporator (Figure 3; IKA, Staufen, Germany) at 40°C under reduced pressure (Table 7). Once the solvents were completely evaporated, extracts were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 20 mg/mL and stored at -20°C.

Ethyl acetate was selected due to its medium polarity, low toxicity and environmentally friendly properties. In addition, this solvent is acceptable for food applications[63]. Its medium polarity (polarity index = 4.4)[64] allows for the extraction of both polar (sulphated polysaccharides, polyphenols, vitamins B and C and phycobiliproteins) and non-polar (chlorophylls, carotenoids and polyunsaturated fatty acids - PUFAs -, α-tocopherol - vitamin E - and vitamin A)[65] components from marine microalgae biomass in order to assess their bioactivity. Moreover, it has been reported that ethyl acetate extracts showed high cytotoxicity for cancer cells lines, while no effect was observed on healthy cell strains[56,66]. Ethanol was chosen because of its slightly higher polarity index (5.5)[61]. It was expected that ethanol would extract more polar compounds but also responsible for antioxidant and anti-proliferative activities (polyphenolics, vitamins and some carotenoids). In addition, ethanol extracts are easier to fractionate than ethyl acetate and even more environment friendly.

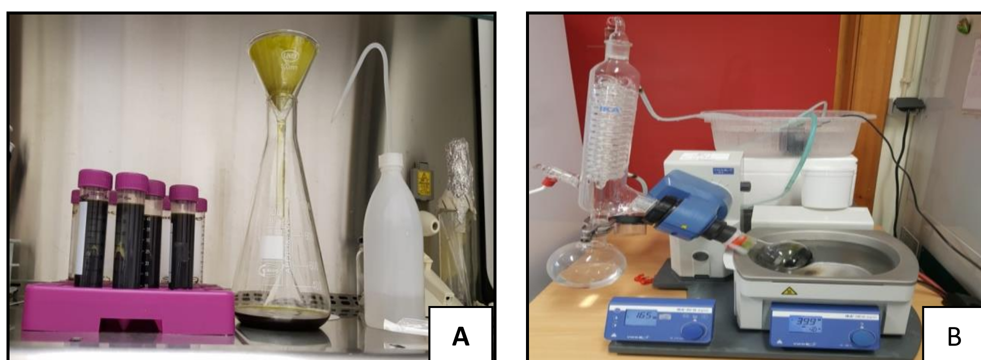


Figure 3. Extract preparation from dried algal biomass. Powder was mixed with solvent and left overnight under constant stirring. Next day the mixture was first centrifuged and filtered (A) and afterwards concentrated in a rotary evaporator (B).

2.3. Selection of extracts for testing

The microalgal extracts (ethanol, ethyl acetate) tested on HepG2 and THP-1 cell lines were selected based on bibliographic research about their reported cytotoxicity on these cell lines and their immediate availability (commercialized species).
At first only ethyl acetate and then ethanol extracts of the following microalgae were selected: *Porphyridium* sp., *Skeletonema costatum, Nannochloropsis* sp., *Phaeodactylum tricornutum, Tetraselmis* sp. CTP4 *and Isochrysis* sp.

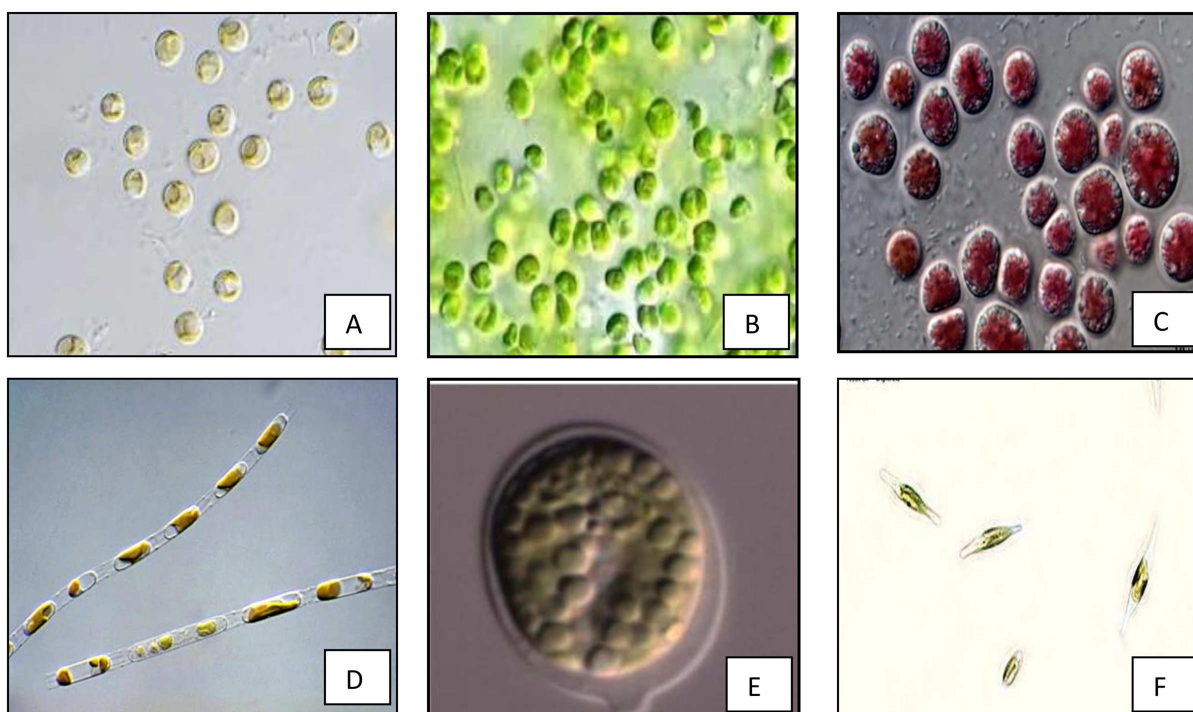


Figure 4.Microalgae species used to test cytotoxicty on HepG2 and THP-1 cells. *Isochrysis galbana* (A) (Source:www.algaeresearchsupply.com); *Nannochloropsis oculata* (B) (source: www.cfb.unh.edu); *Porphyridium cruentum* (C) (source: www.algaeresearchsupply.com); *Skeletonema spp*. (D) (source: www.cfb.unh.edu); *Tetraselmis* sp. *CTP4* (E) (source: Pereira et al, 2015); *Phaeodactylum tricornutum* (F) (source: www.utex.org).

2.4. Bioassays

2.4.1. Cell cultures

Cancer cell lines were selected based on their immediate availability and opposed morphology, having HepG2 as epithelial and THP-1 as monocytic cell line. HepG2 is an adherent cell culture, representative of solid tumours (hepatocellular carcinoma), and THP-1 cells in suspension, representative of bone marrow malignancies (acute monocytic leukaemia). S17 cells are adherent cells, derived from murine bone marrow and were used as healthy control and to assess selectivity of extracts.

The human hepatocellular carcinoma cell line HepG2 (ATCC® HB-8065^{TM}) and human monocytic cell line THP-1 (ATCC® TB-202) were maintained in RPMI-1640 culture media supplemented with 10 % foetal bovine serum (FBS), L-glutamine (2mM), penicillin (50 U/mL) and streptomycin(50 µg/mL). S17 cell line was kindly provided by Dr. Nuno Santos

(CBMR, UAlg) and grown in DMEM culture media supplemented with 10 % FBS, L-glutamine (2 mM), penicillin (50 U/mL) and streptomycin (50 μg/mL). All cell lines were grown at 37°C and 5.0% CO_2 in humidified atmosphere (Binder, Tuttlingen, Germany).

2.4.2. *In vitro* cytotoxic activity

2.4.2.1. MTT assay

In vitro cytotoxic activity of crude ethanol and ethyl acetate extracts was assessed by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay. It is a rapid, quantitative assessment of cell proliferation which measures mitochondrial dehydrogenase performance[67]. During the test, yellow water-soluble MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide, a tetrazole), is reduced to purple formazan due to cleavage of the tetrazole ring by dehydrogenases present in the mitochondria of living cells. This reduction takes place only when mitochondrial reductase enzymes are active. Resulting formazan crystals are soluble in dimethyl sulfoxide (DMSO) and other organic solvents, but are insoluble in water. Therefore, the amount of crystals formed has a positive correlation to the number of live cells. This can be quantified by measuring the absorbance (optical density), between 500 and 600 nm. This method has certain limitations since it is influenced by the physiological state of cells and there is some variance in mitochondrial dehydrogenase activity between different cell types. Nevertheless, the MTT assay has long been used as the "golden standard" of cytotoxicity assays as it is highly sensitive and has been miniaturised for use as a high-throughput screening assay[67,68].
The assay is recommended even in ISO Standard for assessing cytotoxicity (ISO 10993-5: Biological Evaluation of Medical Devices), where in Annex C, the MTT assay is prescribed as a quantitative cytotoxicity method. The MTT assay has several benefits over traditional qualitative cytotoxicity methods: it can detect as few as 950 cells, has high repeatability and a standard microplate reader can be used to analyse the results, increasing its throughput ability[69].
For the purpose of this Master thesis, a short MTT assay (48h of exposure) was performed and consisted of the following steps. On the first day, exponentially growing cells were seeded on 96-well plates at a density of 5×10^3cells/well for adherent cell lines (HepG2 and S-17) and 1×10^4 cells/well for the suspension cell line (THP-1). Cell suspension for plating cells was prepared in the following manner: one flask of each adherent cell line (HepG2, S-17) was first trypsinised adding 1 mL of trypsin/EDTA solution and incubated at 37°C in 5.0% CO_2 for 10 minutes or until cells were completely detached from the bottom.

Afterwards, 3 mL of corresponding culture media was added to the flasks to inactivate trypsin, suspension was gently mixed, and 0.5 mL was transferred to Eppendorf tubes for counting. Then, 10μL was taken and mixed with 0.4 % trypan blue (1:1, v/v) and 10μL was applied to the counting chamber. Cells were counted under a 10× objective using an inverted microscope (Motic, Germany). Procedure for THP-1 cells was the same, without the trypsinization step, as it is not required for cells in suspension. After counting, cells were plated and incubated overnight at 37°C in 5.0% CO_2, to recover and to attach to the bottom of the well (for adherent cell lines). On the second day, the extracts were applied at concentrations ranging from 125 to 3.9 μg/mL and incubated for 48h at 37°C in 5.0% CO_2 (Figure5). A positive control was done in which cells were treated for 48 h with etoposide at the same concentrations of the extracts and negative control cells were left untreated. In addition, dimethyl sulfoxide (DMSO) was tested for toxicity as extracts were dissolved in DMSO in order to apply to cells. For this purpose, one group of control cells was treated with DMSO only, at the highest concentration used in the test wells (0.6 %, v/v). On the fourth day, two hours before the end of the incubation period, 20μL of MTT (5 mg/mL in PBS) were added to each well and further incubated for 2h at 37°C in 5.0% CO_2 (Figure 5). A blank was performed consisting only of complete culture medium and MTT. After the incubation, the medium was carefully removed from the wells and purple formazan crystals were in 150 μL of DMSO (Figure 5). Then, absorbance was measured on a Biotek Synergy 4 spectrophotometer (Winooski, US) at 590 nm.
Cell cultures were handled under Class II Biological Cabinet (Telstar, Terrassa, Spain), applying aseptic technique and using sterile pipette tips, solutions and tubes.

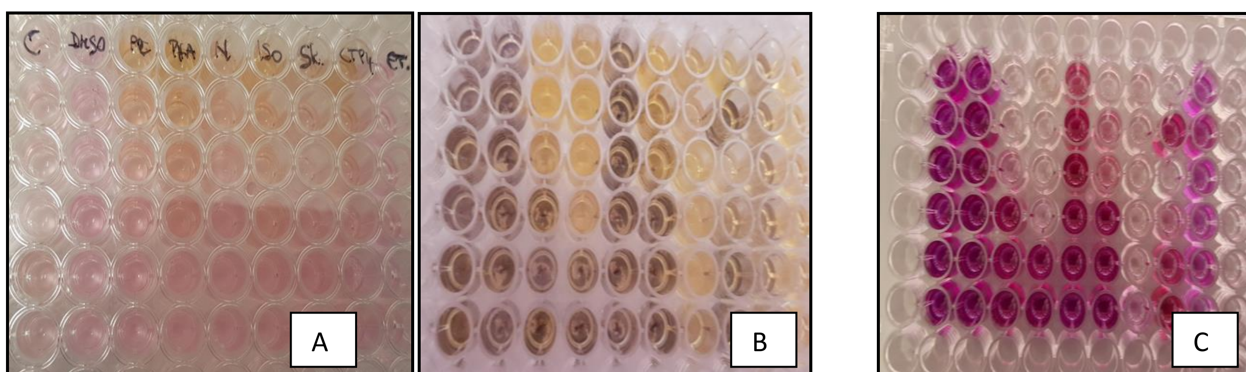


Figure 5. **MTT assay performed on a 96-well plate (HepG2 cell line).** Cells were plated and treated with extracts for 48h (A). After incubation, MTT was added and insoluble formazan crystals were formed (B). To dissolve the crystals, DMSO was added and plates were read at 590 nm. C – negative control (untreated cells); DMSO – cells treated with dimethyl sulfoxide (DMSO); POC, PHA, N, ISO, SK, CTP4 – cells treated with microalgal extracts; ET – cells treated with etoposide. Extracts were applied in decreasing concentration, starting with 125.0 µg/mL in the first well and ending with 3.9 µg/mL in the last well of each column.

2.4.2.2. Calculations and data interpretation

Results of MTT assay were expressed as percentage of cell viability in respect to untreated cells (controls) and as half maximal inhibitory concentration (IC_{50}, µg/mL).

In order to calculate the volume of cell suspension needed to seed appropriate amount of cells, Neubauer chamber was used for counting (VWR International, Leuven, Belgium) (Figure 6) following the equation:

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

where N represents number of cells/mL; A, B, C, and D (Fig. 6) correspond to the number of cells in each quadrant of the counting chamber and 10^4 is a conversion factor[70].
To calculate cell viability (%) after the treatment, the average absorbance of blank wells was calculated first and then subtracted from all the measurements on the 96-well plate, to eliminate the background noise. Afterwards, average of negative control wells (cells without treatment) was calculated representing 100 % of cell viability.

The cell viability of the positive control, DMSO control and cells treated with the extracts was then calculated following the formula:

$$\textit{Cell viability } (\%) = \frac{\text{A (sample)}}{\text{A (negative control average)}} \text{ x } 100$$

where A states for absorbance values at 590 nm.

Obtained values were then analysed using the GraphPad Prism software to obtain half maximal inhibitory concentration (IC_{50}). A dose-response curve by means of non-linear regression (variable slope, four parameters) was plotted to obtain IC_{50} (µg/mL) values. All experiments were conducted at least three times with all conditions performed in triplicates. Only IC_{50} values below 100 µg/mL and acceptable 95% confidence interval (CI) were selected.

In addition, selectivity index (SI) of the extracts was determined using the equation:

$$\text{SI} = \frac{\text{CNT}}{\text{CT}}$$

where CNT and CT correspond to the extract-induced cytotoxicity on non-tumour cells (S-17) and tumour (HepG2 and THP-1) cells, respectively[48,55,71,72].

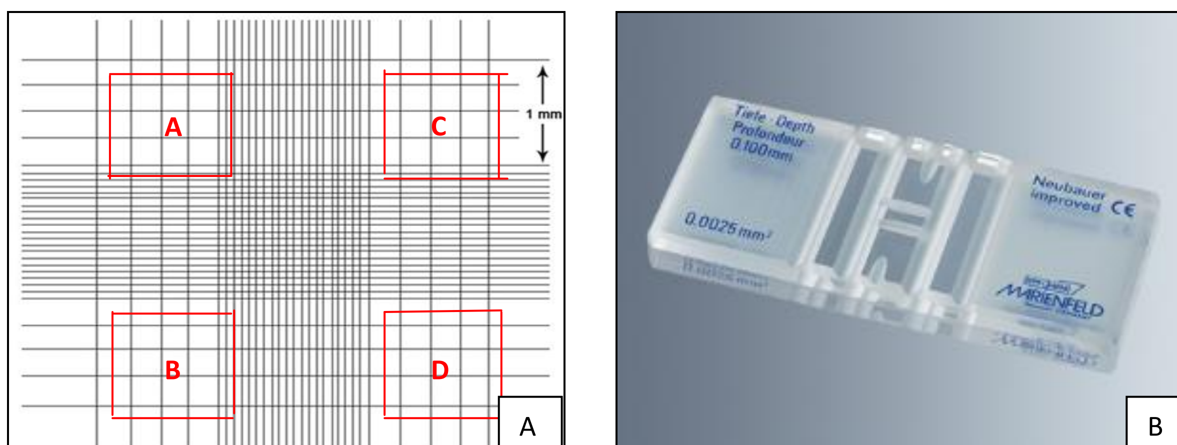


Figure 6. Neubauer counting chamber. Average count of the cells in red marked squares multiplied by 10000 gives number of cells per mL (A) (source: www.biocyclopedia.com; www.vwr.com)

2.5. Extract fractionation

The active crude ethanol extract of *Phaeodactylum tricornutum* was subjected to a bio-guided fractionation procedure in order to concentrate the active (lead) compound responsible for cytotoxic activity. Liquid-liquid extraction (LLE) was chosen as a preference method over silica gel column chromatography for this purpose, firstly because column chromatography can be time- and solvent-consuming while LLE can be performed in one day and reduces the volume of solvent spent; secondly, with LLE only four fractions were obtained, hence decreasing the amount of fractions to be further tested for cytotoxic activity.

Liquid-liquid extraction, also known as partitioning, is a process of separation of a solute (compound) between two immiscible solvents, in a biphasic system. One of the solvents is usually water or the aqueous phase and the other solvent is a non-polar, organic phase. The extraction is performed in a separating funnel and comprises a step of gentle mixing of the two solvents, followed by a step of phase separation. It is important to consider both steps in the selection of solvents and modes of operation. Equilibrium is reached when the chemical potential of the extractable solute is the same in the two phases. Position of the phases in the separating funnel depends on the density of the solvents. The aqueous phase is usually on the bottom as water is more dense than most organic solvents[73]. During LLE, aqueous layer was successively extracted with organic solvents of increasing polarity (e.g. n-hexane – chloroform – ethyl acetate)[74,75].

For the LLE, the crude ethanol extract of *Phaeodactylum tricornutum* (3.5 g) was dissolved in ethanol, introduced in the separating funnel and mixed with MilliQ water (1:4, v/v) to increase the polarity of the mixture making it relatively immiscible with the organic solvents selected. Then, 50 mL of hexane was added to the mixture; the funnel was gently mixed and left to sit until two phases formed. The organic, hexane layer was collected while aqueous layer was introduced again to the funnel and extracted with another two portions of hexane. The process was repeated with dichloromethane and finally ethyl acetate, affording four fractions (hexane fraction, dichloromethane fraction, ethyl acetate fraction and remaining, water-ethanol fraction) (Figure7). Organic fractions were concentrated using a rotary evaporator (40°C) under reduced pressure (Table 7), transferred to pre-weighed vials and dried under a gentle nitrogen flow to determine the yield of the fraction. Fractions were then dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 20 mg/mL. Water-ethanol fraction was freeze-dried and also stored dissolved in DMSO at a concentration of 20 mg/mL.

The four fractions obtained (hexane, dichloromethane, ethyl acetate and water-ethanol) were retested for cytotoxicity and selectivity on HepG2, THP-1 and S-17 cell lines, as described above. The most active fraction was chemically characterised by gas chromatography – mass spectrometry (GC/MS) to tentatively identify major compounds present and possibly responsible for the observed cytotoxicity.

2.5.1. Determination of extract yield

The yield of each fraction was expressed as percentage (%) in respect to initial mass of the extract subjected to fractionation (3.5 g) and calculated from the following formula:

$$(m_1 - m_2)x100$$

where m_1 (g) corresponds to the weight of the extract residue after solvent removal; m_2 (g) is weight of the dry biomass.

Table 7. Properties of organic solvents used for LLE and operating conditions for rotary evaporator[76,61]

Solvent	Formula	Molar mass (g/mol)	Evaporation energy (J/g)	Boiling point at 1013 mbar	Density (g/cm^3) at 20°C	Pressure (mbar)	Polarity index
Ethanol	C_2H_6O	46.0	879	79	0.789	175	5.1
Water	H_2O	18.0	2261	100	0.998	75	9.0
Hexane	C_6H_{14}	86.2	368	69	0.659	360	0.0
Dichloromethane	CH_2Cl_2	84.9	251	39.8	1.325	850	3.1
Ethyl acetate	$C_4H_8O_2$	88.1	394	77	0.895	240	4.4

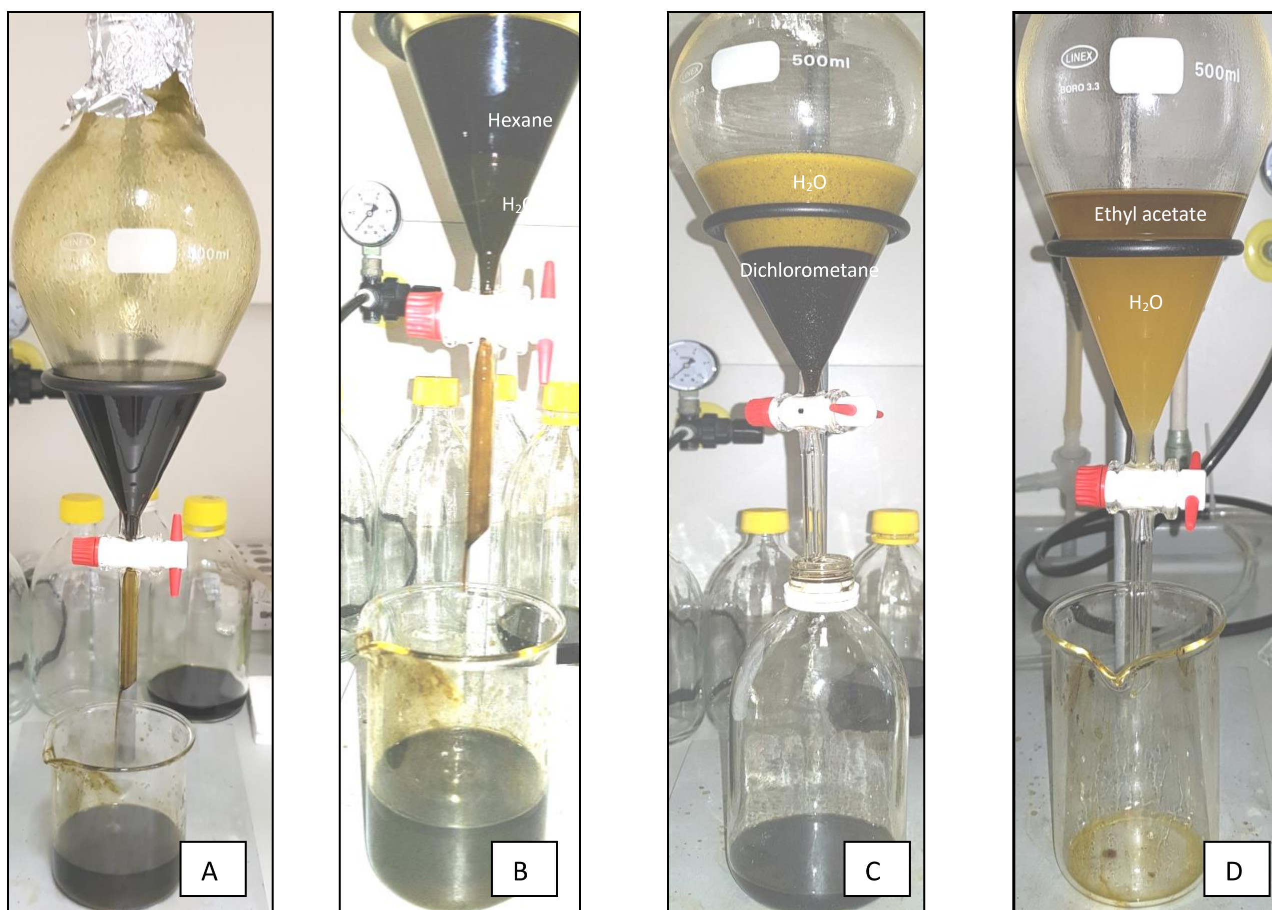


Figure 7. Liquid-liquid extraction (LLE) of *Phaeodactylum tricornutum* ethanol extract. During the LLE, solvents with increasing polarity were used. Dried extract was first dissolved in ethanol and mixed with Milli Q water in a separating funnel (A). Next, hexane was added, funnel was gently shaken and two phases were formed; organic (hexane) fraction in the upper part and aqueous fraction (water and ethanol) in the lower part due to the water density (Table 8) (B). Afterwards, aqueous fraction was introduced again into the funnel and extracted with dichloromethane, which has higher density than water (Table 8) and went to the bottom, while aqueous fraction formed upper layer (C). Last extraction of aqueous phase was done with ethyl acetate, affording two fractions, organic (ethyl acetate) in the upper layer and water with ethanol in the lower layer (D). Each extraction was repeated three times.

2.6. Characterization with gas chromatography – mas spectrometry (GC/MS)

2.6.1. Derivatization of samples

Prior to GC/MS analysis, derivatization was needed to increase the volatility of compounds and improve separation of analytes. This was achieved by adding acetyl chloride (catalyst) and methanol.

Dried fraction (5mg) was treated with 1.5 mL of derivatization solution (methanol/acetyl chloride, 20:1, v/v), in reaction vessels. Afterwards 1 mL of hexane was added, and the mixture was placed in a water bath for 1 hour at 70°C. After cooling in an ice bath (15 min), a mixture of distilled water and n-hexane was added (1:4, v/v), and the mixture centrifuged (2000g, 5 min, RT). The organic phase was collected, and the whole step was repeated one more time. Finally, the organic phase was removed, dried with anhydrous sodium sulphate

and filtered (0.22 μm). Hexane was evaporated under a gentle nitrogen flow until dryness and the fraction resuspended in 500 μl of GC-grade hexane and transferred to vials for GC-MS analysis[77].

2.6.2. GC/MS analysis

Samples were analysed on an Agilent GC-MS (Agilent Technologies 6890 Network GC System) coupled with MS detector (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 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 and quantification of bioactive compounds in fractions, the total ion mode was used.

2.6.2.1. Expression of results

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

2.7. Statistical analysis

Results were expressed as mean ± standard deviation, and all experiments were conducted at least three times, and performed in triplicates. Significant differences were assessed by analysis of variance (ANOVA) using GraphPad Prism v. 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Differences were considered as statistically significant when $p < 0.05$. Significance between means was analysed by Dunnett's and Tukey's multiple comparison tests. Prior to ANOVA, D'Agostino-Pearson test was performed to check for normality of data and homogeneity of variances was assessed with unpaired t-test. The 50% inhibitory concentrations (IC_{50}) were calculated by sigmoidal fitting of the data (dose-response curve) in the GraphPad Prism v. 7.0 program.

3. **RESULTS AND DISCUSSION**

3.1. Initial screening for cytotoxicity against HepG2 and S17 cell lines – ethyl acetate (EA) extracts

In the initial screening, the cytotoxicity of ethyl acetate (EA) extracts from *Phaeodacytlum tricornutum* (PHA), *Porphyridium sp.*(POC), *Isochrysis sp.* (ISO), *Skeletonema costatum* (SKLT), *Nannochloropsis* sp.(NANNO) and *Tetraselmis sp.*CTP4 (T CTP4) was assessed against human hepatocelluar carcinoma cell line (HepG2) and murine bone marrow cells (S17) (non-tumoural) via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium (MTT) reduction assay for 48h. Etoposide, which is used in the treatment of different types of cancer and acts as topoisomerase II inhibitor[36–38] was used as positive control in this study. All extracts, including etoposide, were applied to cells in a range of concentrations from 3.9 µg/mL to 125 µg/mL. In addition, dimethyl-sulfoxide (DMSO) was tested for cytotoxicity and applied at highest concentration used in test wells (0.6%, v/v). As all the extracts were dissolved in DMSO prior the experiment, it was necessary to show that this solvent is not toxic to cells at concentrations used.

Results of average cell viability expressed as percentage in respect to untreated controls are summarised in Annex 1. All extracts, except NANNO and T CTP4, led to a reduction in cell viability in a concentration-dependent manner. The best results were achieved with the application of PHA, POC, SKLT and ISO,where cell viability of HepG2 cells in respect to untreated controls at 125 µg/mL was reduced to 6.44%, 14.2, 4.88 and 4.92%, respectively. In the case of the non-tumoural cell line (S17), the same extracts (PHA, POC, SKLT, ISO) showed less cytotoxicity at the same concentration with cell viabilites of 39.9%, 81.6%, 33.7 and 40.6 %, respectively. This could indicate that some of the extracts showed higher selectivity towards cancer (HepG2) cells. It was also observed that all extracts lost their cytotoxic activity below the 31.25 µg/mL concentration. DMSO showed no toxicity to the cells with average cell viability of 94.9% for HepG2 and 91.6% for S17 cell line, which is statistically similar to that of untreated controls. Due to its medium polarity, DMSO is the prefered solvent in cytotoxicity assays for wide range of compounds, as it can dissolve both polar and nonpolar compounds; in addition, it is miscible with water[78]. Therefore, it is important to determine the safe concentration that could be used in the assay, as response greatly depends on the type of cell line used. For example, for skin fibroblast cells, DMSO concentration must not exceed 0.1% final concentration (v/v)[79]. On the other hand, study with Caco2/TC7 colon tumor cell line showed that even at 10% (v/v) DMSO did not induce

cytotoxicity[78]. Here, we showed that for HepG2 and S17 cell lines, DMSO at final concentration of 0.6 % (v/v) did not induce any observable toxic effect.

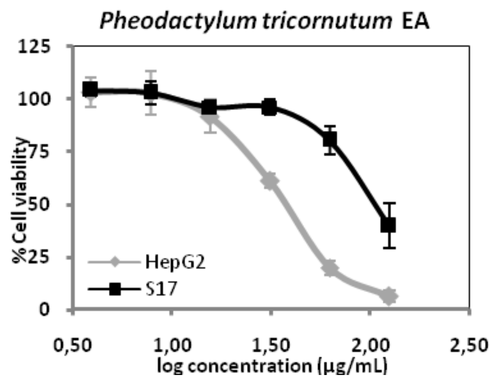
Next step in this study was to calculate the half-maximal inhibitory concentration, IC_{50} (µg/mL). The IC_{50} is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function[52]. In this particular case, it represents the concentration of a drug (extract) that will cause 50% decrease in cell viability *in vitro*. So, the most active extract is the one with the lowest IC_{50} value. Based on the IC_{50} results, the most active extract was identified in terms of bioactivity, and selectivity of extracts was assessed (Table 8). In that sense, the most active extract will be the one with the lowest IC_{50} value. At this step, two extracts, NANNO and T CTP4, were excluded from further analysis – as the decrease in cell viability of HepG2 cells was less than 50% at all concentrations tested (Figure 8).

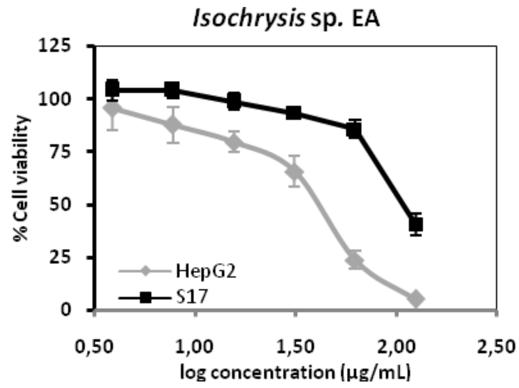
The most active extract was the one prepared from *P. tricornutum* (PHA) with the IC_{50} value of 34.6±3.5 µg/mL for HepG2 cells. This value was similar to that of etoposide (IC_{50} = 29.0±3.2 µg/mL) which is a widely used chemotherapeutic agent. Selectivity between tumoural and non-tumoural cells (S17) was used to assess the effectiveness of the extracts in cancer therapies[80]. This means that promising candidate for anti-cancer therapy needs to be highly selective for cancer cells while maintaining healthy cells, as opposed to chemotherapeutics, which are known for low selectivity, that causes some serious secondary effects[36]. As shown in Table 8, etoposide displayed low selectivity (SI=1.56), which was expected.

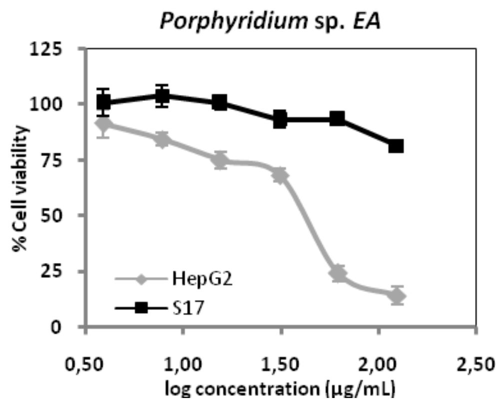
In comparison to S17 cells, PHA showed high selectivity (SI=3.09). Similar result was obtained with *Skeletonema* sp. with calculated IC_{50} of 37.2 ±3.6 µg/mL, which was also comparable to etoposide. However, in this case, the extract showed no selectivity when compared to S17 cells (IC_{50} = 34.8 ± 5.4 µg/mL), meaning that it was highly toxic even for healthy cells, and it was therefore excluded from further evaluation. Extracts obtained from *Porphyridium* sp. (POC) and *Isochrysis* sp. (ISO) had slightly lower cytotoxicity against HepG2 cells with IC_{50} values of 42.3 ±2.7 and 44.7 ± 3.1 µg/mL, respectively. ISO extract displayed low selectivity (SI=1.77) similarly to that one of etoposide (SI=1.56). Interestingly, cytotoxicity of etoposide on HepG2 cell line was much lower than previously reported by Vizetto-Duarte et al. (2016) for the same cell line and under same working conditions. This could be due to the development of resistance of HepG2 cells to etoposide over the course of time. These cells are known to display greater resistance to drugs in general, comparing to other cell lines[25,48]; hence the reason for choosing this cell line for screening of promising compounds in this study. It is noteworthy to mention that development of etoposide resistance

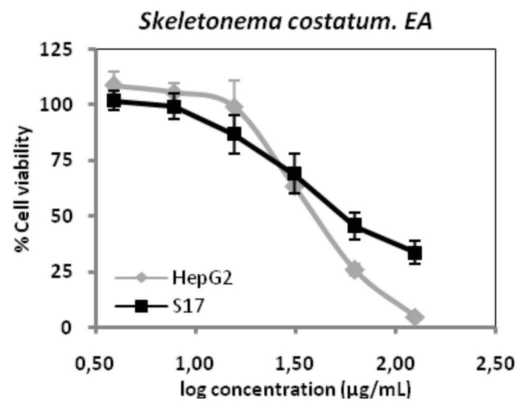
is quite frequent after prolonged exposure of cell lines to this drug which represents also a big problem when treating patients with ovarian or lung cancer using this chemotherapeutic agent[81,82].

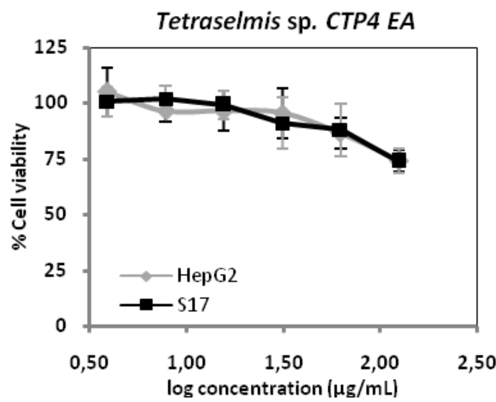
According to the previously established criteria of National Cancer Institute (NCI) in 1990, natural extracts are considered as promising candidates for anticancer drugs when the IC_{50} is less than 30 µg/mL and selectivity index higher than 3[83]. Unfortunately, none of the extracts displayed IC_{50} below the established criteria, but PHA extract had the IC_{50} closest to this value and more importantly, showed high selectivity compared to non-tumoural cell line. Previous studies on MDA-MB-231 breast cancer cells reported cytotoxicity of *C. calcitrans* ethyl acetate (EA) extracts with the IC_{50} of 60 µg/mL after 72 h of exposure.[56]. Moreover, EA extracts obtained from some plants (*Rhaponticum uniflorum*) were successful in an inhibition of cell growth and induction of apoptosis in oral squamous cell carcinoma cell line SSCC15 with reported IC_{50} value of 50µg/mL[84]. Taking into account that both of these cell lines are adherent like HepG2 used in this study, results obtained with PHA extracts (IC_{50} = 34.6±3.5 µg/mL) could be considered as promising. Therefore, it was decided to proceed with this extract in further studies.

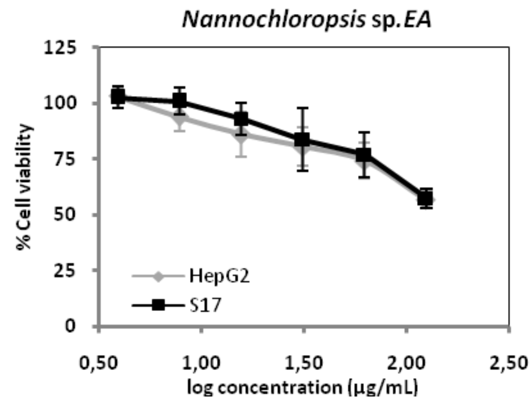












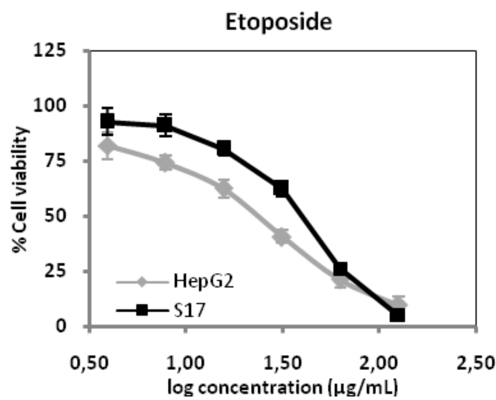


Figure 8. Cell viability (%) after 48 h exposure to different concentrations (3.9 – 125 µg/mL) of the ethyl acetate (EA) extracts of the species *P. tricornutum, Isochrysis* sp., *Porphyridium* sp.*, Skeletonema costatum, Nannochloropsis* sp.*, and* Tetraselmis sp. CTP4) and etoposide, **on HepG2 and S17 cell lines**

Table 8. ***In vitro*** **cytotoxic activity (IC_{50}; µg/mL) of the ethyl acetate (EA) extracts of the species *P. tricornutum, Isochrysis* sp., *Porphyridium* sp., *Skeletonema costatum, Nannochloropsis* sp., *Tetraselmis* sp. CTP4 and etoposide, on HepG2 and S17 cell lines**

Species	Cell line		
	HepG2	**S17**	**Selectivity index**
***P. tricornutum* (PHA)**	$34.6\pm3.5^{a,b,A}$	$107\pm7^{c,B}$	3.09 ± 0.12^{b}
***Porphyridium* sp. (POC)**	$42.3\pm2.7^{b,A}$	$>125^{a,B}$	$>2.7^{c}$
***Isochrysis* sp.(ISO)**	$44.7\pm3.1^{b,A}$	$79.2\pm2.7^{b,B}$	1.77 ± 0.09^{a}
***Skeletonema costatum* SKLT)**	$37.2\pm3.6^{a,b,A}$	$34.8\pm5.4^{a,A}$	n.s
***Nannochloropsis* sp. (NANNO)**	>125	>125	n. s
***Tetraselmis* sp. CTP4 (T CTP4)**	>125	>125	n. s
Etoposide	$29.0\pm3.2^{a,A}$	$45.4\pm0.5^{a,B}$	1.56 ± 0.11^{a}

Results are expressed as mean ± SD of data obtained from 3 different experiments performed in triplicates. Significant differences ($p<0.05$) were assessed with Tukey's test. Different letters (a,b,c) within each column represent significant differences between the species and species vs. etoposide for each cell line. Different letters (A,B,C) within each row represent significant differences between the effect of the extracts to the different cell lines; n. s – non-selective (SI=1).

3.2. Screening for cytotoxicity of *P. tricornutum* (PHA) ethanol extract against HepG2 and S17 cell lines

In the first part of the screening for cytotoxic activity of the ethyl acetate (EA) extracts from different microalgae species, PHA extract was identified as the one with highest bioactivity in terms of IC_{50} value and selectivity comapring to non-cancer cells (IC_{50} = 34.6±3.5 µg/mL; 3.09, respectively). In the next step, the ethanol extract from the same species (*P. tricornutum*) was tested for cytotoxicity against HepG2 and S17 cell lines to assess cell viability and selectivity, in order to compare with EA extract and determine which of them exhibits lower IC_{50}.

As it was explained in the *Material and methods* section, the ethyl acetate (EA) extracts were selected in the first screening phase to assess bioactivity on cancer cells, due to the medium polarity of EA, which allows for the extraction of both polar (sulphated polysaccharides, polyphenols, vitamins B and C and phycobiliproteins) and non-polar (chlorophylls, carotenoids and polyunsaturated fatty acids (PUFA's), α-tocopherol (vitamin-E) and vitamin-A[65] components from marine microalgae biomass to assess their bioactivity on cancer cell lines. Another reson for chosing this solvent was based on previous studies from MarBiotech research group dealing with cytotoxicity of microalgae extracts. It was revealed that less

polar extracts (e.g. acetone, ether, hexane) from *Isochrysis galbana*, *Tetraselmis* sp., and *Scenedesmus* sp., were more toxic to HepG2 cells than more polar ones. In fact, the best result was achieved with the least polar hexane extract of *Tetraselmis* sp., which reduced cell viability down to 10.8%, similar to etoposide[55]..

Apart from its wide usage in pharmaceutical industry[85], EA is present in confectionery for flavoring ice creams and cakes, and perfumes, as well as fruits such as apples[56]. However, it was decided to test ethanol extract of the same species to check if it would show higher bioactivity to cancer cells. Ethanol has a slightly higher polarity index (5.5)[61] than EA, and it was expected that ethanol would extract more polar compounds, also known for anti-proliferative activities (polyphenolics, vitamins and some carotenoids) and lead to lower IC_{50} values for HepG2 cells. In addition, the *Isochrysis* sp. ethanol extract was tested due to the previously reported cytotoxicity of this species against HepG2 cell line[55], as well as due to the medium cytotoxicity displayed (IC_{50} = 44.7±3.1µg/mL) with EA extract; therefore it was investigated whether ethanol could extract more bioactive compounds from the biomass of this species. After 48h of exposure to *P. tricornutum* ethanol extract (PHA ETH), a significantly lower IC_{50} was obtained (19.4 ±2.2µg/mL) for HepG2 cells comparing to the previous assay done with *P. tricornutum* ethyl acetate (PHA EA; 34.6 ± 3.5µg/mL). This value was again similar to that of etoposide (positive control used in this study). Furthermore, the selectivity index was higher in the case of PHA ETH (SI=4.40) comparing to PHA EA (SI=3.09) (Table 9). Looking at the cell viability results (Annex 2; Figure 9), at concentration of 31.2µg/mL only 20.8 % of HepG2 cells survived, which is less than in the previous experiment with PHA EA where at the same concentration the survival rate was more than 50%. For S17 cells, PHA ETH showed higher toxicity than PHA EA with an IC_{50} of 85.6 ±4.4 µg/mL compared to PHA EA (IC_{50} = 107±7 µg/mL) (Table 9). This difference can be attributed to the potent activity of the extract at 125µg/mL, where only 8.08% of cells survived the exposure to PHA ETH, while this number went up to 39.9 % in the case of PHA EA (Annex 2; Figure 9).

With *Isochrysis* sp. ethanol extract (ISO ETH) poor results were obtained with IC_{50} value of 67.8± 2.6 µg/mL for HepG2 cells, which was significantly higher than the previous result from ISO EA (IC_{50} = 44.7±3.1 µg/mL). Moreover, ISO ETH displayed low selectivity (SI= 1.53) similarly to that of etoposide (SI=1.61). Due to these results, both ISO EA and ISO ETH were excluded from further experiments.

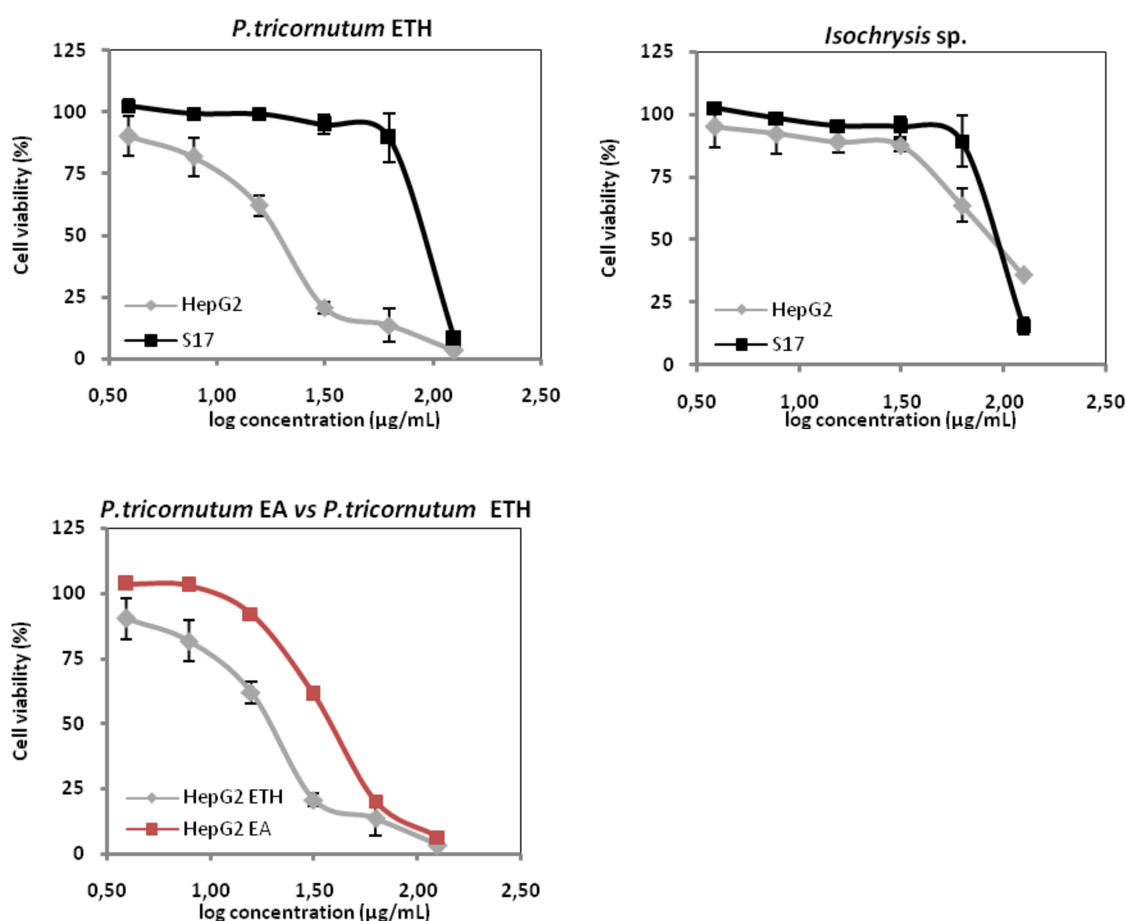


Figure 9. Cell viability (%) after 48 h exposure to different concentrations (3.9 – 125 µg/mL) **of the ethanol (ETH) extracts of the** species *P. tricornutum* and *Isochrysis* sp.**, on** HepG2 and S17 cells.

Table 9. ***In vitro*** **cytotoxic activity (IC_{50}; µg/mL) of ethanol (ETH) extracts of** ***P. tricornutum*****,** ***Isochrysis*** **sp. and etoposide on HepG2 and S17 cell lines**

Species	Cell line		Selectivity
	HepG2	**S17**	**HepG2vs.S17**
P. tricornutum **ETH**	19.4±2.2^{a,A}	85.6±4.4^{a,B}	4.40±0.15^{b}
Isochrysis **sp ETH**	67.8±2.6^{c,A}	104±4^{b,B}	1.53±0.08^{a}
P. tricornutum **EA**	34.6±3.5^{a,b,A}	107±7^{b,A}	3.09±0.12^{c}
Isochrysis **sp EA**	44.7±3.1^{b,d,A}	79.2±2.7^{a,B}	1.77±0.09^{a}
Etoposide	29.0±3.2^{a,A}	45.4±0.5^{c,B}	1.56±0.11^{a}

Results are expressed as mean ± SD of data obtained from 3 different experiments performed in triplicates. Significant differences (p<0.05) were assessed with Tukey's test. Different letters (a,b,c) within each column represent significant differences between the species and species vs. etoposide for each cell line. Different letters (A,B,C) within each row represent significant differences between the effect of the extracts to the different cell lines; n. s – non-selective (SI=1).

Previous studies addressing the cytotoxicity of ethanol extract of freshwater microalga *Micractinium sp*. reported suppression of proliferation in human colon cancer cells HCT116 (adherent cell line) at the concentrations of ≥25 μg/mL in a dose-dependent manner[86]. Another freswater microalga *Chloromonas sp.* showed cytotoxicity against human melanoma (A375) and cervical cancer (HeLa) cells with the IC_{50} value below 25 μg/mL[87]. Ethanol extract from marine microalga *C. calcitrans* displayed high cytotoxicity against human breast cancer cell line MCF-7 with IC_{50} value of 3 μg/mL[88]. These data suggest that results obtained with *P. tricornutum* ethanol extract on HepG2 cells (IC_{50} = 19.4±2.2 μg/mL) are promising and therefore it was decided to test this extract on other cell lines.

3.3. Screening for cytotoxicity of *P. tricornutum* (PHA) ethanol extract against THP-1 cell line

Since *P. tricornutum* ethanol extract (PHA ETH) exhibited the highest cytotoxicity towards HepG2 cells, which are considered as more resistant to drugs comparing to other cell lines, this extract was further evaluated on a human monocytic cell line (THP-1). PHA ETH treatment was again carried out in non-tumoural murine stromal (S17) cells to determine the selectivity index. In addition, cells were also treated with etoposide as a positive control. As shown in Annex 3 and Figure 10, PHA ETH supressed cell growth down to 11.7% at a concentration of 125 μg/mL but already at 62.5μg/mL the survival rate increased to 72.4%, indicating that this extract did not display high toxicity towards THP1. The calculated IC_{50} value of 102±7μg/mL was significantly higher than the values obtained for this extract on both HepG2 and S17 cell lines (Table 10). Selectivity index (SI=0.844) points out that this extract was more selective for non-tumoural cells (S17) than leukaemia THP1 cells, which in turn, points to its uneffectiveness. Nevertheless, more studies should be performed with other non-tumoural cell lines in suspension, as here we only compared THP-1 cells with adherent cell line (S17) and it is expected to obtain different responses for adherent and non-adherent cells. The cell line used in the study as a healthy control was derived from murine bone marrow and that should be considered in the results analysis, as responses to drugs can slightly differ between human and murine cell cultures. Additional human-derived healthy controls should be included in the study.

As reported before[80,89], etoposide was highly toxic to THP1 cells, obtaining significantly lower IC_{50} (0.924±0.241 μg/mL) comparing to S17 cells (45.4±0.5 μg/mL) and displaying extremely high selectivity (SI=49.4) (Table 10).

Table 10. Cytotoxicity (IC_{50}; µg/mL) of *P. tricornutum* ethanol extract and etoposide against HepG2, THP1 and S17 cell lines

Species	Cell line			Selectivity	
	HepG2	S17	THP1	HepG2vs. S17	THP1 vs. S17
***P.tricornutum* ETH**	19.4±2.2^{a,A}	85.6±4.4^{b,B}	102±7^{b,C}	4.40±0.15^{a,B}	0.844±0.072^{a,A}
ETOPOSIDE	29.0±3.2^{a,A}	45.4±0.5^{a,B}	0.924±0.241^{a,C}	1.56±0.11^{b,B}	49.4±1.2^{b,A}

Results are expressed as mean ± SD of data obtained from 3 different experiments performed in triplicates. Significant differences ($p<0.05$) were assessed with Tukey's test. Different letters (a,b,c) within each column represent significant differences between the species and species vs. etoposide for each cell line. Different letters (A,B,C) within each row represent significant differences between the effect of the extracts to the different cell lines; n. s – non-selective (SI=1).

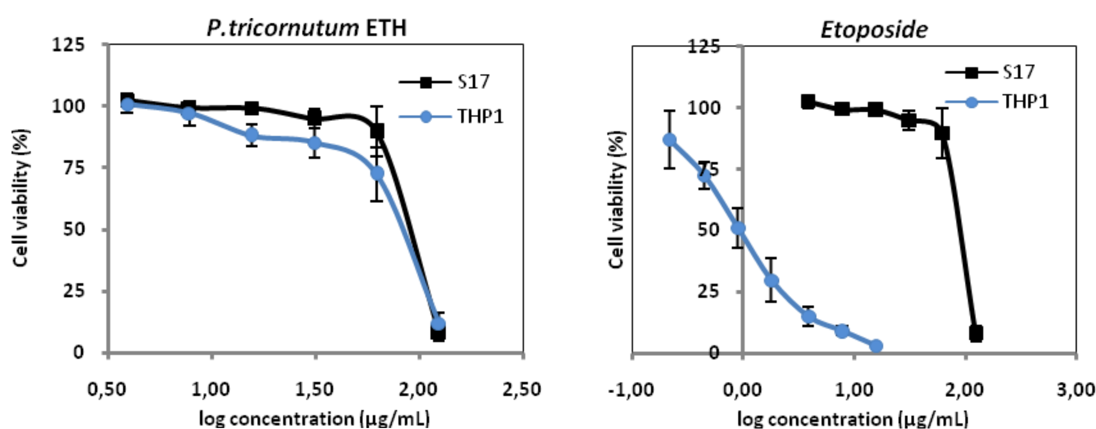


Figure 10. Cell viability (%) after 48 h exposure to different concentrations (0.24– 125 µg/mL) **of the ethanol (ETH) extract of** *P. tricornutum* and etoposide **on** HepG2 and S17 cells .

Although the THP-1 human monocytic leukaemia cell line was firstly derived from a patient with acute monocytic leukaemia, this cell line has been used more frequently as a model for macrophage differentiation in anti-inflammatory assays than as an *in vitro* cancer cell model[27]. Therefore, there are very few works adressing citotoxicity of natural compounds towards this cell line and it is difficult to speculate about the results obtained in this study and to compare with other publications. It was demonstrated in two studies that synthetic compounds, such as chiral hexacyclic steroids and bis(indolyl)methanes, have anti-cancer activity on THP-1 cells with IC_{50} values in the range of 15.7- 47.3 µM for steroids and 6.34- 38.8 µM for bis(indolyl)methanes[80,89]. However, when aqueous acetone extracts from twenty-

one extremophile plant species collected in saline habitats of the South of Portugal (Algarve) were screened for anti-cancer properties against THP-1 cell line, none of them showed toxicity to these cells, with cell viabilities above 46% after exposure to the extracts (125 µg/mL) for 72h[90], which is similar to the results obtained in this study. These results suggest that PHA ETH extract might be more toxic to adherent cell lines than to suspension cell lines. However, only one adherent cell line and one suspension cell line was tested and in order to confirm this, further studies with additional cell lines are needed. Extract tested is a mixture of a large number (and variety) of compounds that may interact with each other and purification by column chromatography or liquid-liquid extraction needs to be performed in order to isolate bioactive compounds with potential anti-cancer activity. Different compounds present in natural extracts are mainly defensive chemicals synthesized by the organism after the predatory attack or as a stress response to the changes in the environment[91]. There are three types of interactions between these compounds found in natural extaracts: additive,synergistic and antagonistic interaction. All three types of interactions are important factors that need to be considered when developing a new drug from natural compounds. Additive interaction means the effect of two chemicals is equal to the sum of the effect of the two chemicals taken separately. This is usually due to the two chemicals acting with the similar mechanism[91]. Synergystic interaction means that the effect of two chemicals taken together is greater than the sum of their separate effect at the same doses because one compound is potentiating the effect of the other one. Synergic interactions are the most important characteristics for developing combined therapies in cancer treatment. Substances from natural origin and their synergy, allow them to exhibit multitarget mechanism of action, as opposed to the conventional “one drug-one target” pharmacological concept. These compounds are able to interact with different targets, modifying different pathways or different steps of the same signalling cascade[92,93]. There are also evidences that combined therapy consisting of natural extracts and some other chemotherapeutic agent are much effective than single-based ones[93]. Antagonistic reaction means that the effect of two chemicals is actually less than the sum of the effect of the two drugs taken independently of each other. This is because one compound directly blocks the effect of the other. Antagonistic reactions form the basis for antidotes of poisonings[91].

3.4. Evalution of cytotoxic activity of fractions obtained from *P. tricornutum* ethanol extract against HepG2, THP-1 and S17 cell lines

In the first phase of this study, the ethanol extract of the marine diatom *P. tricornutum* was identified as the most bioactive in terms of cytotoxicity and selectitvity towards cancer cells. In the second phase, the crude ethanol extract was subjected to bioassay-guided sequential extraction using hexane, dichloromethane, ethyl acetate and water. The chemical nature and the polarity of the solvents used in the extraction process determines the extraction yield, composition, and biological activity of a given extract[55]. Hence, solvents of increasing polarity were used for the fractionation in order to separate and possibly isolate promising compounds responsible for cytotoxicity.

Four fractions were obtained (hexane, dichloromethane, ethyl acetate and water+ethanol) and tested for toxicity against adherent (HepG2) and suspension (THP-1) cancer cell lines via the MTT colorimetric assay with 48 h of treatment. Concentrations were applied in the same range of concentrations 3.90 µg/mL - 125 µg/mL, as before. The effectiveness of the fractions was measured by assessing selectivity and comparing the calculated IC_{50} values with those of etoposide, as showed in previous section. Selectivity was again determined using non-tumoural murine stromal cells (S17).

Dichloromethane (DCM) was the most active fraction causing a significant decrease in cell viability for both adherent (HepG2) and suspension (THP-1) cell lines. As shown in Figure 11 and Annex 4, treatment of HepG2 cells with 15.6, 31.2, 62.5 and 125 µg/mL of DCM extract for 48h significantly decreased cell viability to 82.1%, 44.3 %,16.6% and 7.19%, respectively. For THP-1 cells, at the same concentrations, survival rates were 50.1%, 31.4%, 9.93% and 5.45%, respectively.

The calculated IC_{50} values of 27.5±1.6 µg/mL and 22.3±1.8 µg/mL µg/mL (for HepG2 and THP-1, respectively) were significantly similar (Table 11). Although in both cases the DCM fraction displayed IC_{50} values significantly higher than etoposide, it was more selective than this chemotherapeutic agent with selectivity index >4.54 for HepG2 and >5.60 for THP-1 cells. These results suggest that the DCM extract was equally effective for both adherent and suspension cell lines while at the same time, there was no observable toxicity for non-tumoural cells even when treated with the highest concentration (125 µg/mL). However, as explained before, this fraction is still mixture of a great number of compounds which need to be characterised and tentatively identified before a promising anti-cancer agent can be unveiled. In spite of the large number of publications addressing cytotoxic activity of

microalgae against cancer cells, there are no reports so far on the potential *in vitro* anti-tumoural activity of dichloromethane fractions of the species included in this study (*P. tricornutum*). Anti-cancer activity against HepG2 and IPC-81 rat promyelocytic leukaemia cell lines has been previously described for compounds present in aqueous fraction of *P. tricornutum* methanol extract, inducing more than 30% cell death at concentration of 4mg/mL of dry weight[94]. It has been shown as well that sulphated polysaccharides isolated from *P. tricornutum* ethanol extract possess cytotoxic activity against HepG2 cells when applied at 250 µg/mL[60]. The concentrations reported in these two studies are considerably higher than the ones used in our research and confirmed to be toxic to HepG2 and THP-1 cells. As mentioned before, previous studies dealing with cytotoxicity of microalgae extracts revealed that cytotoxic effect was mainly attributed to the non-polar compounds in the extracts (e.g. carotenoids) that are extracted with less polar solvents like hexane, acetone and ether. Water is extremely polar, therefore these compounds cannot be extracted in aqueous fraction, hence the reason for such high concentrations. In our study, aqueous fraction of *P.tricornutum* was also inactive at 125 µg/mL, as it will be explained in the next paragraph. As for the sulphated polysaccharides isolated from *P.tricornutum*, they were also extracted in hot distilled water. When sulphated polysaccharides were isolated from seaweeds, extraction with hot water was followed by acetone fractionation. This step might be crucial and would lead to more toxic effect on the cancer cells[95].

Water and hexane fractions showed no toxicity against both HepG2 and THP-1 cells, as the reduction in cell viability did not reach even 50% (Annex 4, Figure 10). As we previously decided that IC_{50} values higher than 125 µg/mL are not promising for anti-cancer drug development, they were not subjected to calculation but rather just reported as higher than 125 µg/mL(>125). Ethyl acetate extract was effective only at a concentration of 125µg/mL for both HepG2 and S17, reducing cell viability to 7.85% and 28.9%, respectively. Obtained IC_{50} values of 84.2±3.7 and 81.9±2.0 µg/mL for HepG2 and S17, respectively, were again not significantly different ($p>0.05$). In addition to low toxicity, this extract displayed low selectivity (SI=1.48), similarly to etoposide (Table 11).

Prior to fractionation, *P. tricornutum* ethanol extract was freshly prepared from dry biomass and re-tested for cytotoxicity against all three cell lines. Interestingly, the IC_{50} values for all cell lines were significanlty higher (Table 11) than the values obtained with already prepared extracts in the first screening (Table 10). As shown in Table 11 and Figure 10, in case of THP-1 and S17 cells, this new extract was completely uneffective in a reduction of cell viability less than 50% (IC_{50} > 125.0µg/mL). On the other hand, extract showed cytotoxic

effect against HepG2 cells with IC_{50} of 39.82µg/mL, which was significantly higher comparing to *P. tricornutum* extract initially tested (19.42µg/mL). Besides, the IC_{50} obtained was also significantly higher in comparision to that of etoposide, but still maintaining high selectivity (SI > 3.14), suggesting that the extract did not lose its effectiveness (Table 11). There are a few factors that need to be taken into consideration and could have contributed to the slight loss of the activity between the extracts. The dried biomass was provided by NECTON, where microalgae are cultivated on a large scale for different purposes. *P. tricornutum* is used for aquaculture; therefore, culture conditions are optimized to enhance the production of specific bioactive metabolites relevant for aquculture, such as eicosapentaenoic acid (EPA) (one type of omega-3 polyunsaturated fatty acid) and the vitamins biotin, thiamine and cobalamine. It was previously described that environmental conditions during the growth of this diatom (e.g. temperature, light, salinity, nutrient availability) have a strong influence on the biomass composition and distribution of different metabolites, especially on the lipidic content[96]. This means that the content of potentially cytotoxic compounds relevant for this study, may differ from batch to batch, and differences in cytotoxicity results can be expected.
Taken alltogether, these results, as well as the results presented earlier in this section from dichloromethane fraction, still indicate that *P. tricornutum* ethanol extract has potent anti-cancer activity and can be considered as a promising candidate in anti-cancer therapy. However, additional (further) studies are needed in order to characterise and identify compounds which might be responsible for this cytotoxicity. In that sense, dichloromethane fraction was selected to be analysed with GS-MS.

Table 11. Cytotoxicity (IC_{50}; µg/mL) of fractions (hexane, dichloromethane, ethyl acetate, water+ethanol) from *P. tricornutum* ethanol extract and etoposide against HepG2, THP1 and S17 cell lines

Extract	Yield (%)	Cell line			Selectivity	
		HepG2	S17	THP1	HepG2 vs S17	THP1 vs S17
Ethanol crude extract	22.40%	39.8±4.3^{b}	>125	>125	>3.14^{c}	n.s
Hexane fraction	31.93%	>125	>125	>125	n.s	n.s
Dichloromethane fraction	16.27%	27.5±1.6^{a,A}	>125	22.3±1.8^{a,A}	>4.54^{b,A}	>5.60^{c,B}
Ethyl acetate fraction	5.71%	84.2±3.7^{c,A}	>125	81.9±2.0^{b,A}	1.48±0.13^{a,A}	1.53±0.15^{b,A}
Water+ethanol fraction	43.62%	>125	>125	>125	n.s	n.s
Etoposide		29.0±3.2^{a,A}	45.4±0.5^{B}	0.924±0.241^{c,C}	15.6±0.1^{a,A}	49.4±1.2^{a,B}

Results are expressed as mean ± SD of data obtained from 3 different experiments performed in triplicates. Significant differences ($p<0.05$) were assessed with Tukey's test. Different letters (a,b,c) within each column represent significant differences between the species and species vs. etoposide for each cell line. Different letters (A,B,C) within each row represent significant differences between the effect of the extracts to the different cell lines; n. s – non-selective (SI=1

3.5. Microscopic examination of morphological changes using inverted microscope

After incubation with tested extracts (125 µg/mL) for 48h, morphological alterations in HepG2, S17 and THP-1 cells were observed in comparison to the control (untreated) cells. This examination was performed before adding MTT in order to screen for some potential apoptotic events in the cells. Phenotypically, apoptosis is characterized by cell shrinkage, chromatin condensation, plasma membrane blebbing, DNA fragmentation, loss of contact with adjacent cells and collapse of the cell into small fragments (apoptotic bodies)[97]. Visualization of the control (untreated) cells showed that the cells maintained their original morphology, as in the culture flask (Figure 12); adherent cell lines (HepG2, S17) were attached to the bottom of the wells, forming monolayers. THP-1 had round-shape appearance,without signs of shrinkage.

Microscopic evaluation (Figure 12) revealed that both *P. tricornutum* ethanol extract (PHA ETH) and its dichloromethane fraction (DM) decreased the number of HepG2 and THP-1 cells, and induced cell detachment from the monolayer, in case of HepG2 cells. In addition, cell shrinkage could be observed, especially in THP-1 cell lines. These changes resemble effects caused by apoptosis, suggesting that both extracts tested might have apoptogenic properties; hence, decrease in cell number might be contributed to apoptosis. However, in order to confirm this, additional studies are needed, such is Annexin V staining assay and DNA fragmentation to identify and quantify apoptotic cells[48].

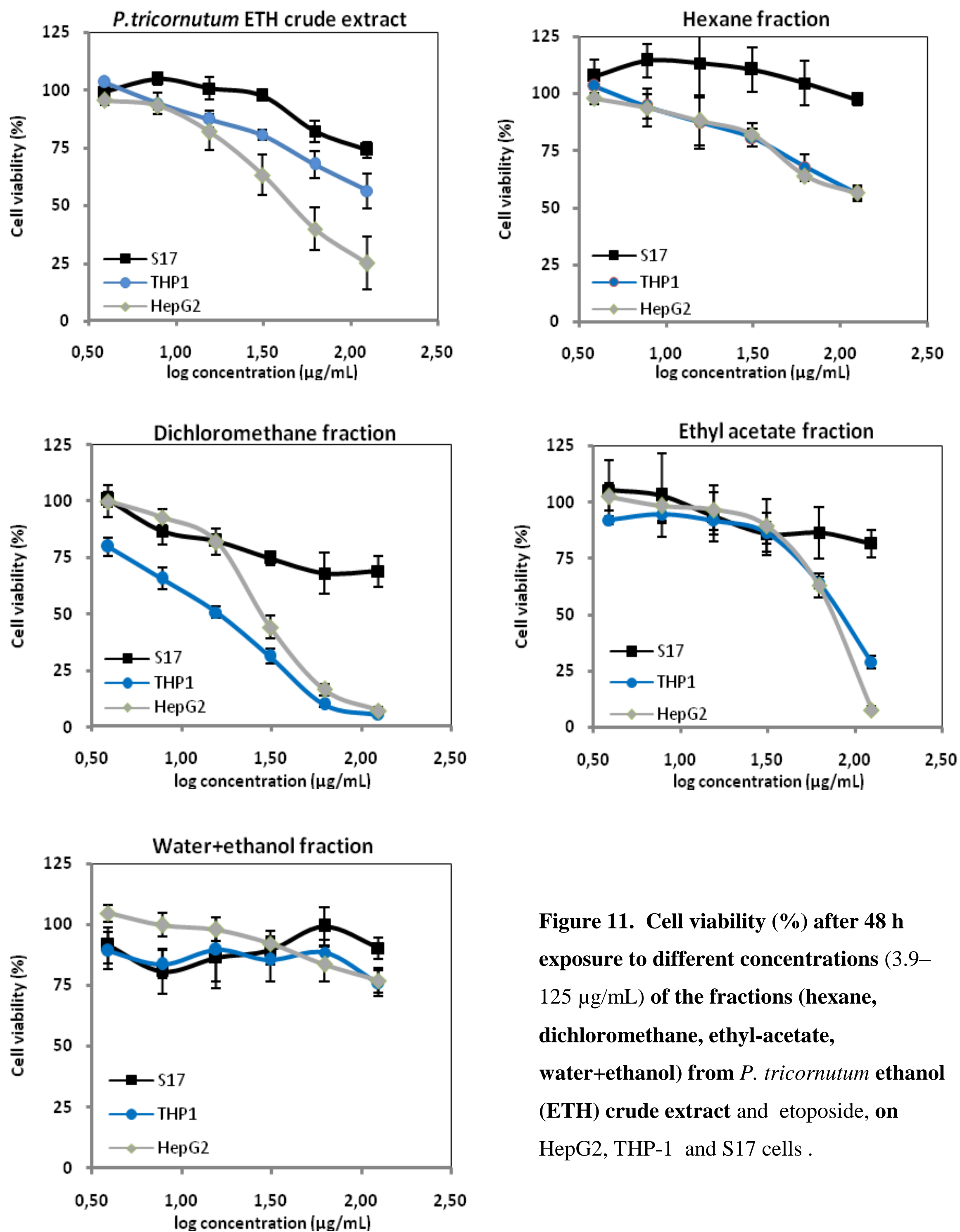


Figure 11. Cell viability (%) after 48 h exposure to different concentrations (3.9–125 µg/mL) **of the fractions (hexane, dichloromethane, ethyl-acetate, water+ethanol) from** *P. tricornutum* **ethanol (ETH) crude extract** and etoposide, **on** HepG2, THP-1 and S17 cells .

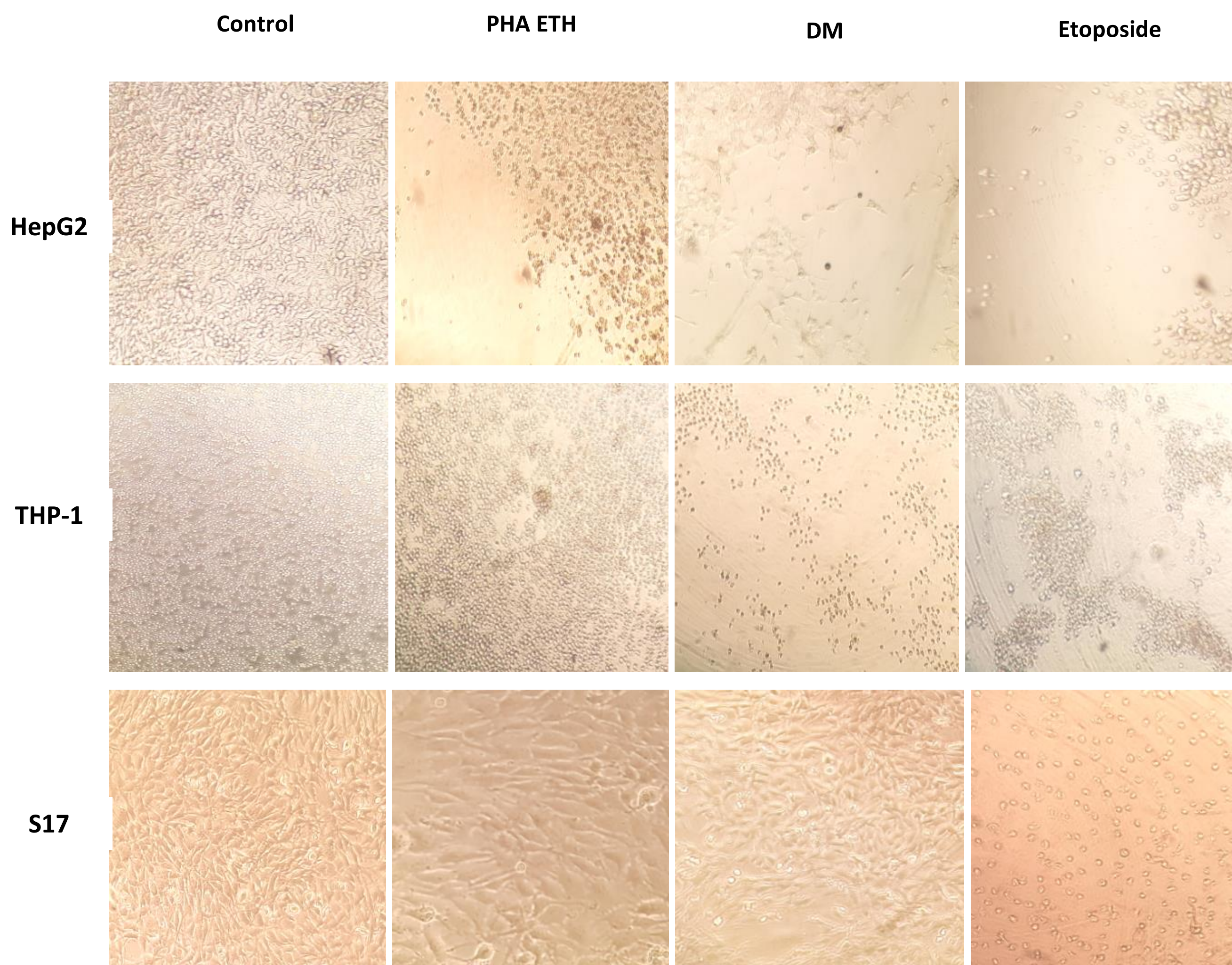


Figure 12. Examination of morphological changes on cells with light inverted microscopy. In order to screen for signs of apoptosis, HepG2, THP-1 and S17 cells were treated with 125 µg/mL of *P.tricornutum* ethanol extract (PHA ETH),dichloromethane fraction of the extract (DM) and etoposide. For THP-1 concentration, etoposide was applied at 7.8 µg/mL.

3.6. Identification of compounds present in the extract using gas chromatography-mass spectrometry (GC/MS)

In the previous section, the dichloromethane fraction was described as the most active with promising IC_{50} values for both HepG2 and THP-1. Besides, this fraction was highly selective for HepG2 cells having no observable toxic effect on non-tumouralS17 cells. In order to identify compounds with potential anti-cancer properties, this extract was analysed byGC/MS. In the GC/MS analysis, a total of 52 peaks were detected, of which 33 were successfully identified (Table 12).

Compound identification was performed by comparative analysis of mass spectral data of the detected compounds with those present in the NIST library. Only match scores above the treshold (>700) were considered as a positive identification.Thirteen of these compounds were previously described inthe literature as promising anti-cancer agents, and could contribute for the cytotoxic activity against HepG2 and THP-1 cells observed in this study. These 13 compounds can be classified into six different categories : saturated fatty acids (SFA) (pentadecanoic acid, hexadecanoic acid, 7-hexadecenoic acid, 9-octadecenoic acid, 10-octadecenoic acid) polyunsaturated fatty acids (5,8,11,14-eicosatetraenoic acid - ETA, 4,7,10,13,16,19-docosahexaenoic acid - DHA, 5,8,11,14,17-eicosapentaenoic acid - EPA, gamma-linolenic acid, sterols (24-methylcholesta-5,22-dien-3β-ol - Brassicasterol) ,vitamins (dl-α-Tocopherol), phenols (2,4-Di-tert-butylphenol) and terpenoid alcohols (phytol).
Phaeodactylum tricornutum is known for its high lipid content and is considered as an important source of the polyunsaturated fatty acids (PUFAs) EPA and DHA; therefore this diatom has an important commercial application, especially in aquaculture. Omega-3 or n-3 fatty acids are the most important PUFAs as they are key nutrients involved in normal growth and development of various human tissues. It is also well known that n-3 PUFAs, particularly EPA and DHA, are essential for the prevention and reduction of risks of some diseases, such as atherosclerosis, cardiovascular disease, inflammation, and mental illness[98]. Several studies reported that EPA and DHA can induce apoptosis in tumor cells *in vitro* and *in vivo*, in a dose- and time-dependent manner. They showed cytotoxicity against a wide range of solid tumors (colorectal carcinoma, esophageal, and gastric, ovarian, lung cancers), as well as towards myeloid and lymphoid leukaemia and lymphomas[99].
Amongst saturated fatty acids (SFAs), hexadecanoic acid waspreviously described as the most abundant in this microalga[98].Looking at the Table 16, it can be seen that the three most abundant peaks detected by GC/MS were identified as EPA, hexadecanoic acid and 9-hexadecenoic acid with 16.67%, 17.44% and 19.56% of total peak area, respectively.
It was published earlier this year that a lipid fraction containing a mixture of PUFAs (ETA and DHA) and SFAs (pentadecanoic acid, hexadecanoic acid, heptadecanoic acid, octadecanoic acid, 9-hexadecenoic acid , 7-methyl-6-hexadecanoic acid, 9-octadecenoic acid and 11-octadecenoic acid) from a sponge *A. sinoxea* was active against human colorectal adenocarcinoma cell line (HT-29) with IC_{50} 26.52± 8.19 μg/mL[100]. Most of these fatty acids were also detected in our sample (Table 12). In another study where plant extracts (*Kigelia. pinnata*) was screened for anti-cancer properties against human colorectal carcinoma cell line (HCT 116) and the most active fraction obtained with column chromatography with ether

:acetone (6:4) as solvent system revealed that the active compound was n-hexadecanoic acid showing high toxicity with an IC_{50} of 0.8 μg/mL. Furthermore, it was proposed that the observed cytotoxic activity of hexadecanoic acid was due to its high affinity towards interaction with DNA-topoisomerase I which makes this fatty acid as a good candidate for anti-cancer therapy [101]. Currently, there are no other evidences of cytotoxicity caused by hexadecanoic acid obtained from *P. tricornutum*.

Apart from a high content of fatty acids, *P. tricornutum* is an important source of sterols. Sterols play relevant roles in cellular defence and signalling, and they are precursors of several hormones and bioactive secondary metabolites. Few reports have dealt with sterol biosynthesis in this alga and the major sterol identified was 24-methylcholesta-5,22-dien-3β-ol (Brassicasterol). Other sterols reported were campesterol, cholesterol and ergosterol[96,102]. In this study, we have indetified only one sterol from *P. tricornutum* - brassicasterol, representing 1.86 % of total peak area (Table 12). Anti-cancer properties were also attributed to this class of compounds. A sterols-rich fraction from *A. sinoxea* was highly toxic to adherent cell lines (human colorectal adenocarcinoma (HT 29), breast cancer (MCF-7)) as well as to suspension cell line (lymphoblastic leukaemia cell line - MOLT-4) with IC_{50} values of 1.20 ±0.24, 4.12 ± 0.40 and 2.47 ± 0.31 μg/mL, respectively. In adition, the fraction was inactive against normal fibroblast cells, exhibiting good selectivity[100].

Microalgae are a great source of vitamins and antioxidants. *P. tricornutum* has high content of α-tocopherol (vitamin E) whch is a potent antioxidant heavily involved in the protection and prevention of lipid peroxidation in membranes. The antioxidant level in natural phytoplankton communities is often species-dependent, varying also with the individual, physiological condition, and their strategies to deal with reactive oxygen species. It was earlier reported that abiotic stress stimulates production of vitamin E in *P. tricornutum* KAC 37 strain.[103] In the GC/MS analysis of the active fraction in this study, α-tocopherol was detected representing 1.16% of total peak area (Table 15). Besides from its cancer-protective properties, Vitamin E showed cytotoxicity in previous studies when tested against keratin-forming tumour cell HeLa (KB), MCF-7 and cervical cancer (CasKi) cells with IC50 of 18.6, 7.5 and 6.0 μg/mL, respectively. These promising results suggests that vitamin E from *P. tricornutum* could be a potential anti-cancer agent. Interestingly, one constituent of α-tocopherol with larger peak area than vitamin E itself (Table 12) , was also detected in our extract, and that was phytol. Phytol is an acyclic monounsaturated diterpene alcohol also present in chlorophyll and used as a precursor for the production of synthetic forms of vitamin E. Phytol is an aromatic ingredient used in many fragrance compounds and it may be found in cosmetic and

noncosmetic products. It has shown that it possesses antioxidant activity, as well as anti-inflammatory and anti-allergic effects[104]. Furthermore, an essential oil from *C. juttae* (rich in phytol, 30%) showed strong cytotoxic acitivity against triple-negative breast cancer cells (MDA-MB-231) in a dose-dependent manner, reducing cell viability to 2% at 100 μg/mL[105]. Furthermore, this compound was described as promising drug for schistosomiasis[106]. Phytol could have had a potential cytotoxic effect on HepG2 and THP-1 cells used here and it is of a special interest since phytol was not reported previoulsy as an abundant *P. tricornutum* metabolite and no cytotoxicity of this metabolite on HepG2 and THP-1 was described before. Despite the great variety of phenols present in *P. tricornutum*, only one was detected in our study - 2,4-bis(1,1-dimethylethyl) phenol also called 2,4-Di-tert-butylphenol (DTBP). This phenol belongs to the group of marine metabolites, mostly synthesized by marine bacteria and some bacteria living in soils; reportedly, it has antibacterial, antifungal and antioxidant properties[107]. In addition, DTBP is a potent anti-tumoural compound, as described previously; when tested on keratin-forming tumour cell HeLa (KB), this phenol displayed high cytotoxicity with an IC_{50} of 0.81 μg/mL. For MCF-7 cells, IC_{50} was 5.75 μg/mL[104]. DTPB has not been previously associated with *P. tricornutum*.

Our GC/MS results indicate that dichloromethane fraction obtained from *P. tricornutum* ethanol extract contains variety of promising compounds with potential anti-cancer properties. At this stage of this research, it is difficult to point out one specific compound responsible for observed cytotoxicity. However, the most abundant compounds present in this fraction (5,8,11,14,17-eicosapentaenoic acid - EPA) , hexadecanoic and 9-hexadecenoic acid) are more likely responsible for the observed effect. Sterols are potent anti-cancer compounds, and it is also possible that Brassicasterol caused cell death in cancer cells in spite of the low amounts detected in this fraction. Nevertheless, synergic effect of all compunds present should not be neglected. In order to investigate this,further studies are needed;the dichloromethane fraction should be re-fractionated again, to separate between the fatty acid fraction, the sterol fraction and phenols fraction and tested again for cytotoxicty. With this procedure,then umber of compounds would be narrowed down in each fraction and it would be easier to understand the mechanism behind the cytotoxictiy,and to identify one or more compounds with anti-tumoural properties. To our knowledge, this is the first study to report the cytotoxicity of *P.tricornutum* against HepG2 and THP-1 cells.

However, pharmaceutical industries are constantly searching for a drug candidates in compounds that have not been previously identified and characterized by NIST library. As mentioned in the first paragraph of this section, out of 52 detected peaks, 33 were

successfully identified using NIST library, with 99.6% of total peak area. Among the unidentified compounds, the largest peak represented only 1.05% of total peak area, belonging to a compound with retention time of 39.942 min. This means that almost all volatile or semi-volatile compounds present in the extract were successfully identified and almost all were previously reported as cytotoxic.Taking this into consideration, *P.tricornutum* extract may not be a promising anti-cancer drug candidate. Anyhow, further studies are needed to confirm this and either descart this microalga as a potential source of new compouds with cytotoxic activities, or find some compounds with desired properties. With GC/MS, only volatile compounds in extract were detected.Using some other methods like liquid chromatography coupled with mass spectrometry(LC/MS), some new, non-volatile compounds responsible for cytotoxic effect could be identified.

P.tricornutum is not the only microalga that has anti-cancer properties. This area is still being investigated by researchers and there is still a great number of unexplored microalgae species that could be a source of promising anti-tumoural compounds. Microalgae that come from extreme environments (very acidic or alkaline waters, high temperature, light, salinity,polar environments and metal concentration)could be of a special interest due to the adaptative mechanism that these species developed to cope with these extreme environmental conditions[108]. Marine organisms have emerged as sources of „alternative“ therapies for cancer in the past few years and some anti-tumoural drugs from marine origin have been introduced to the market. One example is Ara-C (Cytarabine, Alexan R, Udicil R) isolated from the Caribbean sponge *Cryptotethya crypta* and another one is trabectedin, from the marine tunicate *Ecteinascidia turbinata*[1]. Nevertheless, microalgae have some advantages in marine drug discovery than other marine organisms. They produce cytotoxic compounds as metabolites and once these compounds are identified and characterized, growth conditions of specific microalgae species can be optimized in order to produce more biomass and therefore, more compound of the interest.In case of sponges and tunicates, cytotoxic compounds are usually produced by bacteria or other microorganisms that live inside these organisms, which means that they need to be isolated and grown industrially in order to produce the desired compounds[100,109]. Microalgae are photosynthetic organisms that use solar energy and fix CO_2, without the necessity of additional energy source which would impose higher costs of production. Bacteria on the other hand, require large bioreactors that consume a lot of energy and in addition, they need a sugar source for their cultivation, which is a large drawback comparing to microalgae. In addition, compounds obtained from other marine organisms are sometimes so complicated that is almost impossible to synthesize them synthetically, which is

another disadvantage of using other marine organisms than microalgae in marine cancer drug research[46] .

Table 12. Characterisation of compounds presentin dichloromethane fraction by (GC/MS)

RT	Tentatively indetified compound	% total area
22.589	9-Hexadecenoic acid	19.6
23.071	n-Hexadecanoic acid	17.4
30.668	5,8,11,14,17-Eicosapentaenoic acid (EPA)	16.7
22.199	γ-Linolenoic acid	7,.2
21.963	3,7,11,15-Tetramethyl-2-hexadecen-1-ol (Phytol)	5.35
22.065	6,9,12,15-Hexadecatetraenoic acid	2.79
19.493	Cyclononasiloxane	2.07
26.974	9,12-Octadecadienoic acid	1.98
34.453	4,7,10,13,16,19-Docosahexaenoic acid(DHA)	1.88
29.505	Cyclononasiloxane	1.75
41.555	Tetrapentacontane, 1,54-dibromo-	1.62
23.981	Citronellol	1.60
27.144	9-Octadecenoic acid	1.45
40.532	Tetrapentacontane, 1,54-dibromo-	1.42
32.074	Eicosanoic acid	1.38
28.771	1,3-Dioxolane	1.39
39.637	Octadecane	1.30
24.097	24-methylcholesta-5,22-dien-3β-ol (Brassicasterol)	1.21
41.568	dl-α-Tocopherol	1.16
39.942	Unknown	1.05
23.529	Pregna-5,9(11)-dien-20-ol-3-one ethylene	1.03
26.294	Tetrahydro-2H-pyran	0.98
38.824	Tetratetracontane	0.92
39.721	Ergosta-5,22-dien-3-ol, acetate (Brassicasterol acetate)	0.76
26.696	6,9,12,15-octadecatetraenoate (Stearidonic acid)	0.70
17.286	2- Propenoic acid (Acrylic acid)	0.69
27.273	10-Octadecenoic acid	0.58
20.933	3-Hexadecyne	0.53
30.532	5,8,11,14-Eicosatetraenoic acid (ETA)	0.52
26.02	4,7,10,13,16,19-Docosahexaenoic acid(DHA)	0.47
20.615	Pentadecanoic acid	0.38
12.585	2,4-bis(1,1-dimethylethyl) Phenol/ 2,4-Di-tert-butylphenol	0.35
15.588	Cyclooctasiloxane	0.31
30,98	cis-8,11,14,17-Eicosatetraenoic acid(ETA)	0.30
SUM		99.6

4. CONCLUSIONS

P. tricornutum is a rich source of lipids, especially of n-3 polyunsaturated fatty acids (PUFAs) (eicosapentaenoic acid – EPA, and docosahexaenoic acid - DHA) and fucoxanthin. Accordingly, *P. tricornutum* has emerged in the biotechnology field for various applications, s such as a biofuel precursor and recombinant protein expression host due to its biosynthetic capacity and high growth rates. This microalga is also a promising future source of the carotenoid fucoxanthin which could be used both as an antioxidant and a pigmenter in fish aquaculture[98]. However, there are only two reports so far addressing anti-cancer properties of this diatom.

Here, we show for the first time that *P. tricornutum* ethanol extract has strong cytotoxic activity against human hepatocellular carcinoma (HepG2) and human monocytic leukaemia (THP-1) cell lines, especially its dichloromethane fraction. Calculated IC_{50} values for HepG2 were similar to that of etoposide ($p>0.05$), a commonly used chemotherapeutic drug in the treatment of different types of cancer. Furthermore, the extract showed high selectivity towards HepG2 cells, with no observed toxic effect on healthy control cells (S17).

This is a very important point, since in order for a therapeutic agent to be truly effective, it should be toxic to cancer cells without harming normal cells; conversely, conventional chemotherapeutics (e.g. etoposide) kill cancer cells, but also strike healthy cells, causing adverse effects and severe morbidity.

GC/MS analysis of the dichloromethane fraction successfully identified 99.6% of compounds present in the fraction using NIST library. Among these,13 compounds could potentially (according to the literature)be reponsible for cytotoxicity against HepG2 and THP-1 cells. Additional studies are needed to isolate specific active compound(s) and to confirm its anti-cancer properties on the cells used in this study. For this purpose, the fractionshould be further fractionated, to obtain sub-fractions potentially more active with a more limited number of compunds. Despite the identified compounds with anti-cancer properties, this extract may not be the best to look for a promising drug lead due to the fact that almost all compounds in the extract are already described and pharmaceutical industries search for drug leads in new compounds with original structures. GC/MS analysis detected only volatile compounds, meaning that additional analysis is required (e.g., LC/MS) to screen for non-volatile compounds with cytotoxic effect on cancer cells.

There are also certain limitations in this study which need to be taken into consideration. More healthy control cells are needed for future studies as here we only report cytotoxicity on

S17, which are bone marrow stromal cell, originated from mouse and that are adherent cells. Both HepG2 and THP-1 are human-derived cell lines; therefore human-derived healthy control cell lines would be also needed to propperly assess selectivity. Concerning the results obtained with THP-1, a suspension cell line healthy control should be used because S17 is adherent cell line, and cells with different morphology respond to chemotherapeutics differently. THP-1 cells were more susceptible to etoposide than HepG2, but more resistant to the treatment with the extract.Additional leaukaemia cell lines should be tested, as THP-1 are more aproppriate for anti-inflammatory assays. Furthermore, we are not sure about the mechanism of cytotoxicity that this microalga exhibited towards selected cell lines. In order to investigate whether reduction in cell viability was due to apoptosis, additional assays detecting apoptotic cells should be performed in the future.

Despite the fact that no new structures were identified in the extracts and fractions prepared from *P.tricornutum*, microalgae can be considered a promising source of antitumoural compounds and further studies should include screening other species for cytotoxic properties against cancer cells.

5. ANNEX

Annex 1. Cell viability (%) of HepG2 and S17 cell lines after exposure to ethyl acetate (EA) extracts of selected microalgal species

		Concentration (µg/mL)							
Extract	**Cell line**	**3.9**	**7.8**	**15.6**	**31.25**	**62.5**	**125**	**Control**	**DMSO**
PHA	**HepG2**	104±7	103±10	92.0±7.7	61.2±3.5	20.1±3.3	6.44±2.73	101±6	95.0±7.4
	S17	104±3	103±5	96.2±3.0	96.1±3.6	80.6±6.9	39.9±10.7	100±6	91.6±6.9
POC	**HepG2**	91.6±6.2	84.6±3.1	75.1±3.8	68.2±2.9	24.2±3.4	14.2±3.8		
	S17	101±6	104±5	101±3	93.5±3.7	93.3±2.0	81.6±2.1		
ISO	**HepG2**	95.6±10.5	87.8 ±8.6	79.6±4.8	65.7±7.1	23.6±4.2	4.92±1.45		
	S17	104±5	104±3	98.9±3.9	93.3±2.6	85.6±4.3	40.6±5.1		
SKLT	**HepG2**	109±6	106±4.	99.4±11.9	68.6±3.4	26.0±2.6	4.88±0.52		
	S17	102±4	99.5±5.8	87.1±8.6	69.2±9.2	45.5±6.0	33.7±5.2		
NANNO	**HepG2**	103±3	94.0±7.0	86.2±10.2	80.7±8.6	74.2±8.0	56.6.±3.8		
	S17	103±5	101±6	93.2±7.1	83.8±14.0	77.1±10.0	57.5±4.2		
T CTP4	**HepG2**	105±11	96.0±5.0	96.6±8.9	95.6±11.2	86.6±7.0	74.2±5.0		
	S17	100±7	101±6	99.1±6.5	91.0±11.3	87.9±11.7	74.0±5.5		
Etoposid	**HepG2**	82.1±6.2	74.2±4.5	62.4±5.6	40.6±5.2	21.2±5.0	9.64±2.81		
	S17	92.9±3.5	91.0±4.0	80.6±4.4	62.4±4.6	62.4±4.6	5.03±0.77		

DMSO, dimethyl sulfoxide;

Annex 2. Cell viability (%) of HepG2 and S17 cell lines after exposure to ethanol (ETH) extracts from *P. tricornutum* (PHA) and *Isochrysis* sp. (ISO)

		Concentration (µg/mL)						Control	DMSO
Extract	**Cell line**	**3.9**	**7.8**	**15.6**	**31.25**	**62.5**	**125**		
PHA	**HepG2**	90.2±8.0	81.8±7.9	62.0±4.2	20.8±2.3	13.7±6.6	3.36±0.66	100±1	94.9±7.5
	S17	102±2	99.4±1.9	99.2±1.9	94.9±4.0	89.6±10.1	8.08±3.08	100±4	96.5±5.6
ISO	**HepG2**	95.2±1.7	92.4±2.3	89.1±0.9	87.8±1.2	63.7±4.2	35.8±4.3		
	S17	102±4	98.5±8.9	95.6±5.1	95.2±8.3	89.4±10.2	15.6±4.8		
Etoposide	**HepG2**	82.1±6.2	74.2±4.5	62.4±5.6	40.6±5.2	21.2±5.0	9.64±2.81		
	S17	92.3±3.5	91.0±3.9	80.6±4.4	62.4±4.6	26.1±3.3	5.03±0.77		

DMSO, dimethyl sulfoxide;

Annex 3. Cell viability (%) of THP1 and S17 cell lines after exposure to ethanol (ETH) extract from *P. tricornutum* (PHA) and etoposide

Concentration (µg/mL)	PHA	Etoposide	Control	DMSO	PHA	Etoposide	Control	DMSO
	THP-1 cell line				S17 cell line			
0.24	-	87.1±11.9	101±5	89.4±3.8			100±6	91.6±6.9
0.49	-	72.5±5.4						
0.98	-	51.2±8.2						
1.95	-	29.8±8.7						
3.9	101±4	14.9±4.0			103±2	92.86±3.48		
7.8	97.1±4.8	9.15±1.80			99.4±1.9	91.05±3.95		
15.6	88.1±4.5	3.14±1.50			99.2±1.9	80.57±4.40		
31.2	85.31±6.0	-			94.9±4.0	62.38±4.56		
62.5	72.4±10.8	-			89.5±10.1	26.09±3.28		
125	11.7±4.5	-			8.07±3.08	5.03±0.77		

DMSO, dimethyl sulfoxide;

Annex 4.Cell viability (%) of HepG2, THP1 and S17 cell lines after exposure to fractions obtained from *P. tricornutum* (PHA) ethanol extract

		Concentration (µg/mL)						Control	DMSO
Extract / Fraction	**Cell line**	**3.9**	**7.8**	**15.6**	**31.25**	**62.5**	**125**		
Ethanol crude extract	**HepG2**	95.7±2.3	93.9±1.9	82.0±7.8	63.3±8.8	39.9±9.2	25.1±11.4	100±6	96.6±7.4
	S17	99.3±3.6	1055±2	101±5	97.6±1.9	82.2±4.7	74.2±3.3	102±4	92.9±5.8
	THP-1	103±1	94.5±4.7	87.4±3.7	80.7±2.3	67.5±5.9	56.4±7.6	101±5	89.4±3.8
Hexane fraction	**HepG2**	97.9±2.5	93.8±8.4	88.1±10.6	82.1±5.3	63.8±2.3	56.4±2.5		
	S17	108±7	114±7	113±15	110±10	104±9.9	97.4±2.1		
	THP-1	96.1±4.2	95.0±5.4	83.8±11.7	82.3±4.0	83.0±5.6	67.2±3.3		
Dichloro-methane fraction	**HepG2**	99.9±7.2	92.8±3.4	82.1±5.7	44.3±5.0	16.6±2.7	7.22±1.84		
	S17	101±3	86.7±5.6	82.5±1.2	74.7±3.2	68.1±9.3	69.0±7.0		
	THP-1	79.8±4.2	65.8±5.0	50.9±2.6	31.4±3.3	9.9±1.0	5.45±0.57		
Ethyl acetate fraction	**HepG2**	103±6	98.5±6.7	96.7±11.0	89.7±11.7	63.0±5.6	7.85±1.43		
	S17	105±13	103±8	93.6±10.9	86.0±9.5	86.5±11.4	81.8±6.1		
	THP-1	92.0±1.7	94.8±3.8	91.8±3.9	86.5±4.9	64.2±2.6	28.9±2.6		
Water+ Ethanol fraction	**HepG2**	105±3	99.8±4.9	97.9±4.9	92.1±5.4	83.7±7.0	76.9±5.1		
	S17	91.4±7.3	80.7±9.2	86.4±12.5	89.3±5.6	99.2±8.0	90.4±4.4		
	THP-1	89.0±7.6	83.4±5.7	89.6±13.0	85.3±8.9	88.5±5.1	75.7±5.2		
Etoposide	**HepG2**	82.0±6.2	74.2±4.5	62.4±5.6	40.6±5.2	21.2±5.0	9.64±2.81		
	S17	92.9±3.5	91.0±3.9	80.6±4.4	62.4±4.6	26.1±3.3	5.03±0.77		
	THP-1	14.9±4.0	9.2±1.8	3.1±1.5	-	-	-		

DMSO, dimethyl sulfoxide;

6. BIBLIOGRAPHY

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