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Toxicological Assessment of Marine Cyanobacterial Extracts in Human Tumor Cell Lines – Proteomic and Gene Expression Approach

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TESE DE MESTRADO APRESENTADA

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Toxicological Assessment of Marine Cyanobacterial Extracts in Human Tumor Cell Lines – Proteomic and Gene Expression Approach.

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Abstract

Cyanobacteria are known to synthesize secondary metabolites that may have potential as drugs for the treatment of human diseases such as cancer. At the Laboratory of Ecotoxicology Genomics and Evolution, LEGE – CIIMAR, studies concerning the bioactive potential of marine cyanobacteria isolated from the Portuguese coast have been performed. Recent results revealed significant cytotoxicity of extracts of the *Cyanobium* sp. strain LEGE06113 and the *Synechocystis salina* strain LEGE06155, in human cancer cell lines. In this work we aimed to proceed to the characterization of the mechanisms involved in the reported cytotoxicity, by using a molecular and proteomic approach. Cytotoxicity of strains was studied on the RKO human colon cancer cell line by employing real-time PCR to analyze gene expression and two-dimensional gel electrophoresis for protein expression.

Cyanobacterial cultures were performed and extracts obtained from lyophilized biomass. *In vitro* cultures of the human cell lines were established and conditions for the mRNA expression and for the cell's proteome analysis optimized. Cellular viability of cells exposed to the cyanobacteria extracts was evaluated by the 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) assay. The mRNA expression of a set of target genes (CCNB1, CCNE, P21CIP, BAD, BCL-2) was analyzed through real-time PCR in order to check for differences in their expression on cells exposed to the different cyanobacterial strains. Patterns of proteins with isoelectric points between 4-7 and molecular masses of 19-117 kDa were separated with high resolution in two-dimensional polyacrylamide gels (2DE).

The MTT results confirmed that an ethyl acetate fraction (fraction B) of the cyanobacteria strains induced a decrease in cells viability. Real-time PCR results using multiple reference gene normalization, showed that CCNB1 (cell cycle) and BCL-2 (apoptosis) demonstrated differences in mRNA expression. LEGE06113 fraction B increased the BCL-2 mRNA expression while LEGE06155 fraction B decreased the CCNB1 mRNA expression. This result supported the hypothesis that there are differences in the mechanisms of cytotoxicity of the two fractions, and indicated an interaction with the progression of the cell cycle. The proteomic results also demonstrated the cytotoxic effect of the

cyanobacterial strains, with different protein patterns expressed in the treatment groups compared with control. Qualitative and quantitative differences were detected in many protein spots from the 2DE gels, according to the cyanobacterial strain.

Conjugating these two types of molecular analysis, the observed cytotoxicity can be due to influences in the cell cycle regulation or apoptosis, but further investigations are needed to identify additional biochemical pathways in the RKO cell line due to the action of the cyanobacterial fraction. The further identification of protein function, using mass spectrometry, will complement our understanding of the molecular mechanisms underlying cytotoxicity. Combining the gene expression and the proteomics, new insights in the molecular mechanisms leading to growth inhibition in human carcinoma cells are acquired and may be useful in the future to understand the underlying molecular mode of action – an important prerequisite for development of future cancer drugs.

Resumo

As cianobactérias são conhecidas por sintetizar metabolitos secundários que poderão ter potencial como drogas no tratamento de doenças do ser humano, tais como o cancro. No Laboratório de Ecotoxicologia, Genómica e Evolução, LEGE – CIIMAR, estudos relativos ao potencial bioactivo de cianobactérias marinhas isoladas da costa Portuguesa foram realizados. Resultados recentes revelaram uma citotoxicidade significativa de extractos da estirpe *Cyanobium* sp. LEGE06113 e da estirpe *Synechocystis salina* LEGE06155,em linhagens de células cancerígenas humanas. Neste trabalho o objectivo consistiu em continuar com a caracterização dos mecanismos envolvidos na citotoxicidade reportada, usando uma abordagem molecular e proteómica. A citotoxicidade das estirpes foi examinada na linhagem celular humana de carcinoma de cólon RKO empregando PCR em tempo real (RT-PCR) para a análise da expressão génica e electroforese de duas dimensões para a expressão proteica.

Realizaram-se culturas de cianobactérias e os seus extractos foram obtidos a partir da biomassa liofilizada. As linhagens tumorais humanas foram mantidas em culturas *in vitro* e condições para a análise de expressão de mRNA e do proteoma celular, optimizados. A viabilidade celular das células expostas aos extractos das cianobactérias seleccionadas foram avaliadas usando o teste de brometo 3-(4.5-dimetiltiazol-2-il)-2.5-difeniltetrazolio (MTT). A expressão do mRNA de um conjunto de genes alvo (CCNB1, CCNE, P21CIP, BAD, BCL-2) foi analisada através de RT-PCR para verificar possíveis diferenças na sua expressão em células expostas a diferentes extractos de cianobactérias. Padrões de proteínas com pontos isoeléctricos entre 4-7 e massas moleculares de 19-117 kDa foram separados com alta resolução em géis de poliacrilamida (2DE).

Os resultados da análise de MTT confirmaram que a fracção de acetato de etilo (fracção B) das estirpes induziu uma diminuição na viabilidade celular. Os resultados do RT-PCR usando normalização de múltiplos genes referência, mostrou que CCNB1 (ciclo celular) e BCL-2 (apoptose) demonstraram diferenças na expressão de mRNA. A fracção B de LEGE06113 aumentou a expressão de mRNA do BCL-2, enquanto a fracção B de LEGE06155 diminuiu a expressão de mRNA do CCNB1. Este resultado suporta a hipótese de que há

diferenças nos mecanismos de citotoxicidade das duas fracções, e indicou uma interacção com o progresso do ciclo celular. Os resultados da proteómica também demonstram o efeito citotóxico das estirpes de cianobactérias, com diferentes padrões de proteínas expressos nos grupos de tratamento em comparação com o controlo. Diferenças qualitativas e quantitativas foram detectadas em várias proteínas nos géis de 2DE, de acordo com a estirpe de cianobactérias.

Conjugando estes dois tipos de análise molecular, a citotoxicidade observada pode dever-se a influências na regulação do ciclo celular ou apoptose, mas posteriores investigações são necessárias para identificar as vias bioquímicas adicionais na linhagem celular RKO devido à acção dos extractos de cianobactérias. A posterior identificação da função das proteínas, usando espectrometria de massa, irá complementar o nosso conhecimento dos mecanismos moleculares subjacente à toxicidade. Combinando a expressão genética e a proteómica, novos conhecimentos nos mecanismos moleculares que conduzem à inibição do crescimento em células de carcinoma humano são adquiridos e podem ser úteis no futuro para entender o modo de acção molecular subjacente – um pré-requisito importante para o desenvolvimento de futuros medicamentos contra o cancro.

Table of Contents

Figure index

Table index

List of abbreviations

2DE – Two-Dimensional gel electrophoresis

ACTB – Beta Cytoskeletal Actin

APS – Ammonium persulfate

BAD – BCL2-Associated Agonist Of Cell Death

BAX – Bcl-2-associated X protein

BCL-2 – B-Cell CLL/Lymphoma

BCL-w – BCL2-Like 2

BCL-xL – BCL2-Like 1

CCNB1 – Cyclin B1

CCNE – Cyclin E1

CDK – Cyclin-dependent kinase

cDNA – complementary DNA

CHAPS – 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate

CIIMAR – Interdisciplinary Center of Marine and Environment Research

DFS-*Taq* – DNA Free Sensitive *Taq* DNA polymerase

DMEM – Dulbecco's Modified Eagle Medium

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

dNTP – deoxyribonucleotide

DTT – Dithiothreitol

ESTSP – School of Health Technology of Porto

gDNA – genomic DNA

HepG2 – Liver hepatocellular carcinoma cell line

HPRT1 – Hypoxanthine Phosphoribosyltransferase

HT-29 – Colon adenocarcinoma cell line

IEF – First-dimension isoelectric focusing

LECEMA – Laboratory of Cellular, Molecular and Analytical Studies

LEGE – Laboratory of Ecotoxicology, Genomics and Evolution

LPS – Lipopolyssacharides

M – Mitosis

mRNA – messenger RNA

MTT – 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide

NPKS – Non-ribossomal peptides

NRPS – Non-ribossomal peptide synthetases

NTC – Negative control

P21CIP – Cyclin-Dependent Kinase Inhibitor 1A

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

pI – Isoelectric point

PKS – Polyketides synthases

PSI – Photosystem I

PSII – Photosystem II

PUMA – p53 upregulated modulator of apoptosis

RB flasks – Round bottom flasks

RKO – Colon carcinoma cell line

RNA – Ribonucleic acid

RPL8 – Ribosomal protein L8

RT-PCR – Real-Time Polymerase chain reaction

sALCL – systemic anaplastic large-cell lymphoma

SB – solubilization buffer

SD – Standard deviation

SDS-PAGE – Sodium dodecyl sulphate polyacrylamide gel electrophoresis

T47D – Human ductal breast epithelial tumor cell line

TEMED – Tetramethylethylenediamine

UB2E2C3 – Ubiquitin-Conjugating Enzyme E2C

Appendixes

1. Introduction

In the last years, and from a large diversity of organisms, the marine environment has provided a long list of natural compounds, with pharmacological potential in the treatment of several human diseases (Borowitzka, 1995; Kinghorn *et al.*, 2009; Mishra and Tiwari, 2011). Effectively, the sea is the major component of our planet and contains an enormous diversity of organisms with a rich capacity of adaption to different and extreme conditions in a variety of habitats. In what concerns to marine natural products, the most studied species have been mainly sponges, tunicates and microalgae. Most recently marine cyanobacteria have been also in focus (Sponga *et al.*, 1999; Tan, 2007; Cooper and Yao, 2012).

1.1. Cyanobacteria

Cyanobacteria are very interesting prokaryotic gram-negative organisms, with the ability to perform photosynthesis identically to plants. Gram-negative bacteria are characterized by a layered and complex cell wall constituted by a thin inner layer of peptidoglycan and an outer layer, which is divided in an outer membrane and a periplasmic space. This outer membrane is chemically characterized by the presence of lipopolysaccharides (LPS), phospholipids and proteins while the periplasmic space is composed by a matrix with a high number of proteins and enzymes for nutrient acquisition, peptidoglycan synthesis and modification of toxic compounds (Ferreira and Sousa, 1998; Madigan *et al.*, 2003). Cyanobacteria are the only oxygenic photosynthetic bacteria, with both photosystems (PSII and PSI), using H_2O as a photoreductant in the electron transport chain and consuming CO_2 as a carbon source. Moreover, cyanobacteria can also perform anaerobic respiration and nitrogen fixation (Whitton and Potts, 2002; Cohen and Gurevitz, 2006).

Morphologically, cyanobacteria can be unicellular, colonial or filamentous, presenting absence of nucleus, chloroplast and mitochondria, so photosynthesis takes place in the thylakoids, a membrane system with photosynthetic pigments. This prokaryotic group has an asexual reproduction,

normally by fission, budding, trichome rupture (hormogonium) or akinete germination (Ferreira and Sousa, 1998).

Since cyanobacteria have characteristics from both bacteria and plants, they can be classified by the International Botanical Code of Nomenclature or International Code of Nomenclature of Bacteria. In accordance with the Botanical classification, the phylum of Cyanobacteria is divided into 5 orders: *Chroococcales*, *Pleurocapsales*, *Oscillatoriales*, *Nostocales* and *Stigonematales*. In the nomenclature of bacteria Cyanobacteria are classified into 5 subsections, within the Eubacteria domain (Castenholz and Waterbury, 1989; Castenholz and Boone, 2001). Both classifications are valid and a correspondence between them is possible (Table 1).

Table 1 – Main characteristics and genus of the 5 orders or subsections of the phylum Cyanobacteria. Adapted from Catenholz and Bone, 2001.

Orders/Subsections	Main Characteristics	Genus
I / Chroococcales	Unicellular or not aggregated filamentary. Reproduction by binary fission in one, two or three plans. Rarely form akinetes.	*Chroococcus, Cyanobacterium, Cyanobium, Cyanothece, Dactylococcopsis, Gloeobacter, Gloeothece, Microcystis, Prochlorococcus, Synechococcus, Synechocystis*
II / Pleurocapsales	Unicellular or not aggregated filamentary of cells united by exterior walls or a gel matrix. Reproduction by multiple fission. The daughter cells are smaller than the stem cells. Rarely form akinetes.	*Cyanocystis, Dermocarpella, Myxosarcina, Stanieria, Xenococcus*
III / Oscillatoriales	Filamentous. Reproduction by binary fission in one plan. Unbranched and uniseriate trichomes. Trichomes without heterocysts or akinetes.	*Arthrospira, Lyngbya, Leptolyngbya, Microcoleus, Oscillatoria, Planktothrix, Pseudanabaena, Trichodesmium*
IV / Nostocales	Filamentous. Reproduction by binary fission in one plan. Unbranched and uniseriate trichomes.Trichomes can differentiate in heterocysts or akinetes.	*Anabaena, Anabaenopsis, Aphanizomenon, Cylindrospermopsis, Gloeotrichia, Nodularia, Nostoc, Rivularia*
V / Stigonematales	Filamentous. Reproduction by binary fission periodically or in more than one plan. Multiseriated trichomes or trichomes with various types of branches.	*Loriella, Geitleria, Nostochopsis, Westiella*

Cyanobacteria have a wide distribution around the world, and exist in terrestrial, freshwater and marine ecosystems. They have the capability of

adapting to a variety of environmental conditions, surviving and gaining tolerance in hostile environments, such as ice fields, hot springs and deserts (Uzair, 2011; Biondi *et al.*, 2008; Vasconcelos, 2006; Martins *et al.*, 2005).

The presence of specialized cyanobacterial cells confers tolerance and adaptation under adverse conditions. Examples for these cells are heterocysts used for nitrogen fixation in nitrogen depleted areas and akinetes, which have the ability to store reserve substances (Ferreira and Sousa, 1998).

The most common environment in which cyanobacteria seem to dominate is the aquatic one. In brackish, marine and freshwater ecosystems, these organisms can be present in either benthic or planktonic photosynthetic communities. In recent years, the study of marine cyanobacteria in particular has been coming into focus, due not only to the formation of cyanobacterial blooms in coastal areas but specially due to the potential as producers of interesting natural compounds. If in on one hand certain environmental conditions such as high luminosity, temperature and nutrient concentrations led to cyanobacteria proliferation and a high toxicological risk to humans and other organisms due to the potential production of toxins (Vasconcelos, 2006; Martins *et al.*, 2005), on the other hand marine cyanobacteria have revealed high potential as pharmacological agents (Mayer and Gustafson, 2008).

1.2. Bioactive Compounds from Marine Cyanobacteria

Although the occurrence of cyanobacteria can be considered a threat to ecosystems and public health due to the potential production of toxins, an increased number of studies assign to marine cyanobacteria the capacity to produce secondary bioactive compounds, which have pharmacological interest for the treatment of human diseases, such as cancer (Oftedal *et al.*, 2010; Bain *et al.*, 2007; Martins *et al.*, 2007; Selheim *et al.*, 2005; Rocha *et al.*, 2001).

Studies in this subject have been focusing mainly on filamentous strains from the genus *Lyngbya*, *Microcoleus* and *Symploca* that grow in high densities in tropical and subtropical regions (Figure 1) (Schlegel *et al.*, 1999). In contrast, many marine cyanobacterial genera, namely the picoplanktonic forms, have

been largely overlooked, due to their occurrence at lower densities. The low concentrations in the environment lead to difficulties in the collection process and isolation (Martins *et al.*, 2005). However, when compared to other organisms such as macroalgae and invertebrates, cyanobacteria have a potential advantage for this type of work since they are easily cultivated in laboratory for biomass production (Borowitzka, 1995).

Some examples of the less studied cyanobacteria genera are *Cyanobium*, *Synechocystis*, *Synechococcus* and *Leptolyngbya*. Several strains of these genera were isolated from rocky beaches along the Portuguese coast and are maintained in the LEGE cyanobacteria culture collection at CIIMAR. From 28 strains a research work based on screening the cytotoxicity of extracts in human cancer cell lines was conducted (Costa *et al.*, submitted). This cytotoxicity screening revealed several strains as promising for the isolations of bioactive compounds. From the *Cyanobium* strain LEGE06113 the natural compound Hierridin B was isolated and was found to exhibit selective activity towards the colon adenocarcinoma cell line, HT-29, but further investigations are necessary to elucidate this activity (Leão *et al.*, 2013).

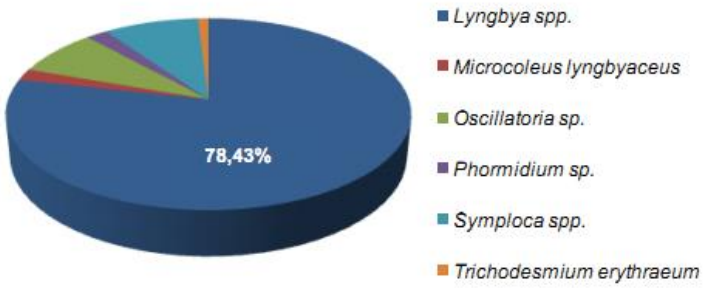

Figure 1 – Marine cyanobacteria from the genus *Oscillatoriales*, indicating the major species that product bioactive compounds, in a total of 800 compounds (Gerwick *et al.*, 2008).

Since an increasing number of studies confirm the pharmacological potential of several bioactive compounds from cyanobacteria, it is essential to study the molecular and biochemical pathways responsible for the formation of secondary metabolites. A widely discussed issue is the biosynthesis pathway to originate these secondary compounds, since they can either be polyketides (PKS), non-ribossomal peptides (NPKS), or have mixed structural and chemical characteristics (Barrios-Llerena *et al.*, 2007). The non-ribossomal peptide biosynthesis is achieved by the multiple-enzyme complex family NRPS (non-ribossomal peptide synthetases). The polyketide synthases (PKS) are also

multifunctional synthases organized into repeated functional units and each unit catalyzes all the steps of enzyme reactions for the polyketide chain elongation (Dittmann *et al.*, 2001). The combination of these biosynthetic enzymatic pathways leads to the production of an incredible diversity of metabolites (Nunnery *et al.*, 2010).

Some compounds from marine cyanobacteria have already been isolated and tested in clinical trials to evaluate their capacity to act against human diseases such as cancer. The most famous example of success of a cyanobacterial compound is brentuximab vedotin, which acts as a microtubule disruptor and is used in the clinical treatment of Hodgkin lymphoma and systemic anaplastic large-cell lymphoma (sALCL) (de Claro *et al.*, 2012). Unfortunately, many other compounds that entered clinical trials were dropped out, since some of them developed undesired secondary effects on cancer patients such as Dolastin 10 that caused neurological consequences (Simmons *et al.*, 2005). With the confirmation of its therapeutically potential, natural compounds can give rise to synthetic analogous with the purpose of improving the pharmacological properties to succeed the treatments. Using the example of dolastin 10, the analog TZT-1027 was created to improve its capacity as an anticancer compound (Watanabe *et al.*, 2007). In table 2, information is gathered from several marine cyanobacterial compounds that were described to have anticancer activity.

On studies realized until the present, the main targets for these metabolites with anticancer activities are the disruption of microtubules and actin filaments; inhibitors of protein synthesis and modulation of cellular death (Tan, 2010). But interactions on cell cycle control and growth factors also need attention since may take an important role in cell survival and development. Apoptosis seems to be the main process through which cyanobacterial metabolites act in cancer cells. However the mechanisms leading to apoptosis are not always known (Costa *et al.*, 2012).

Although this work focused on the anticancer activities, through cytotoxic effects, the marine cyanobacterial bioactive compounds have also been reported to act as antibacterial, antiviral, antifungal and algaecide (Figure 2) (Mayer *et al.*, 2011; Martins *et al.*, 2008; Zainuddin *et al.*, 2002; Schlegel *et al.*, 1999).

If the therapeutic potential of the bioactive compounds is confirmed, there are economic advantages in their use to treat certain diseases, since the culture and extraction of these natural compounds can be more simple, compared to other organisms (Uzair *et al.*, 2011). Also, almost every new discovered cyanobacterial strain has new and unique secondary metabolites (Tan, 2007).

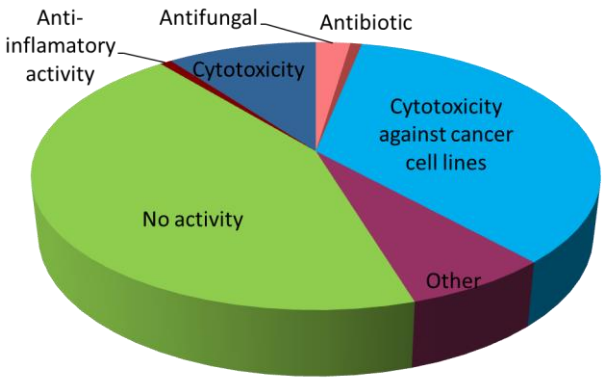

Figure 2 – Bioactivities reported from a study involving128 marine cyanobacteria nitrogen-containing compounds (Tan, 2007).

Moreover the use of these natural bioactive compounds is an important reason to continue respecting and protecting the environment, since its contamination is negatively affected by drug excipients, being an actual problem among societies around the world.

Table 2 – Marine cyanobacterial bioactive compounds with potential anticancer activity. Adapted from Costa *et al.*, 2012.

Compound	Source	Chemical Classification	Anticancer activity	Reference
Apratoxins	*Lyngbya*	Cyclic depsipeptide	Cytotoxicity against a panel of cancer cell lines	(Luesch *et al.*, 2001, 2002)
Aurilides	*Lyngbya majuscula*	Cyclic depsipeptide	Cytotoxicity against NCIH460 and neuro-2a mouse neuroblastoma cell line	(Han *et al.*, 2006)
Belamide A	*Symploca sp.*	Linear tetrapeptide	Disruption of microtubule network in A10 cells	(Simmons *et al.*, 2006)

Bisebromoamide	*Lyngbya sp.*	Peptide	Cytotoxic against a panel of cancer cell lines	(Teruya *et al.*, 2009a)
Biselyngbyaside	*Lyngbya sp.*	Glicomacrolide	Cytotoxicity against a panel of cancer cell lines	(Teruya *et al.*, 2009b)
Calothrixins	*Calothrix*	Pentacyclic indolophenanthridine	Active against human HeLa cancer cells and apoptotic killing of human Jurkat cancer cells	(Chen *et al.*, 2003)
Caylobolide	*Lyngbya majuscula*	Macrolactone	Cytotoxicity against human colon tumor cells	(MacMilliam *et al.*, 2003)
Coibamide A	*Leptolyngbya sp.*	Cyclic depsipeptide	Cytotoxicity against NCIH460 lung and mouse neuro-2a cells	(Medina *et al.*, 2008)
Cryptophycin 1	*Nostoc spp.*	Cyclic depsipeptide	Cytotoxicity against human tumor cell lines and human solid tumors	(Wagner *et al.*, 1999)
Curacin A	*Lyngbya majuscula*	Lipopeptide	Anti-proliferative and cytotoxic activity against colon, renal and breast-cancer derived cell lines.	(Gerwick *et al.*, 1994)
Dolastatin 10	*Symploca sp.*	Linear Pentapeptide	Anti-proliferative activity against murine PS leukemia cells	(Kalemkerian *et al.*, 1999)
Hectochlorin	*Lyngbya majuscula*	Cyclic depsipeptide	Cytotoxicity against colon, melanoma, ovarian and renal cell lines	(Marquez *et. al.*, 2002)
Homodolastatin 16	*Lyngbya majuscula*	Cyclic depsipeptide	Activity against esophageal and cervical cancer cell lines	(Davies-Coleman *et al.*, 2003)
Isomalyngamide	*Lyngbya majuscula*	Fatty acid amides	Against tumor cell migration through the β1 integrin-mediated antimetastatic pathway	(Chang *et al.*, 2011)
Jamaicamides	*Lyngbya majuscula*	Polyketide-Peptides	Cytotoxicity against H460 human lung and neuro-2a mouse neuroblastoma cell lines	(Edwards *et al.*, 2004)
Lagunamides	*Lyngbya majuscula*	Cyclic depsipeptide	Cytotoxic against a panel of cancer cell lines	(Tripathi *et al.*, 2012)
Largazole	*Symploca sp.*	Cyclic depsipeptide	Anti-proliferative activity	(Zeng *et al.*, 2010)
Lyngbyabellin A	*Lyngbya majuscula*	Cyclic depsipeptide	Disruption of cellular actin microfilaments in A10 cells and cytotoxicity	(Luesch *et al.*, 2000)

			against various cell lines	
Majusculamide C	*Lyngbya majuscula*	Cyclic depsipeptide	Induction of filamentous F-actin loss against A10 cells	(Pettit *et al.*, 2008)
Malevamide D	*Symploca hydnoides*	Peptide ester	Toxicity against P388, A549, HT29, and MEL28 cell lines	(Horgen *et al.*, 2002)
Malyngamides	*Lyngbya*	Fatty acid amines	Cytotoxicity to NCIH460 human lung tumor and neuro-2a cancer cell lines	(Gross *et al.*, 2010)
Malyngolide dimmer	*Lyngbya majuscula*	Cyclodepside	Toxicity against H460 human lung cell lines	(Guitierrez *et al.*, 2010)
Obyanamide	*Lyngbya confervoides*	Cyclic depsipeptide	Cytotoxic against KB cells	(Williams *et al.*, 2002a)
Palau'amide	*Lyngbya sp.*	Cyclic depsipeptide	Cytotoxic against KB cells	(Zou *et al.*, 2005)
Palmyramide A	*Lyngbya majuscula*	Cyclic depsipeptide	Sodium channel blocking activity in neuro-2a cells and cytotoxic activity in H460 human lung carcinoma cells	(Taniguchi *et al.*, 2010)
Pitipeptolides	*Lyngbya majuscula*	Cyclic depsipeptides	Cytotoxicity against HT29 colon adenocarcinoma and MCF7 breast cancer cells	(Montaser *et al.*, 2011a)
Pitiprolamide	*Lyngbya majuscula*	Cyclic depsipeptide	Cytotoxic activity against HCT116 colon and MCF7 breast cancer cell lines	(Montaser *et al.*, 2011b)
Somocystinamide A	*Lyngbya majuscula*	Lipopeptide	Stimulates apoptosis in a number of tumor cell lines	(Wrasidlo *et al.*, 2008)
Symplocamide	*Symploca sp.*	Cyclic peptide	Cytotoxic to NCIH460 non-small lung cells and neuroblastoma cells	(Linington *et al.*, 2008)
Symplostatin 1	*Symploca hydnoides*	Linear Pentapeptide	Cytotoxic to NCIH460 non-small lung cells and neuroblastoma cells	(Mooberry *et al.*, 2003)
Tasiamide	*Symploca sp.*	Cyclic peptide	Cytotoxic against KB and LoVo cells	(Williams *et al.*, 2002b)
Tasipeptins	*Symploca sp.*	Cyclic depsipeptides	Cytotoxic against KB cells	(Williams *et al.*, 2003a)
Ulongapeptin	*Lyngbya sp.*	Cyclic depsipeptide	Cytotoxic against KB cells	(Williams *et al.*, 2003b)
Veraguamides	*Symploca cf. hydnoides*	Cyclic depsipeptides	Cytotoxicity to the H460 human lung cancer cell	(Mevers *et al.*, 2011)

			line	
Wewakazole	*Lyngbya sordida*	Cyclic dodecapeptide	Cytotoxicity to the mammalian cell line	(Malloy *et al.*, 2011)
Wewakpeptins	*Lyngbya semiplena*	Depsipeptides	Cytotoxicity to NCIH460 human lung tumor and neuro-2a mouse neuroblastoma cells	(Han *et al.*, 2005)

1.3. Cancer

Cancer has been the human disease with an increased incidence along the last decades, both in developing as well as in developed countries. The process of cancer development is termed carcinogenesis where the main actors are a group of abnormal cells with uncontrolled growth, and the capacity to multiply and invade surrounding, and sometimes, distant tissues. The severity of cancer is due to this invasive capacity, because it can spread throughout the entire organism and in that case, treatment is very difficult to achieve (Hodgson, 2010).

The development of this severe disease is mainly through the accumulation of mutations in critical genes, which can be a result of deficient DNA replication or repair, oxidative damage and, in most cases, DNA damage caused by environmental carcinogens. Many cancers originate from a single cell (monoclonal) and by an accumulation of critical mutations in target genes within this cell, providing a growth advantage and allowing the mutant clone to multiply to other tissues (Figure 3). Nevertheless, many cancers require a number of years to accumulate multiple critical mutations and to expand to other tissues to yield a clinically detectable cancer (Hodgson, 2010).

The main genes allocated in tumor development are proto-oncogenes, oncogenes and tumor suppressor genes. The proto-oncogenes are genes found in normal cells and are involved in the regulation of cell growth. When mutations occur in the proto-oncogenes and they gain functions due to that alteration, they are designated as oncogenes. An example of a functional mutation in a proto-oncogene is that its gene product continually stimulates cell proliferation. The other family of genes, tumor suppressor genes and their proteins function as negative regulators of cell growth (Hodgson, 2010). So,

the activation of oncogenes and the mutation of tumor suppressor genes are major events leading to carcinogenesis (Bain *et al.*, 2007). Also, these two families of genes can inhibit the mechanism of programmed cell death (apoptosis), therefore providing, along with the enhanced cell growth, a selective advantage to cancer progression.

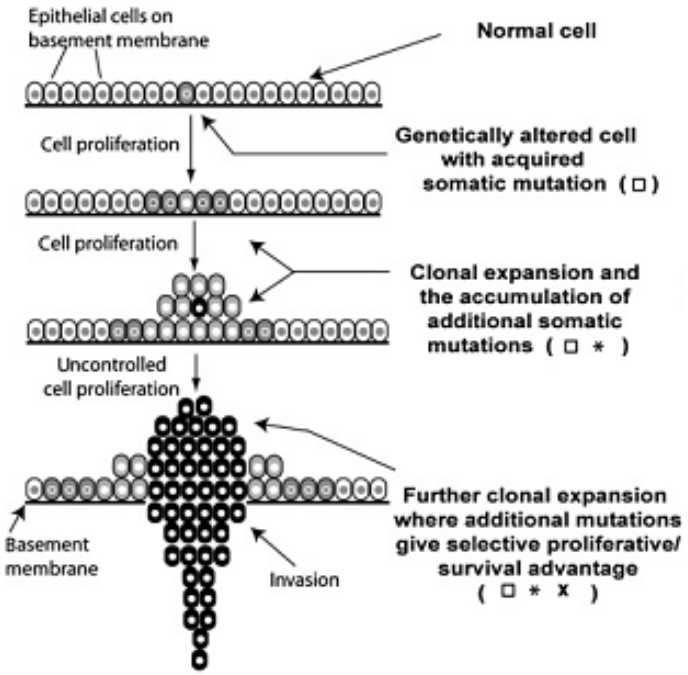

Figure 3 – Monoclonal origin of cancer, showing the accumulation of several somatic mutations in a single cell, providing a selective advantage and expansion to other tissues and tumor development. Adapted from Hodgson, 2010.

Neoplasm is an abnormal mass with uncontrolled and uncoordinated growth that continues after the stimulus has ceased. Within neoplasms, they can be further divided into benign and malignant, but cancer usually is used to refer to malignant neoplasms (Table 3) (Hodgson, 2010).

Table 3 – Main characteristics of the different types of neoplasms, benign and malignant.

Benign Neoplasm	Malignant Neoplasm
Generally of slow growth	Slow or fast growth
Well differentiated	Undifferentiated
No capacity for invasion	Capacity for invasion
Rare and normal mitosis	Frequent and abnormal mitosis
No metastasis	Metastasis

Concerning the type of cancer incidences, the most common cancers among women are breast, lung and colon/rectum, while in men are lung, prostate and colon/rectum cancers (figure 4). Although, there is one type of cancer that owns the spotlight regarding this disease, lung cancer. There is an increased mortality rate in lung cancer both in women and men and is mainly due to cigarette smoking (Hodgson, 2010).

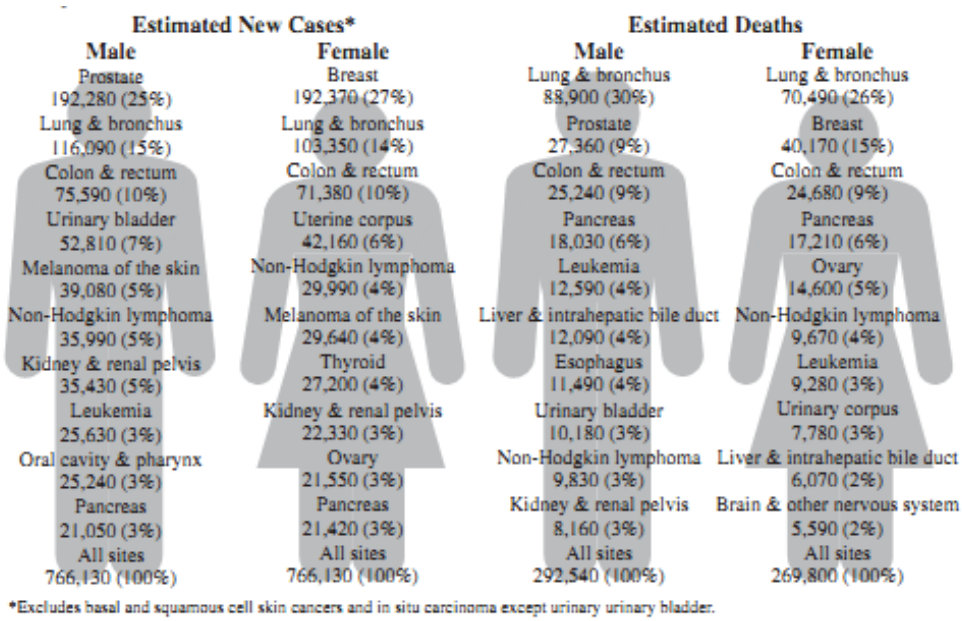

Figure 4 – Cancer cases and deaths by sites and sex, estimates for the year 2009 (**Hodgson, 2010**).

Nowadays, cancer is still the leading cause of death in many countries, such as the USA, thus it is important to continually study the mechanisms underlying this disease and to obtain new compounds for treatment. Also, the increased chemoresistance of the compounds used in cancer treatment provides the need to explore new bioactive compounds. Secondary metabolites produced by a variety of organisms such as cyanobacteria in the environment are a rich and promising source of bioactive compounds for the treatment of cancer.

2. Objectives

Some studies already performed in Laboratory of Ecotoxicology Genomics and Evolution (LEGE) at CIIMAR reported bioactivity in cyanobacterial extracts from marine cyanobacteria of the strains *Synechocystis* and *Synechococcus* in mice (Martins *et al.*, 2005; Selheim *et al.*, 2005) invertebrates (Martins *et al.*, 2007), cells and bacteria (Martins *et al.*, 2008). Results from a screening on the anticancer potential of cyanobacteria strains isolated from the Portuguese coast revealed an ethyl acetate fraction (Fraction B) of strains LEGE06113 (*Cyanobium sp.*) and LEGE06155 (*Synechocystis salina*), as cytotoxic to several human cancer cell lines. Taking these results as a starting point we aimed in this work to assess the anticancer potential of these cyanobacterial strains. We analyzed cytotoxicity in three human tumor cell lines and started identifying some of the key mechanisms that interfere with the cellular viability in one selected cell line.

The main objectives were:

- To evaluate the viability of cells exposed to fraction B (100 µg/mL) of strains LEGE06113 (*Cyanobium* sp.) and LEGE06155 (*Synechocystis salina*);
- To analyze the expression of gene markers of cellular differentiation, tumor growth and apoptosis;
- To analyze the proteome of the tumor cells in order to detect alteration on the proteome profile.

3. Material and Methods

3.1. Cyanobacterial Strains and Culture

For this work two cyanobacterial strains were selected, LEGE06113 from the genus *Cyanobium* and LEGE06155 from the genus *Synechocystis*. These strains are part of the LEGE cyanobacteria culture collection and were isolated from water samples and solid materials from the Portuguese coast.

Cyanobacterial strains were cultured in Z8 medium complemented with NaCl (20g/L), at 25°C and with the photoperiod of 14h/10h of light and dark respectively, using a light intensity of 10 µmols photons s^{-1} m^{-2}. Cultures were performed in 6L flasks with 4L of medium and the period of growth was approximately of one month with constant aeration. In the exponential phase, cells were harvested trough centrifugation at 4600 rpm and 4°C, frozen and freeze-dried. Lyophilized material was stored at -20°C.

3.2. Cyanobacterial Extracts and Fractioning

The cyanobacterial extracts were performed with the lyophilized material. A crude extract was prepared with a dichloromethane:methanol (2:1, v/v) solution and thereafter fractioned using a polarity gradient from 100% hexane, to 100% ethyl acetate to 100% methanol (fractions A, B and C respectively) in a Si column chromatography. This process allows the isolation of compounds with different polarities, where the fraction A is nonpolar and the polarity increases from fraction to fraction, with Fraction C having higher polarity compounds.

On the first extraction, originating the crude extract, 1g of lyophilized material of each strain was extracted for 10 minutes with 50mL of a dichloromethane:methanol (2:1) solution, stirring periodically with a spatula (Fig. 1).

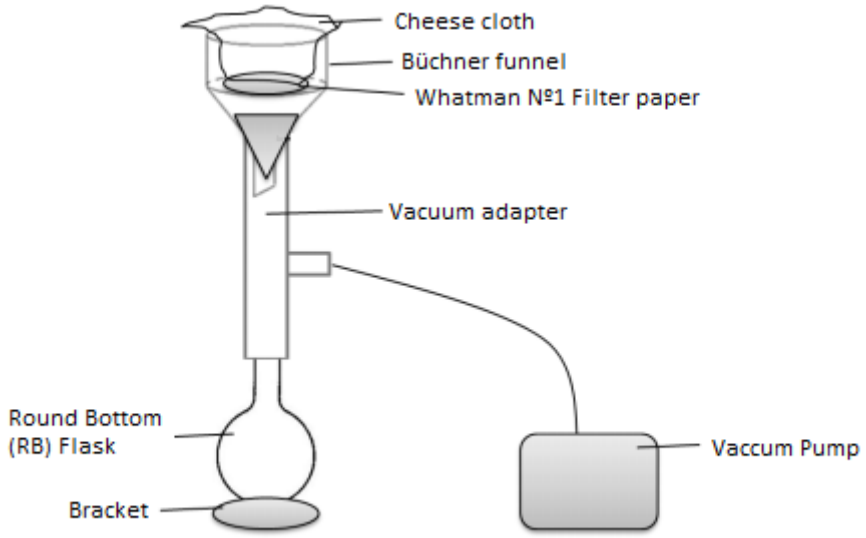

Figure 5 – System assembled for the extraction of the crude extract, from the lyophilized cyanobacterial material (Costa, 2011).

Next, the solution was placed into the Bücher funnel, under vacuum. When all the liquid material reached the round bottom (RB) flask the process was stopped and the residual mass in the cheese cloth was collected for new extraction, for another 10 minutes. This process of extraction was repeated 3 times at room temperature and 3 more times in a hotplate, at approximately 40°C. In the end of all the extractions, the RB flask was removed from the system and the solvents were evaporated in the rotary evaporator at -7°C and at the dichloromethane pressure. The RB flask was washed with a mixture of isooctane:ethanol (1:1, v/v) to dissolve the pellet, using ultrasounds, and transferred to a 22mL clear glass vial (previously weighted). Then the mixture was evaporated using a N_2 stream drying apparatus, and finally the glass vial weighted to obtain the total mass of the crude extract.

The following step was the fractioning of the crude extract to form 3 fractions A, B and C. A specific volume of hexane was used to dissolve the lyophilized biomass at a concentration of 50 mg/mL. To perform the fractioning a silica (Si) chromatography column of 2g or 5g was used, depending on the total cyanobacterial biomass (50 mg or 150 mg, respectively), and the following system was assembled, as shown in figure 6.

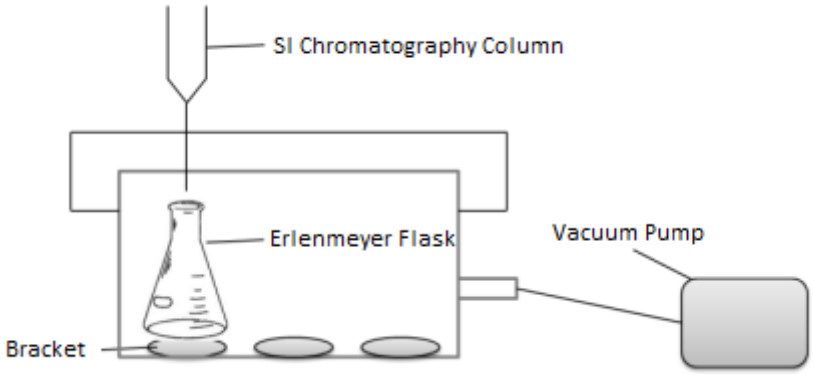

Figure 6 – System assembled for the fractioning of the crude extract to originate Fraction A (hexane), Fraction B (ethyl acetate) and Fraction C (methanol) (Costa, 2011).

Silica on the SI chromatography column was activated with hexane. The crude extract was placed in the column, followed by hexane, which was collected in an Erlenmeyer flask, until a yellow coloration reached ¾ of the column. In that condition, a sequential extraction of the SI column was performed, as shown in table 1, yielding the Fraction A (Hexane), Fraction B (Ethyl acetate) and Fraction C (methanol).

Table 4 – Solvents volumes added to obtain the 3 fractions, A, B and C (Costa, 2011).

Fractions		Column 2g				Column of 5g			
		Hexane	Ethyl Acetate	Methanol	Total	Hexane	Ethyl Acetate	Methanol	Total
Fraction A	100% Hexane	4000 µL	0 µL	0 µL	4 mL	10000 µL	0 µL	0 µL	10 mL
	20% Ethyl Acetate in Hexane	3200 µL	800 µL	0 µL	4 mL	8000 µL	2000 µL	0 µL	10 mL
	40% Ethyl Acetate in Hexane	2400 µL	1600 µL	0 µL	4 mL	6000 µL	4000 µL	0 µL	10 mL
Fraction B	60% Ethyl Acetate in Hexane	1600 µL	2400 µL	0 µL	4 mL	4000 µL	6000 µL	0 µL	10 mL
	80% Ethyl Acetate in Hexane	800 µL	3200 µL	0 µL	4 mL	2000 µL	8000 µL	0 µL	10 mL
	100% Ethyl Acetate	0 µL	4000 µL	0 µL	4 mL	0 µL	10000 µL	0 µL	10 mL
	75% Ethyl Acetate in Methanol	0 µL	3000 µL	1000 µL	4 mL	0 µL	7500 µL	2500 µL	10 mL
Fraction C	75% Methanol in Ethyl Acetate	0 µL	1000 µL	3000 µL	4 mL	0 µL	2500 µL	7500 µL	10 mL
	100% Methanol	0 µL	0 µL	4000 µL	4 mL	0 µL	0 µL	10000 µL	10 mL
	100% Methanol	0 µL	0 µL	4000 µL	4 mL	0 µL	0 µL	10000 µL	10 mL

All the solvents in the fractions were evaporated in the rotary evaporator, at the pressure of the less volatile solvent and the sediment present in the RB flasks was resuspended with the less volume possible of isooctane:methanol (1:1, v/v). This mixture was then transferred to 15mL glass vials previously

marked and weighted, evaporated with N_2 stream drying apparatus and stored at -20°C.

To perform the toxicity tests, the different extracts (crude, fraction A, B and C) were dissolved in isooctane:methanol (1:1, v/v) to a final concentration of 10 mg/mL. Afterwards, 1 mL of this combination was transferred to a 4 mL glass vial (previously marked and weighted) and evaporated with N_2 stream drying apparatus to obtain its biomass. Finally, a volume of DMSO at 100%, was added to each glass vials to a final concentration of 100 µg/mL.

3.3. Cell Culture and Cytotoxicity Assays

Cytotoxic assays were initially performed with the cell lines HepG2 (liver hepatocellular carcinoma cells), RKO (colon carcinoma cells), T47D (breast carcinoma cells). Cell lines were selected according to previous studies.

Cells were cultured in DMEM Glutamax medium (Dulbecco's Modified Eagle Medium DMEM GlutaMAX™ - Gibco-Invitrogen), supplemented with 10% (v/v) fetal bovine serum (Gibco – Invitrogen), 5 mL fungizone (Gibco - Invitrogen) and 5mL of penicillin-streptomycin (Pen-Strep 100IU/mL and 10 mg/mL, respectively) (Gibco – Invitrogen). Cells were maintained in a humidified incubator at 37°C, with 5% CO_2.

3.3.1. Cytotoxicity Assays

The cytotoxicity of cyanobacteria crude extract and fractions was assessed by the reduction of 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT), a yellow tretazole soluble in water, to purple formazan crystals that are insoluble in water. The reduction of MTT to formazan is directly proportional to the mitochondrial activity and consequently cell viability.

Initially for the MTT analysis, cells were seeded in 96-well plates, at a cell density of $5x10^5$ cell/mL for the HepG2 cell line and $1x10^6$ cell/mL for the RKO and T47D and cells were incubated with 0,5 mg/mL MTT for 4 hours, before absorbance reading. Comparing to the MTT conditions from a previous work,

only the cell density was different, using $5x10^5$ cell/mL and $1x10^6$ cell/mL instead of $3,3x10^4$ cell/mL. But since results were not in accordance to the previous work and the high cell density was needed for the posterior mRNA expression and proteomic analysis, other MTT assay conditions were selected. Then, cells were seeded in 24-well plates at a concentration of $5x10^5$ cell/mL for the HepG2 cell line and $1x10^6$ cell/mL for the RKO and T47D tumor cell lines. After the period of incubation with extract (24h) at a concentration of 100 μg/mL, cells were incubated with 0,5 mg/mL MTT (Sigma), for 1 hour. At the end of this period, the medium was aspirated and the formazan crystals formed dissolved in 500 μL 100% DMSO (Panreac). The absorbance reading was made at 570 nm in a "GEN 5™ – Multi – detection Microplate Reader" (Biotek).

3.3.2. Exposure to Cyanobacterial Fraction B

For the following molecular analyses only the tumor cell line RKO was selected and cells were cultured in 24-well plates at a density $1x10^6$ cells/mL. The extract selected for this experiment was fraction B of each cyanobacterial strain (LEGE06113 and LEGE 06155), since in previous and present work it was the extract that presented higher cell toxicity. After 24 hours, of culture, for adhesion of the cells to the wells, cells were exposed to cyanobacterial fraction B, at a final concentration of 100 μg/mL for 24 hours and maintained in a humidified incubator at 37°C, with 5% CO_2. Afterwards, cells were trypsinized (0,25% Trypsin-EDTA, Gibco – Invitrogen), collected to 1.5 mL eppendorfs, centrifuged at 1200 rpm for 5 minutes and stored as cell pellet at -80°C for posterior analysis.

3.4. Gene Expression Analysis

3.4.1. RNA Isolation and cDNA

To investigate the mRNA expression, total RNA was extracted from the cells derived from the exposure to cyanobacterial fraction B, using the RNAspin Mini RNA Isolation Kit (GE Healthcare), according to the manufacturer included instructions and outlined in figure 7. The chosen protocol was "Total RNA purification from cultured cells and tissue" and during the process, RNA is

absorbed on silica membranes, in a column format. For the elimination of traces of gDNA, a DNAse digestion step was included. RNA was eluted following the "High yield and high concentration" step.

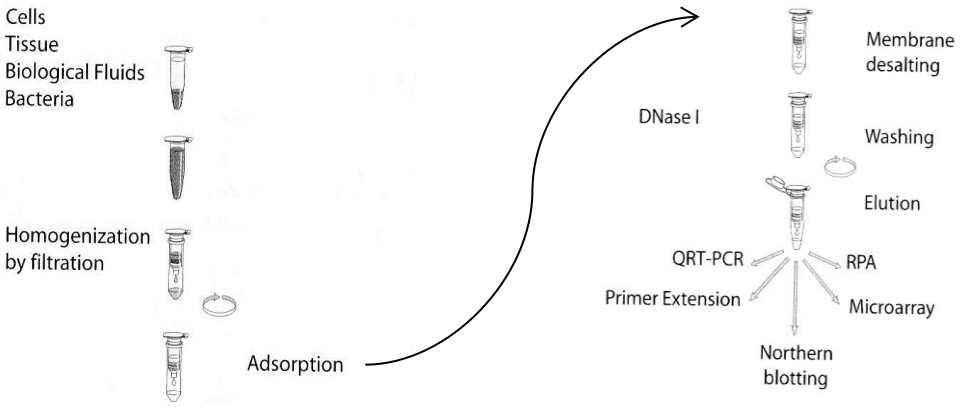

Figure 7 – Overview of the RNAspin Mini Kit procedure.

After this process, RNA was quantified with the Qubit Fluorometer (Invitrogen), using two standards and following the manufacturer instructions. The quality of the extracted RNA was accessed with agarose gel electrophoresis. RNA quality is good when the bands are sharp, with the 28S band being about twice as intense as the 18S band, as shown in figure 4.

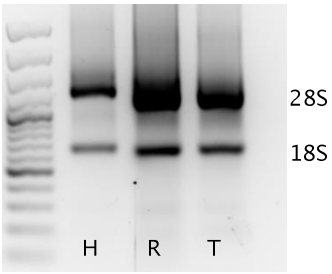

Figure 8 – Representation of total RNA, demonstrating the 28S and 18S band in the tumor cancer cell lines, HepG2 (H), RKO (R) and T47D (T).

Transcription of RNA into cDNA was performed by reverse transcription (RT) using the iScript Select cDNA Synthesis Kit (BioRad) and the oligo (dT) primer. 1 µg of total RNA was reverse-transcribed in a 20 µL reaction, and cDNA was stored at -20°C.

3.4.2. Evaluation of Primer

The cDNA products were used for analysis of primer performance of a set of selected human gene primers (RPL8, CCNE, CCNB1, P21CIP, BCL-2, BAD, ACTB, HPRT1, UBE2C3) as described in table 5. Primers concentration was of 200 nM and primer were designed on two different exons crossing an intron sequence in order to avoid the amplification of genomic DNA. For all PCR reactions, cDNA was diluted at a factor of 1:10 and 3 µL of the diluted cDNA were mixed with 22 µL of PCR mix containing: 10x buffer with 1.5 mM $MgCl_2$ (2,5 µL), 0,5 µL dNTP, 0,25 µL forward and reverse primer and 0,2 µL DFS *Taq* Polymerase (Bioron). The amplification process was carried out in a gradient cycler (Biometra T-Gradient), performing a temperature gradient PCR between 55 and 65°C following the cycling conditions: 4 min at 94°C, 30 sec at 94°C, 30 sec at 55°C-65°C, 30 sec at 72°C, 10 min at 72°C; the number of cycles was 40. The temperature gradient was performed to select the suitable annealing temperature of each gene primer, for the subsequent Real Time PCR analysis. Furthermore, specific bands in agarose gels with the desired product length confirmed the specificity of the PCR reactions.

3.4.3. Real-Time PCR

To quantify differences in mRNA expression, an analysis was performed by real-time PCR using the iQ5 Real-time PCR machine (Bio-Rad). The final reaction volume was of 20 µL with 1x iQ SYBR Green Supermix (Bio-Rad), 10 µM forward and reverse primer (given the final concentration of 200 nM each) and 2µL cDNA sample (1:10 diluted). The samples were run with a duplicate negative control (NTC) to assure the absence of contaminations, and with a duplicate of standard cDNA pool from RKO cells without treatment (1:10 diluted).

Standard curves were analyzed from the diluted cDNA pool and the resulting amplification efficiency of the primers should be between 80 and 110%. The view of the melt curve from the RT-PCR analysis monitored the specificity of the performed reaction.

For the correct RT-PCR mRNA expression in the BioRad iQ5 software, the step point should be on the “annealing temperature” of each gene, the analysis

mode is “PCR base line substracted” and the baseline threshold was selected at the value of 60.

Table 5 – Selected primers main information.

Abbreviation	P21CIP	CCNB1	CCNE	RPL8
Name	Cyclin-Dependent Kinase Inhibitor 1A	Cyclin B1	Cyclin E1	Ribosomal protein L8
Function	Binds and inhibits cyclin-dependent kinase activity, and blocking cell cycle progression.	Essential for the cell cycle control at G2/M (mitosis) transition.	Regulates the subunit of CDK2, required for the cell cycle G1/S transition.	Encodes a ribosomal protein that is a part of the 60S subunit.
Primer sequence	Forward 5'GGGACAGCAGAGGAAGAC3' Reverse 3'CGGCGTTTGGAGTGGTAG5'	Forward 5'CTAAGATTGGAGAGGTTGATGTC3' Reverse 3'CAGGTAATGTTGTAGAGTTGGTG5'	Forward 5'GTTCTCGGCTCGCTCCAG3' Reverse 3'CGGTCATCATCTTCTTTGTCAGG5'	Forward 5' GTGTGGTGGCTGGAGGTG3' Reverse 3'CGATGTGCTGGTGGTTGC5'
Efficiency	96%	91%	86,4%	101,4%
R2	0,979	0,996	0,931	0,999
Annealing temperature	55°C	57°C	57°C	57°C

BCL-2	UBE2C3	BAD	ACTB	HPRT1
B-Cell CLL/Lymphoma	Ubiquitin-Conjugating Enzyme E2C	BCL2-Associated Agonist Of Cell Death	Beta Cytoskeletal Actin	Hypoxanthine Phosphoribosyltransferase
Acts as anti-apoptotic regulators that are involved in a variety of cell activities. The proteins encoded reduce the release of pro-apoptotic cytochrome c from mitochondria and block caspase activations. Have two transcripts: alpha and beta. (We used the alpha transcript)	Encodes the enzyme class of ubiquitin-conjugating enzymes (E2), (modificates proteins with ubiquitin, which target them for degradation).	Regulates cell apoptosis, forming heterodimers with BCL-xL and BCL-2 and reversing their death repressor activity.	Major constituent of the contractile apparatus and one of two nonmuscle cytoskeletal actins.	Converts hypoxanthine to insonie monophosphate and guanine to guanosine monophosphate. Central role on generating purine nucleotides.
Forward 5'GTGTGGAGAGCGTCAACC3' Reverse 3'CTTCAGAGACAGCCAGGAG5'	Forward 5'AGGAGGCTGATGAAGGAG3' Reverse 3'TTCGGTGGTTTGAATGGG5'	Forward 5'GAGGATGAGTGACGAGTTTGTG3' Reverse 3'CGGGATGTGGAGCGAAGG5'	Forward 5'CACCACACCTTCTACAATGAG3' Reverse 3'ATAGCACAGCCTGGATAGC5'	Forward 5'TGGCGTCGTGATTAGTGATG3' Reverse 3'CAGAGGGCTACAATGTGATGG5'
108,2%	99,1%	107,1%	94,6%	96,5%
0,970	0,988	0,995	0,997	0,995
55°C	57°C	59°C	57°C	57°C

3.4.4. Multiple Reference Gene Normalization

To perform the gene normalization and the selection of a normalization factor, the NormFinder Software was used. NormFinder is an algorithm used to identify the optimal combination of reference genes among a set of candidate genes (Andersen *et al*., 2004). It classifies the normalization candidate genes according to their expression stability in a given sample and experimental design.

In this work, the set of reference genes (RPL8, ACTB, HPRT1 and UBE2C3) were selected to perform the analysis of reference gene selection for subsequent normalization; with 4 input genes the NormFinder software gives a normalization factor for the 2 most stable expressed genes in a given sample set. The selected genes were RPL8 and HPRT1, and the geometrical mean of both was used for the normalization.

3.4.5. Statistical Analysis

The statistical analysis was performed using the software "GraphPad Prism" (version 6.02). The verification of normality and homogeneity of variances was achieved by applying the Kolmogorov-Smnirnov test and Shapiro-Wilk for normality and the Bartlett's test for equal variances. When these parameters were fulfilled, the *One-way* ANOVA was performed to compare the gene expression between the different experimental conditions. The *post-hoc* Tukey's test was applied for multiple comparisons among means and the differences were considered significant when the $p \leq 0,05$. If conditions for parametric test were not fulfilled, non-parametric statistical tests were used Kruskal-Wallis and Dunn's test as a *post-hoc* test, and differences were considered significant when $p \leq 0,05$.

3.5. Proteomic Analysis

The proteome analysis was performed with the purpose of knowing the main differences in protein expression regarding the effect of each cyanobacterial fraction in the selected cell line.

3.5.1. Protein Extraction and Quantification

The protein extraction was performed by adding to the cell pellet a volume of solubilization buffer containing urea (7M), thiourea (2M), CHAPS (4%, w/v), dithiothreitol (65mM) and ampholytes (0.8%, v/v, pH 4-7), at a ratio of 80 µL to 15mg of cell biomass. The homogenization process took 1h and then the homogenate was centrifuged at 16000xg, for 20 minutes, at 20°C. The supernatant was collected and proteins were quantified using the Bradford method (Bradford, 1976). The protein samples were stored at -70°C.

3.5.2. Two-dimensional Electrophoresis (2DE)

The protein samples were separated by two-dimensional electrophoresis (2DE), separating proteins by their isoelectric point and molecular weight. Samples with 150 µg protein were diluted in 125 µL of solubilization buffer (SB) and centrifuged at 1000xg for 10 minutes. Then, the protein samples were loaded in 7 cm, pH 4-7 IEF gel strips (Bio-Rad) and proteins separated by isoelectric focusing (IEF) with the following program: 12h at 50V (strip rehydration); step 1 – 250V for 15 minutes; step 2 – 2h voltage gradient to 4000V (linear ramp); step 3 – 4000V until achieving 20000V/h (linear ramp). When the rehydration step was over, paper strips were placed above the electrodes to remove the excess of salts from the samples. At the end of the first dimension, the IEF gel strips were stored at -20°C until performing the second dimension, SDS-PAGE. Before the second dimension electrophoresis IEF gel strips need to be equilibrated using two solutions, Equilibration Buffer 1 with 12.5 mg/mL dithiothreitol (DTT) and Equilibration Buffer 2 with 25 mg/mL iodoacetamide in urea (6 M), glycerol (30%, v/v), SDS (2%, w/v), both during 15 minutes with slow agitation and with 2 mL of each solution to an IEF strip.

For the second dimension, 12% (w/v) acrylamide SDS-PAGE gels were prepared as follows: 3.44mL of H_2O; 3.96mL acrylamide 30% (w/v); 2.5mL gel buffer (1.5M Tris-HCl, pH 8.8); 0.1mL SDS 10% (10g in 100mL H_2O); ammonium persulphate (50 µL at 10% - 0.05µg/0.5mL) and 5 µL TEMED (amounts required for 2 minigels). The IEF strips were placed on top of the gels and proteins were separated, according to their molecular weight, following the program of 50V for 30 minutes and 150V until the proteins reached the end of the gel, as schematized in figure 9.

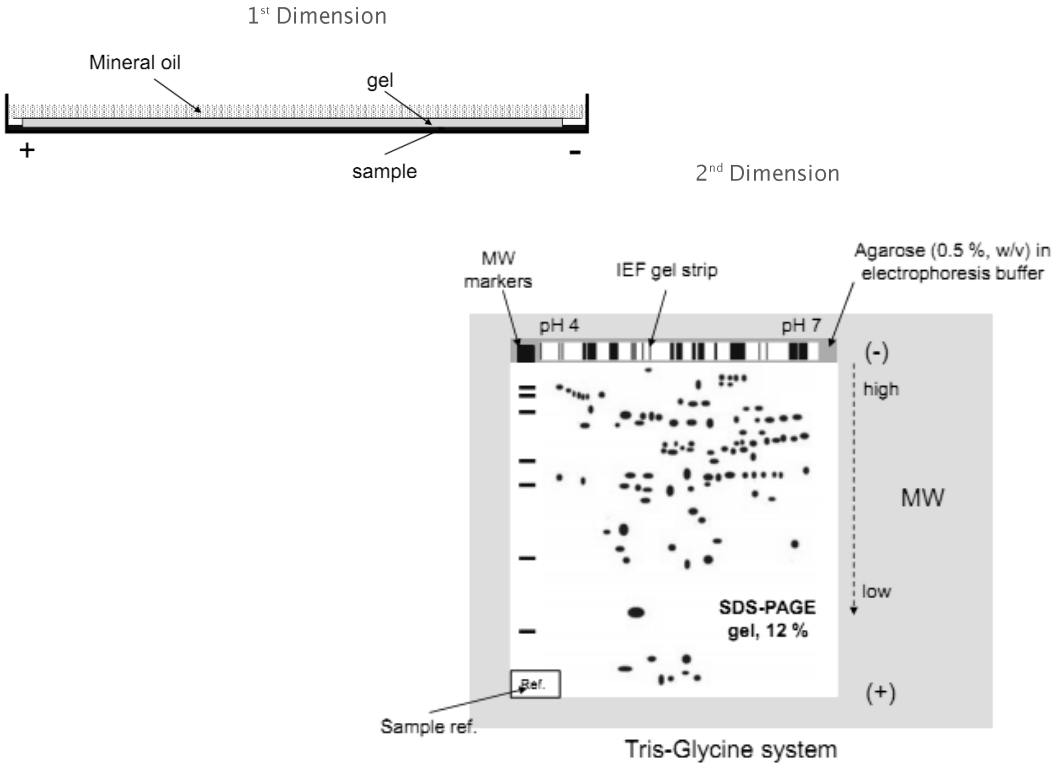

Figure 9 – Illustration of the Two-Dimensional Electrophoresis (2DE). The first dimension is the isoelectric focusing (IEF) where proteins are separated by their isoelectric point and the second dimension is the SDS-PAGE, where proteins are separated according to their molecular weight.

3.5.3. Gel Staining

Coomassie Blue Colloidal staining was the process selected to visualize proteins. After the second dimension, gels were fixed in the fixation solution consisting in methanol (40%, v/v), acetic acid (10%, v/v)) for at least 1h and during that hour another solution was prepared mixing 4 volumes of the staining solution (98%, w/v of Solution A [Ortho-phosphoric acid (2%, w/v); ammonium sulphate (10%, w/v)] and 2%, w/v Solution B [Coomassie Blue G250

(5%, w/v)]) with 1 volume of methanol, with constant agitation. When the step of fixation was over, gels were briefly washed with mili Q water and then stained with 50 mL of the staining solution, overnight (increased sensitivity). In the end of the staining procedure, gels were briefly washed with mili Q water to remove the colloidal particles and stored in the storage solution with ammonium sulphate (20%, w/v) at -20°C.

3.5.4. Gel Image Acquisition and Protein Expression Analysis

Gel images were acquired in the GS-800 calibrated densitometer (Bio-Rad) and protein spots detected automatically with the PDQuest 2-D analysis software (Bio-Rad), reproducing the sensitivity parameters for all the gel images. The spot detection and matching was manually revised in the software. When the protein expression was analyzed, a master gel was created in the software, which contained all the spots detected in the 2DE gel images. The master gel is created when the matchset is performed by the software, which allows us to make quantitative and qualitative comparisons of spots between gels, calculate values of molecular weight and isoelectric points. The spot expression (presence/ absence) and intensity variations were also analyzed, by comparing each protein spot between all the gels in the experimental groups. The quantitative variations were statistically evaluated by the *t*-student and Mann-Whitney tests ($p \leq 0.05$).

4. Results and Discussion

4.1. Cytotoxic Assays

For the cytotoxicity confirmation three tumor cell lines were initially used, HepG2, RKO and T47D, but only the tumor cell line RKO was selected for the following mRNA expression and proteomic analyses. Between the cyanobacteria crude extract and fractions A, B and C, fraction B revealed to be the most interesting as already described by Ribeiro, 2012 (results in Appendix II).

The results regarding cell viability of the T47D cell line exposed to fraction B, at a concentration of 100µg/mL in 24-well plates and at a concentration of $1x10^6$ cell/mL are presented in figures 10 and 11, according to the cyanobacterial strain, LEGE06113 and LEGE06155, respectively. The negative control consisted in growth medium with 1% DMSO and the positive control 20% DMSO with growth medium. As expected, a reduction of cell viability occurred demonstrating cytotoxicity of the fraction on the tumor cancer cell line. Results with LEGE06155 strain were more pronounced and reduced cell viability was time dependent.

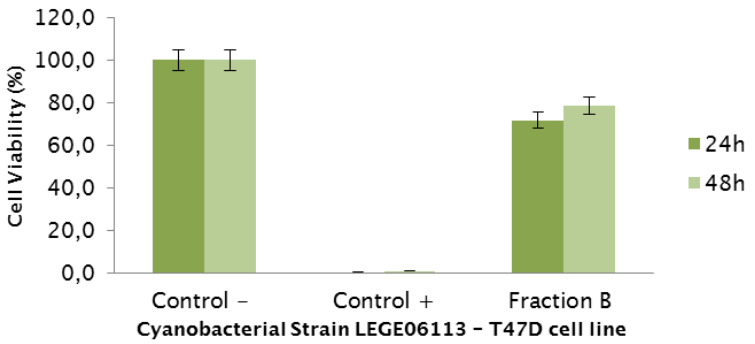

Figure 10 – Cell viability from the Fraction B of the cyanobacterial strain LEGE06113, in the tumor cell line T47D (breast carcinoma), at a concentration of 100µg/mL. The negative control corresponds to 1% DMSO (5 µL) and the positive control to 20% DMSO (100µL).

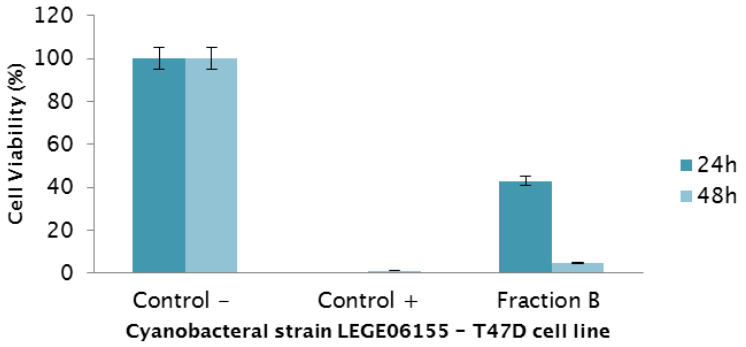

Figure 11 – Cell viability from the Fraction B of the cyanobacterial strain LEGE06155, in the tumor cell line T47D (breast carcinoma), at a concentration of 100µg/mL. The negative control corresponds to 1% DMSO (5 µL) and the positive control to 20% DMSO (100µL).

The results regarding the cell viability of RKO cell line exposed to fraction B, at a concentration of 100µg/mL in 24-well plates and at a concentration of $1x10^6$ cell/mL are presented in figures 12 and 13, according to the cyanobacterial strain, LEGE06113 and LEGE06155 respectively. In this cell line, results are similar to the T47D (Figure 10 and 11). Also a decrease in the cell viability occurred. However, with the fraction B from the cyanobacterial strain LEGE06155, the decreased of cell viability at 24h was less in the RKO that the T47D cell line, 40% and 60%, respectively. Regarding time exposition and the cyanobacterial fraction from LEGE06113, a partial recovery of cell viability after 48h was also observed.

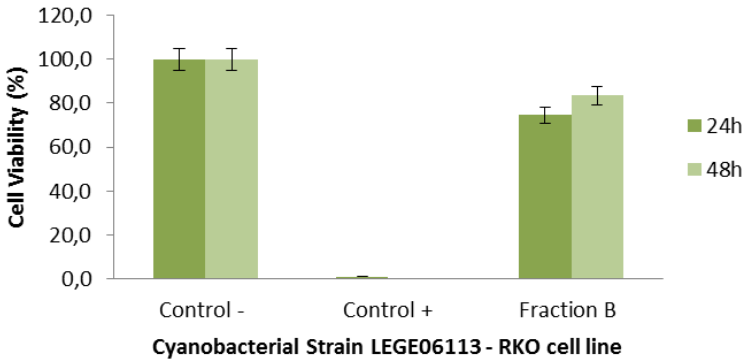

Figure 12 – Cell viability from the Fraction B of the cyanobacterial strain LEGE06113, in the tumor cell line RKO (colon carcinoma), at a concentration of 100µg/mL. The negative control corresponds to 1% DMSO (5 µL) and the positive control to 20% DMSO (100µL).

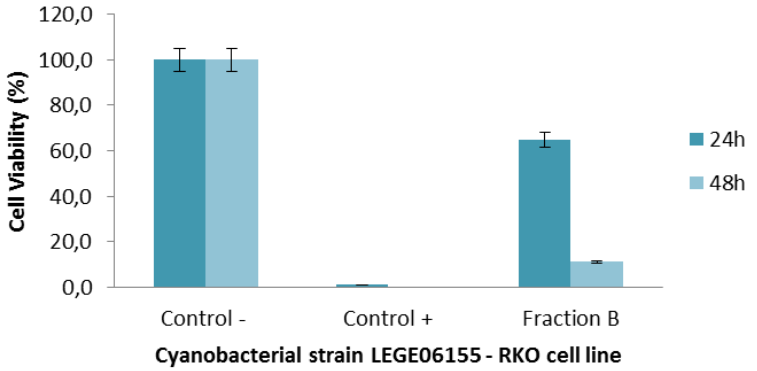

Figure 13 – Cell viability from the Fraction B of the cyanobacterial strain LEGE06155, in the tumor cell line RKO (colon carcinoma), at a concentration of 100µg/mL. The negative control corresponds to 1% DMSO (5 µL) and the positive control to 20% DMSO (100µL).

The results regarding the cell viability of HepG2 cell line exposed to fraction B, at a concentration of 100µg/mL in 24-well plates and at a concentration of $5x10^5$ cell/mL are presented in figures 14 and 15, according to the cyanobacterial strain, LEGE06113 and LEGE06155 respectively. In this tumor cell line, the decrease in cell viability from LEGE06113 was less pronounced relatively to the other two tumor cell lines, and with little variation between the two exposure times, 24h and 48h (Figures 14 and 15). Regarding the 24h exposure to LEGE06155 fraction, cell viability decreased only 20% comparing with negative control, however after 48h cell viability was reduced to 40%.

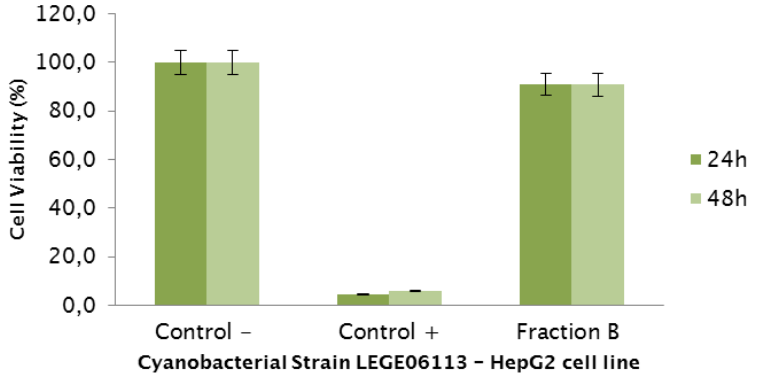

Figure 14 – Cell viability from the Fraction B of the cyanobacterial strain LEGE06113, in the tumor cell line HepG2 (liver carcinoma), at a concentration of 100µg/mL. The negative control corresponds to 1% DMSO (5 µL) and the positive control to 20% DMSO (100µL).

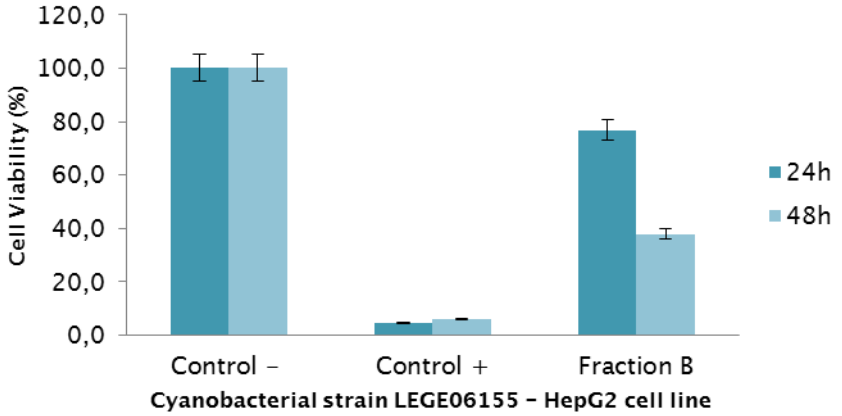

Figure 15 – Cell viability from the Fraction B of the cyanobacterial strain LEGE06155, in the tumor cell line HepG2 (liver carcinoma), at a concentration of 100µg/mL. The negative control corresponds to 1% DMSO (5 µL) and the positive control to 20% DMSO (100µL).

Analyzing all the results from the cytotoxicity assays, it was confirmed that with both cyanobacterial strains a cell viability decrease occurs, more or less depending on the tumor cell line and cyanobacterial strain. The most cytotoxic fraction was from the cyanobacterial strain LEGE06155, with a more pronounced increase in cell death in all three tumor cell lines, both at 24h and 48h. In previous work from Costa *et al.*, 2013, (submitted) the conditions for the MTT assay used a cell density of $3{,}3\times10^{4}$ cells per well in 96-well plates, and with the cyanobacterial strain LEGE06155 a reduction in cell viability to 0% occurred in some cases (Appendix II). Since the difference between the two MTT conditions was the cell density used, further analysis would help to understand this peculiar phenomenon. Although cyanobacterial fractioning of a crude extract resulted in three different fractions (A, B and C) with different polarities, only the fraction B was selected for the cytotoxicity assays, since was shown previously to have higher cytotoxic effect in several tumor cell lines (Ribeiro, 2012), as also seen in Appendix II. This bioactivity can be explained by the fact that this fraction contains compounds with intermediate polarities or by the interaction between compounds chemically active among the B fraction. Almost all bioactive compounds extracted from cyanobacteria are peptides, highlighting the potential from this cyanobacterial fraction (table 2).

Considering the tumor cell cytotoxicity assays only the tumor cell line RKO was selected for the mRNA expression and proteomic analysis of the cyanobacterial

strains. RKO showed more interesting results, i.e. was sensitive to both cyanobacterial extracts but not with the highest mortality rate, thus enabling enough cells for the mRNA expression and proteomic analyses.

The effect of increased cell viability after 48h time exposure had already been reported in Ribeiro (2012) for the RKO cell line, where the inhibitory effect of cyanobacterial extracts was stronger after 24h time exposure, followed by a recovery of cell viability. But further analyses are needed to investigate in more deep the time-dependent effect of cyanobacterial extracts, the susceptibility of other cells lines and cell modifications in each time exposure.

4.2. mRNA Expression Analysis

The analysis of mRNA expression was focused on understanding the events that occur within the cell nucleus, allowing the comprehension of the mechanisms and consequences on the cell functioning, when gene expression was compromised, due to exposure to cyanobacterial extracts.

The target genes selected for this analysis were CCNE (Cyclin E1), CCNB1 (Cyclin B1), P21CIP (Cyclin-Dependent Kinase Inhibitor 1A), BCL-2 (B-Cell CLL/Lymphoma) and BAD (BCL2-Related Protein A1), comprising genes from the cell cycle regulation and apoptosis. The statistical results are presented in table 6, which show the results from the column statistics (mean and standard deviation, SD), the normality tests (Kolmogorov-Sminorv; Shapiro-Wilk) and the *One-way* ANOVA analysis, with Bartlett's test and *post-hoc* Tukey's. The mRNA expression was analyzed in three different groups: a control group where RKO cells were exposed to 1% DMSO; the group A where cells were exposed to the cyanobacterial fraction B from LEGE06113; and group B with cell exposure to the LEGE06155 cyanobacterial fraction B.

Table 6 – Main statistical results from the RT-PCR analysis, comprising results from the normality test and *one-way* ANOVA. The chosen significance level was p≤0,05 for the normality test, for the *one-way* ANOVA and for the homogeneity of variances with Bartlett's test. Group A represents cell exposed to LEGE06113 fraction B; group B represents cells exposed to LEGE06155 fraction B.

		Column Statistics		Normality tests		One-way Anova		
Gene		Mean	S.D.	Kolmogorov-Smirnov	Shapiro-Wilk	p-value	Bartlett's test	Tukey's M.C. test
CCNE	Control	1,250	0,230	p > 0,10	0,548			
	A	1,118	0,226	0,028	0,012			
	B	1,401	0,869	p > 0,10	0,218			
CCNB1	Control	1,062	0,293	0,043	0,041			
	A	0,848	0,225	p > 0,10	0,654			
	B	0,646	0,176	p > 0,10	0,656			
P21CIP	Control	1,026	0,244	p > 0,10	0,908	0,846	0,054	vs B - No
	A	0,994	0,225	p > 0,10	0,997			vs Ctrl - No
	B	0,946	0,486	p > 0,10	0,676			vs A - No
BCL-2	Control	1,213	0,779	p > 0,10	0,124	0,04	0,881	vs B - No
	A	2,365	0,937	p > 0,10	0,068			vs Ctrl - Yes
	B	1,62	0,901	p > 0,10	0,239			vs A - No
BAD	Control	0,887	0,226	p > 0,10	0,910	0,292	0,926	vs B - No
	A	0,868	0,206	p > 0,10	0,626			vs Ctrl - No
	B	0,735	0,195	p > 0,10	0,997			vs A - No

Using the *one-way* ANOVA analysis, the data need to fulfill two assumptions, normality and homogeneity of variances. Examining the results from the statistical analysis, two of the five target genes, CCNE and CCNB1, did not show a Gaussion distribution. In this case, either a data transformation can be used to fulfill the assumptions to use an ANOVA test, or a nonparametric analysis is needed using the Kruskal-Wallis test for the analysis of variance and Dunn's multiple comparison test as *post-hoc* test. The nonparametric analysis was performed for the CCNE target gene, while for CCNB1, a square root (sqrt) transformation was performed and a new parametric statistical analysis was achieved. Outcomes presented in table 7 and 8.

Table 7 – Statistical results from the CCNB1 transformed data, comprising results from the normality test and *one-way* ANOVA. The chosen significance level was p≤0,05 for the normality test, for *one-way* ANOVA and for the homogeneity of variances with Bartlett's test. Group A represents cell exposed to LEGE06113 extract; group B represents cells exposed to LEGE06155.

		Column Statistics		Normality tests		One-way Anova		
Gene		Mean	S.D.	Kolmogorov-Smirnov	Shapiro-Wilk	p-value	Bartlett's test	Tukey's M.C. test
CCNB1	Control	1,023	0,135	0,09	0,069	0,003	0,799	vs B - Yes
	A	0,913	0,130	p > 0,10	0,398			vs Ctrl - No
	B	0,798	0,107	p > 0,10	0,888			vs A - No

Table 8 – Multivariance nonparametric statistical analysis of CCNE. The chosen significance level was p≤0,05 for the Kruskal-Wallis test and *post-hoc* Dunn's test.

		Kruskal-Wallis	
Gene		p-value	Dunn's M.C. test
CCNE	Control	0,783	vs B - No
	A		vs Ctrl - No
	B		vs A - No

Considering all the statistical results, two genes, CCNB1 and BCL-2, showed statistically significant differences in each one of the treatment groups compared to the control group. For the cell cycle target gene (CCNB1), the significant differences in mRNA expression where present when RKO cells were exposed to the LEGE06155 fraction B, with a *p*-value of 0,003. On the other hand, the mRNA expression of the apoptosis target gene (BCL-2) was significantly altered in response to the exposure to the LEGE06113 fraction B, with a *p*-value of 0,04. These expression levels according to the cyanobacterial strain are presented in figures 16 and 17 with also the statistical results from remaining target genes.

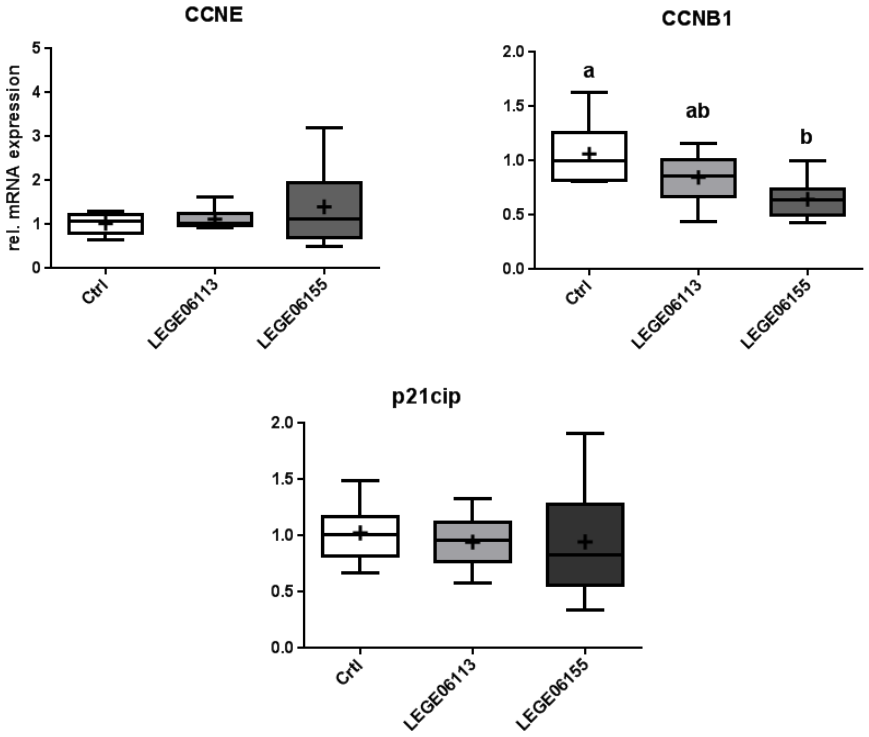

Figure 16 – Relative mRNA expression from selected cell cycle genes, CCNE, CCNB1 and P21CIP. CCNB1 showed significant mRNA expression according to the fraction B of the cyanobacterial strains.

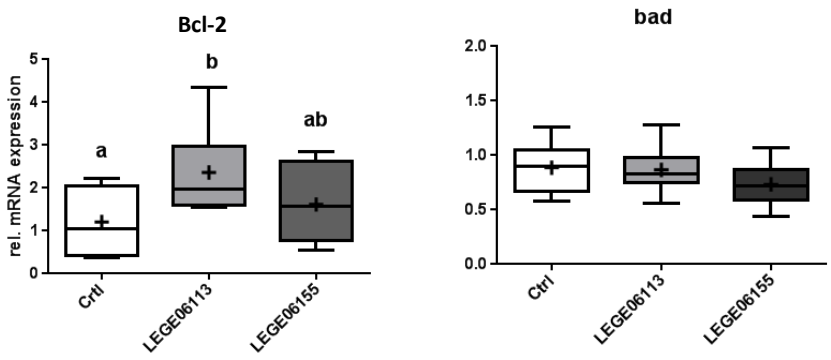

Figure 17 – Relative mRNA expression from selected apoptosis genes, BCL-2 (anti-apoptotic) and BAD (pro-apoptotic). BCL-2 showed significant mRNA expression according to the fraction B of the cyanobacteria strains.

The target gene CCNB1 acts in the regulation of the cell cycle at the G2/mitosis transition. The cell cycle is characterized by two processes, mitosis and interphase which in turn is divided into three phases, G1, G2 and S. In the G1 phase, the cells are preparing for DNA replication, followed by the S phase, where DNA is replicated and in the G2 phase, mechanisms are initiated that will lead to mitosis, the process of nuclear division (figure 18). Sometimes, cells that are in the G1 phase can enter the phase G0, where cells remain in a quiescent state (Vermeulen *et al.*, 2003).

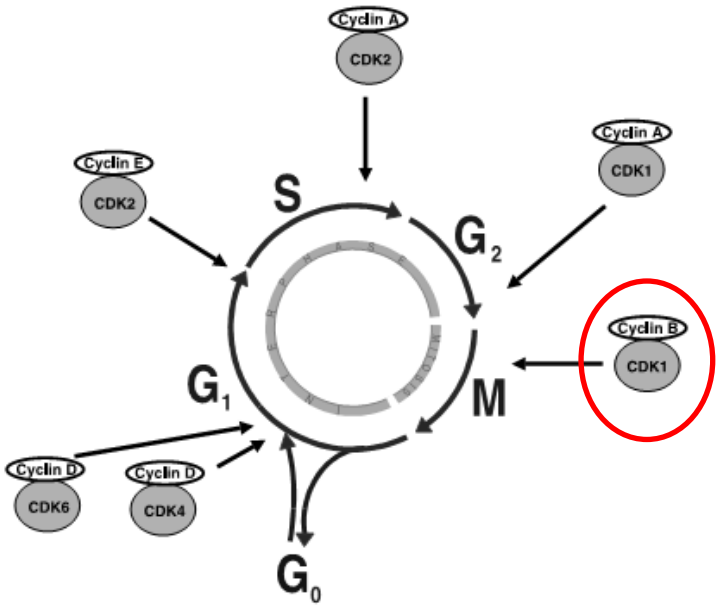

Figure 18 – Different phases of the cell cycle, representing the Cyclin-dependent kinase (CDK) and correspondent cyclins present in each phase of the cycle (Vermeulen *et al.*, 2003).

The cell cycle regulation is performed by a family of serine/threonine protein kinases, named cyclin-dependent kinase (CDK). In a specific point of the cell cycle different cyclins activate CDKs, which in turn induce downstream processes by phosphorylating selected proteins. Therefore, cyclin protein levels rise and fall during the cell cycle while the CDK remain stable, being only activated or deactivated (Vermeulen *et al.*, 2003).

In this work, cyclin B activates CDK1, which is needed for mitosis. This result of altered CCNB1 mRNA expression is novel and interesting, since increasingly studies are focusing on the interaction of bioactive compounds and its cytotoxicity in the cell cycle. As evidenced in figure 18, CCNB1 is involved in progression of the cell cycle to mitosis. The observed decreased in CCNB1 mRNA expression in the presence of the fraction B from the cyanobacterial strain LEGE06155 may be part of a mechanism leading to the cytotoxicity in the RKO cell line. The lower expression of this target gene in the transition of G2 to mitosis, reduce the cells ability to progress in the cell cycle. Consequently, alteration in the progression of the cell cycle leads to fewer cell divisions, hence fewer cells. However more analysis are needed to better understand the implications of this cyanobacterial fraction in the cell cycle regulation, mainly in the G2/M phase in RKO cells, in order to analyze if it affects the activation of CDK1, or the CDK1/cyclinB interaction or yet the phosphorylation ability of activated CDK1. A recent study analyzed the capacity of apoptosis induction from a marine bioactive compound, Salarin C, which was a potent inhibitor of cell proliferation (Ben-Califa *et al.*, 2012). Interestingly, and in accordance to the presented results, the percentage of cells in G2/M phase were strongly reduced.

On the other hand, the other target gene with differential mRNA expression in the presence of a cyanobacterial extract is BCL2-A, being part of the apoptotic gene family. Apoptosis is a controlled cellular death mechanism with a specific set of proteins which signals cell-disassembling biochemical processes (O'Brien *et al.*, 2008). This mechanism can be activated by two pathways, the death receptor pathway (extrinsic) or the mitochondrial pathway (intrinsic) (Sankari *et al.*, 2012).

The BCL-2 protein family has an important role in apoptosis regulation, comprising both pro-apoptotic (BAX, BAD, PUMA) as well as anti-apoptotic (BCL-2, BCL-W, BCL-xL) genes (Fu *et al.*, 2013). The disturbance in conjugation the proteins of these two types of genes cause deregulation in the apoptosis process, a phenomenon common in carcinogenesis. BCL-2 acts as an anti-apoptotic regulator and its protein reduces the release of pro-apoptotic cytochrome c from mitochondria and block caspase activations (intrinsic pathway, figure 19). Cory *et al.* (2003) revealed that anti-apoptotic proteins from the Bcl-2 family interact with oncogenesis by protecting cells from apoptotic stimulus and not by facilitating proliferation. Thought the overexpression of anti-apoptotic proteins affected the cell cycle, mainly in the progression to S phase, inhibiting beginning of the cell cycle (Fu *et al.*, 2013). In this line of argumentation, besides the "traditional" role of BCL-2 as an anti-apoptotic gene, BCL-2 may interact with the progression of the cell cycle (inhibition) if present in higher quantities.

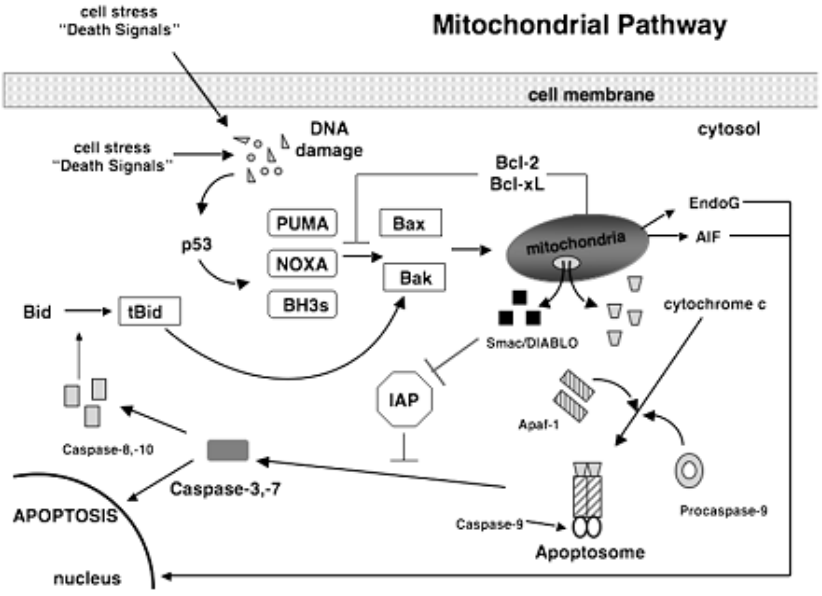

Figure 19 – Mitochondrial apoptotic pathway of intrinsic pathway. Main mechanisms and genes involved in this death cell regulation system, within the cell membrane (O'Brien *et al.*, 2008).

The increased BCL-2 mRNA expression could indicate that higher level of this gene may have contributed to an alteration of the cell cycle regulation. For this work two genes of apoptosis were analyzed when cells where exposed to cyanobacterial fractions, but interactions could occur in many more

candidates, as seen in figure 19. Nonetheless, the LEGE06113 fraction B exhibited reduction in RKO cell viability, so the induction of apoptosis via other genes/proteins cannot be ruled out, since just BCL-2 and BAD were analyzed. Further investigations are needed to better understand the implications that this cyanobacterial fraction possesses in the apoptosis mechanism.

A recent work based on the cytotoxicity of cyanobacterial strains in human cancer cell lines, explored the death cell mechanism that lies behind such cytotoxicity (Ribeiro, 2012). An apoptosis/necrosis assay by staining with propidium iodide (PI) and Hoechst 33342 was performed to study the mechanism of cell death. The fraction B from the cyanobacterial strain LEGE06155 was tested in HT-29 cells (colon adenocarcinoma cell line) and the results pointed to a necrotic cellular death, since only a propidium iodide staining (necrotic assay) was present when cells were exposed to the fraction. These results further support the importance of detecting which cell death mechanism is triggered by the cyanobacterial extract, commonly observed as increased cytotoxicity.

4.3. Proteomic Analysis

The 2DE analysis was performed to the tumor cell line, RKO when exposed to fraction B from the cyanobacterial strains LEGE06113 and LEGE06155. As shown in figure 20 the method, allowed the cells proteome to be resolved between isoelectric points 4-7 and 19-117 kDa molecular mass. An average number of 925 protein spots were detected in each gel. A total of 1450 proteins were analyzed in the different experimental groups. In figure 20 is illustrated the cells proteome from each group condition, control with 1% DMSO (C) and each cyanobacterial strain fraction B, LEGE06113 (A) and LEGE06155 (B), respectively.

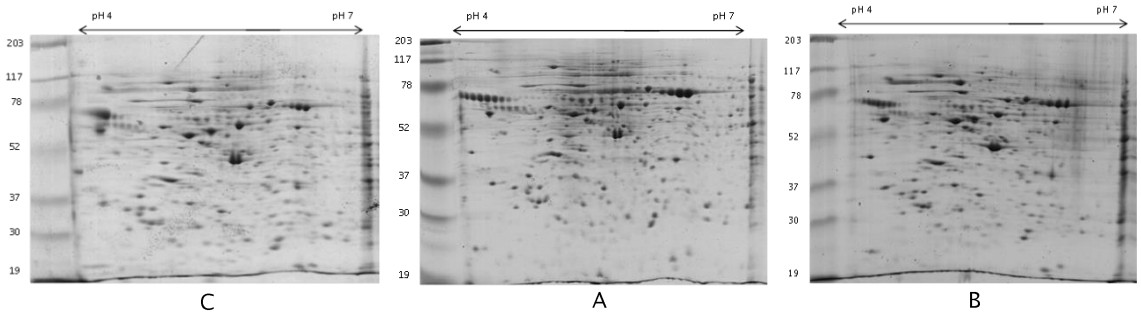

Figure 20 – Two-dimensional electrophoresis from the RKO cell line exposed to the cyanobacterial strains from the cytotoxicity assay, after 24h exposition with 1% extract. Group C corresponds to the control group where RKO cells were exposed to 1% DMSO; Group A corresponds to the condition where RKO cells were exposed to fraction B from the cyanobacterial strain LEGE06113; Group B concerns RKO cells exposed to fraction B from the cyanobacterial strain LEGE06155.

Analyzing the different replicate gels from each condition group an evident result is that there are differences between the proteomes of control and the treated groups and, likewise, between groups treated with the different cyanobacterial fractions B (Figure 20). The main differences in protein expression are quantitative and were detected applying statistics (quantitative variations).

The statistical analyses regarded the comparison of the intensities of the protein spots between two experimental conditions, control cells versus cells exposed to LEGE06113 fraction (Figure 20 A), control cells versus cells exposed to LEGE06155 fraction (Figure 20 B), and both exposure conditions. The statistical tests performed were student *t*-test and Mann-Whitney U test and only differences confirmed by both tests were taken into account to describe quantitative variations in protein expression.

Other differences observed in protein expression were qualitative variations, since there were proteins only present in one experimental group. The proteins differently expressed in one experimental condition (A) are different from the proteins expressed in the other experimental condition (B), suggesting that the fractions from the two cyanobacterial strains, act differently on the same cell line. Figure 21 illustrates the qualitative differences detected in this work. In this case one protein is expressed in cells exposed to LEGE06155 fraction (Figure 21 B), whereas is absent in the control cells (Figure 21 C). The absence of a protein in 2DE gels can result from two situations: the protein is not

expressed under the specific experimental condition, or is expressed at a level that is below the limit of detection of the coomassie staining.

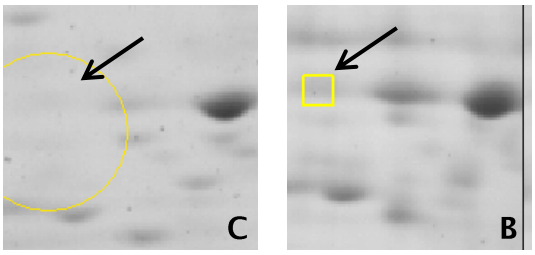

Figure 21 – Zoomed 2DE gel images of RKO cells from the control (C) group and exposed to the cyanobacterial fraction B LEGE06155 (B), showing one protein spot present in the fraction condition (B) and absence in the control group (C) (qualitative variation).

Figure 22 illustrates the quantitative variations of 4 proteins that are expressed in the three experimental groups (A, B and C). Here the intensities of the 4 proteins in the 2DE gels are more intense in the treatment groups (A and B) compared to the control group, suggesting an overexpression of the proteins in the cells exposed to the cyanobacterial fraction. Thought the cyanobacteria fraction B from LEGE06155 has a more pronounced effect in the spot intensity, except in the protein spot 5104, where the spot has the same intensity with both cyanobacterial strains.

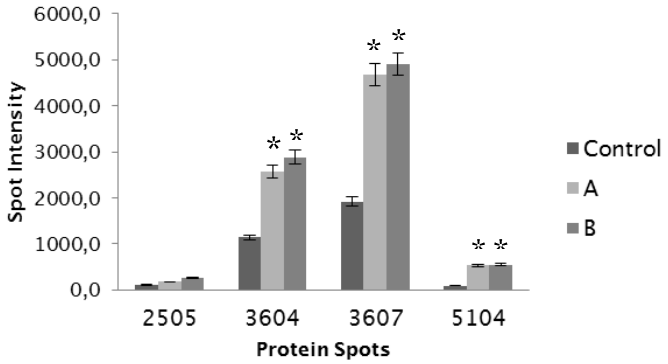

Figure 22 – Protein spot intensities for 2DE experimental gels presented in the Control vs A and Control vs B analysis. Significant variations in spot intensities relatively to control, for of $p \leq 0,05$ (*).

The overall variations detected in the proteome of RKO cells, using 2DE gels, are presented in table 9.

Table 9 – Number of differentially expressed proteins detected by the 2DE gels, after comparison of the experimental groups Control, A and B. In the qualitative analysis, the numbers refer to the proteins present in the cyanobacteria fraction condition (A or B) and absent in the control group. The ↓/↑ respects to the down-regulated or up-regulated proteins.

	SPP		Total SPP	
	Quantitative	Qualitative		
Control vs A	24	1	25	↑ 20
				↓ 5
Control vs B	6	3	9	↑ 9
				↓ 0
A vs B	7	1	8	↑ 1
				↓ 7

When the proteome of the control cells is compared with the proteome of the cells exposed to the LEGE06113 fraction B (Control vs A), 24 proteins were found to have statistically different expression. Moreover 1 protein was found to be present only in the proteome of the cells exposed to LEGE06113 fraction B, however in one control gel replicate this protein was detected by the software. From this total, 20 were up-regulated in the presence of the LEGE06113 fraction, and 5 were down-regulated. Concerning the analysis of the proteome of control RKO cells and cells exposed to LEGE06155 fraction B (Control vs B) only 6 proteins were considered to have statistically different expression. Only 3 proteins were present only in the proteome of the cells challenged with LEGE06155 fraction B. All the 9 proteins were up-regulated in the presence of the LEGE06155 fraction. Finally, comparing the proteomes of the cells from group A and B, (A vs B), statistically different expression is found for 7 proteins, and 1 protein presented qualitative variation among the cyanobacterial strains. These 7 proteins were up-regulated in the presence of LEGE06113 fraction B and the other protein was only expressed in the presence of LEGE06155 fraction B.

The proteomic results reveal interesting biochemical events in RKO cells when exposed to cyanobacterial strains. The proteins are down- or up- regulated, and new proteins are expressed. Some of these events may provide a mechanistic explanation for the bioactivity of the cyanobacteria fraction and the loss of viability of RKO cells. However posterior analyses are required to identify these differently expressed proteins, for example using MALDI-

TOF/TOF mass spectrometry. The protein and gene expression data can be combined to provide a more complete view of the specific cellular mechanisms underlying the cytotoxic effects and subsequently to differentiate between the apoptosis and necrosis processes.

5. Conclusion

The marine environment is a rich source of natural compounds with potential pharmacological applications namely in what concerns to cancer treatment. The cytotoxic properties of several marine cyanobacteria compounds against cancer cell lines can be explored in order to develop new tools for the control of different types of cancer. Recent studies have led to the development of innovative drugs with novel mechanisms of action, using these bioactive natural compounds to fight cancer, acquiring along the way more and more information about carcinogenesis and the mechanisms that characterize each type of cancer affecting humans.

In this work we found that the cyanobacteria strain *Synechocystis salina* (LEGE06155) is potentially promising for the isolation of anticancer compounds. Both cyanobacterial strains included in this study showed different cytotoxic effects when exposed to three different cell lines (HepG2, RKO and T47D), mainly in the fraction B (ethyl acetate solvent). From the molecular and proteomic study performed with the RKO cell line we can conclude that different molecular mechanisms are involved in the bioactivity of LEGE06155 and LEGE06113, since the mRNA and protein expression was differently altered. The mRNA expression was altered in cell cycle and anti-apoptosis regulation, in the CCNB1 gene with LEGE06155 fraction B and BCL-2 gene with LEGE06113 fraction B, respectively. These results highlight the possibility of interaction between cell cycle regulation and apoptosis mechanisms. From the proteomic analysis, differential proteins were expressed in response to the different cyanobacterial strains used (25 proteins with LEGE06113 fraction and 9 proteins with LEGE06155 fraction). These results indicate differences between the mode of action of the cyanobacteria fraction B in the cells proteome.

The proteomics is a breaking area in cancer investigation since it allows identifying variations in protein expression as well as chemical modifications (posttranslational modifications). The combination of mRNA expression and proteomics is important since it will allow the identification of the pathways involved on the cytotoxicity induced by the cyanobacterial compounds. With this knowledge it will be possible to identify the main process leading to

cellular death. The knowledge of the main molecular targets of cyanobacterial compounds may also serve to guide the development of more effective therapeutics against cancer.

Far from being completed this work represents a contribution to unravel the mechanisms involved in the cytotoxicity induced by cyanobacteria on tumor cell lines. As future work we can suggest testing the cytotoxic potential of LEGE06155 in normal cell lines. The results will allow the confirmation if the cytotoxicity exerted is only present in tumor cell lines or if it has toxicity in normal and healthy tissues. For the complementation of the mRNA expression results, a flow cytometry analysis and evaluation of the mechanisms of cellular death (apoptosis, necrosis or autophagy), are needed to better characterize the molecular/cellular pathways. Finally, regarding the proteomic analysis, assessment of the function of the differential expressed proteins would be the next step to take, providing an additional insight into the molecular pathways involved in cellular death.

Although many active natural and synthetic compounds fail to be accepted as drugs due to the toxicity and unfavorable activities, many are useful as drug knowledge indicators or become important cell biology research tools. The broad utilization of such natural substances could be a factor in innovative healing and treatment of various diseases that severely affect humans, since not all bioactive compounds are necessarily considered toxic or toxins.

6. References

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7. Appendix

Appendix I – Z8 medium (Kotai, 1972)

Table 1 – Solutions A and B from the Z8 medium.

Solution A (10 ml/L)		**Solution B** (10 ml/L)	
Reagent	g/L	Reagent	g/L
$NaNO_3$	46.7	K_2HPO	3.1
$Ca(NO_3)_2.4H_2O$	5.9	Na_2CO_3	2.1
$MgSO_4.7H_2O$	2.5		

Table 210 – Fe-EDTA Solution from the Z8 medium.

Fe-EDTA Solution			
Reagent		10 ml/L	
$FeCl_3$*		10	
EDTA-Na**		9.5	
***Solution $FeCl_3$**		****Solution EDTA-Na**	
Reagent	100 ml	Reagent	100 ml
FeCl3.6H2O	2.8 g	EDTA	3.9 g
HCl (0.1 N)	100 ml	NaOH (0.1 N)	100 ml

Table 3 – Micronutrient solution from the Z8 medium.

Micronutrient Solution (1ml/L)			
Reagent		ml/L	
1 to 12		10	
13 to 14		100	
Reagent	g/L	Reagent	g/L
1 – $Na_2WO_4.2H_2O$	0.33	8 – $CuSO_4.5H_2O$	1.25
2 – $(NH_4)_6Mo_7O_{24}.2H_2O$	0.88	9 – $NiSO_4(NH_4)_2SO_4.6H_2O$	1.98
3 – KBr	1.2	10 – $Cr(NO_3)_3.9H_2O$	0.41
4 – KI	0.83	11 – V_2O_5	0.089
5 – $ZnSO_4.7H_2O$	2.87	12 – $Al_2(SO_4)_3K_2SO_4.24H_2O$	4.74
6 – $Cd(NO_3).4H_2O$	1.55	13 – H_3BO_3	3.1
7 – $Co(NO_3)_2.6H_2O$	1.46	14 – $MnSO_4.4H_2O$	2.23

Appendix II – MTT viability assays (3,3x10⁴ cells per well; 96 well plates; 4h exposition to MTT and 10% MTT).

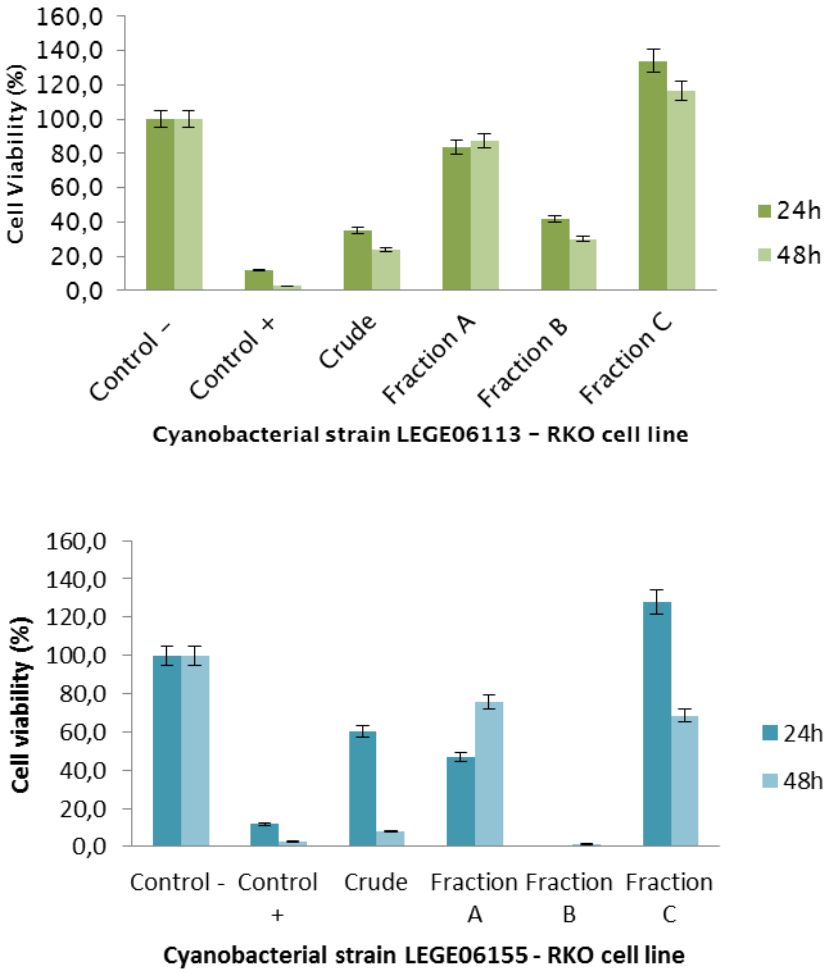

Figure 1 – Cell viability from Crude extract and three fractions (A, B and C) of the cyanobacterial strain LEGE06113 and LEGE06155 (respectively), in the tumor cell line RKO (colon carcinoma), with two exposure times, 24h and 48h at 3,3x10⁴ cells per well. Negative control corresponds to 1% DMSO and positive control to 20% DMSO.

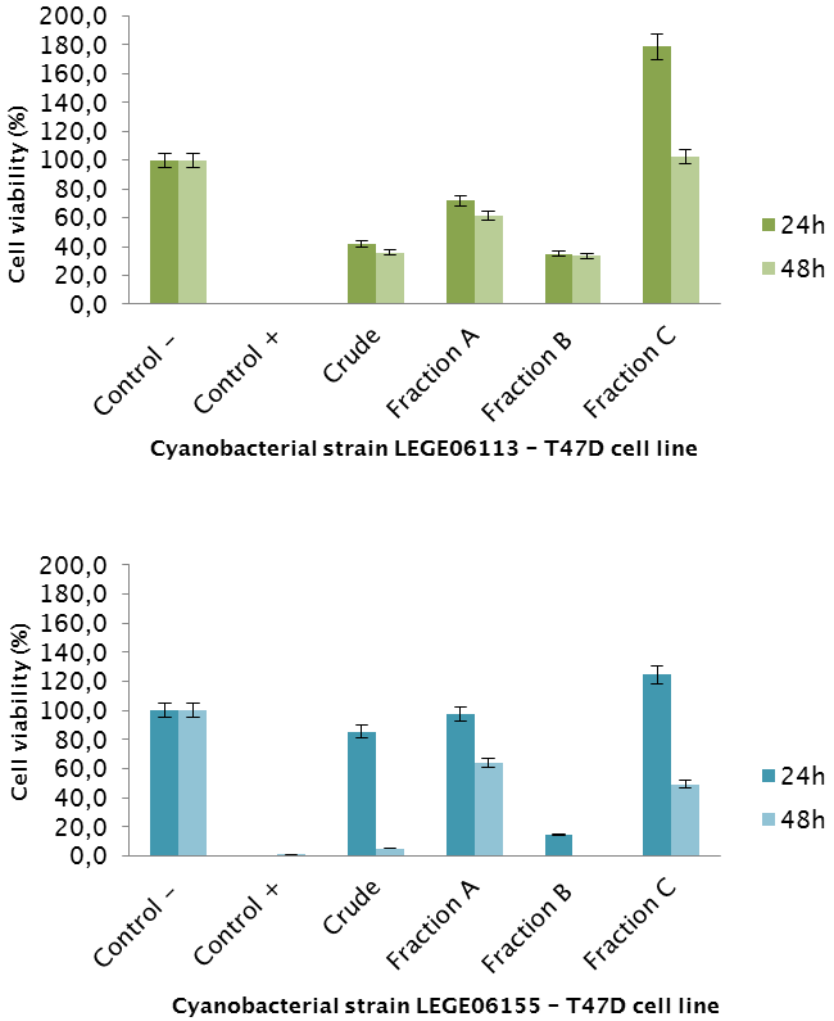

Figure 2 – Cell viability from Crude extract and three fractions (A, B and C) of the cyanobacterial strain LEGE06113 and LEGE06155 (respectively), in the tumor cell line T47D (breast carcinoma), with two exposure times, 24h and 48h at $3{,}3 \times 10^4$ cells per well. Negative control corresponds to 1% DMSO and positive control to 20% DMSO.

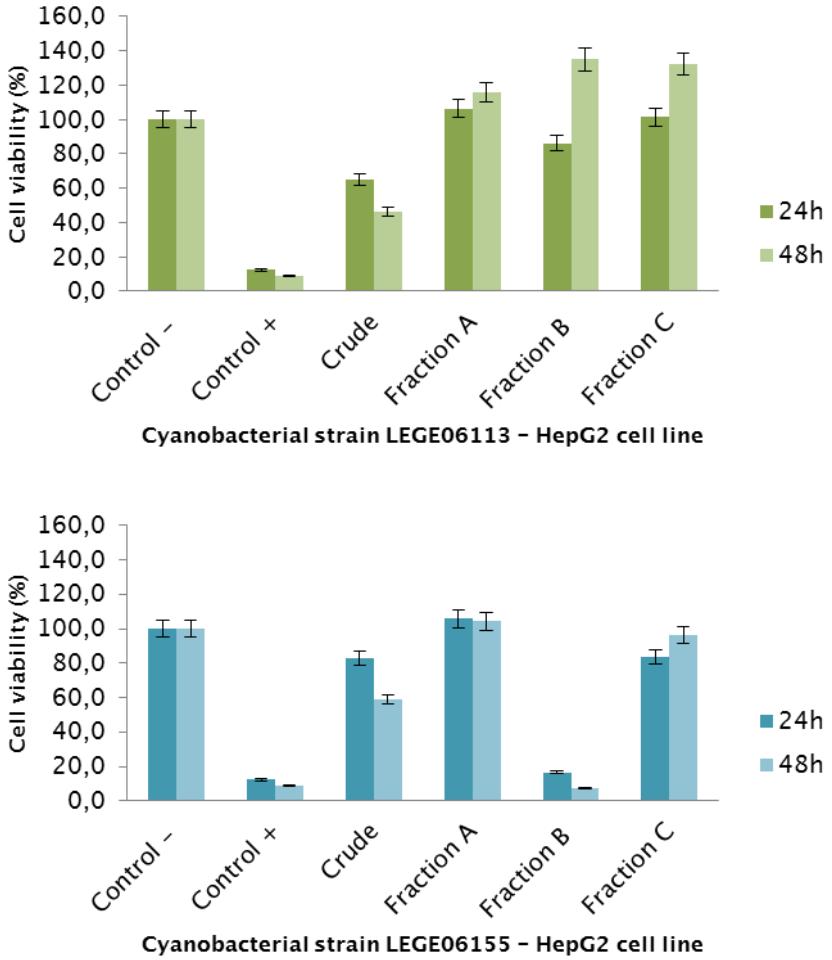

Figure 3 – Cell viability from Crude extract and three fractions (A, B and C) of the cyanobacterial strain LEGE06113 and LEGE06155 (respectively), in the tumor cell line HepG2 (liver carcinoma), with two exposure times, 24h and 48h at $3{,}3 \times 10^4$ cells per well. Negative control corresponds to 1% DMSO and positive control to 20% DMSO.

Appendix III – Differential protein expression.

Table 4 – Normalized intensity values of proteins (SSP numbers) differentially expressed between Control (Ctr 1–4) and group A (LEGE06113 fraction B) (A 1–4) with a significance level of $p \leq 0,05$.

Final SSP	Ctr(1)	Ctr(2)	Ctr(3)	Ctr(4)	A(1)	A(2)	A(3)	A(4)
1505	605,8	1516,4	1303,7	780,2	2548,5	2627,8	1716,9	2548,4
1507	1714,3	1199,2	942,0	557,4	2209,4	29031,0	1850,5	2319,2
1604	1975,2	1697,2	1613,4	1158,7	3695,3	5728,8	3242,5	4171,3
2102	586,5	612,0	506,1	380,2	806,5	851,6	756,2	1005,8
2106	1408,9	977,4	1264,4	587,1	1660,2	1634,7	1696,3	1854,0
2505	115,2	128,3	134,1	63,1	183,0	176,0	193,7	159,5
3203	754,0	729,4	860,0	304,5	1107,5	1088,4	1138,0	1004,0
3601	616,3	663,3	707,0	336,4	1264,1	1148,6	885,4	1342,9
3604	1472,2	1111,5	1268,0	722,5	2585,1	2708,2	1949,4	3054,3
3607	3776,9	1800,0	1370,6	751,2	4731,4	4039,2	5157,6	4778,9
4101	969,4	714,1	739,6	423,4	1015,3	1208,8	1242,6	1139,5
4131	494,6	298,1	470,4	111,1	569,8	656,1	634,8	588,1
4205	2680,5	1994,4	1414,2	974,8	669,8	901,5	938,6	533,9
4809	443,6	296,3	393,3	166,4	276,6	718,7	531,1	414,7
5104	90,6	123,2	163,5	6,7	546,2	522,6	553,4	511,8
5203	470,9	332,5	390,8	137,4	379,6	230,8	227,3	176,5
5506	960,1	955,7	780,8	206,7	584,1	1016,2	574,1	531,2
5813	176,7	86,7	108,0	67,4	161,4	271,6	294,8	161,2
6229					307,6	310,3	295,6	307,6
6606	699,6	1035,0	1097,3	599,7	328,5	270,7	275,3	274,6
7008	588,2	498,5	506,4	242,1	1301,4	1206,5	1418,2	1866,9
7105	10305,6	3830,8	7970,6	4282,3	987,7	885,6	655,5	914,8
7703	104,2	28,0	85,1	43,5	587,2	269,0	207,8	253,1
7704	187,3	83,8	75,3	42,0	238,5	132,4	106,4	140,7
8228	151,1	68,3	132,3	16,7	242,4	327,4	319,1	332,5

Table 5 – Normalized intensity values of proteins (SSP numbers) differentially expressed between Control (crt 1–4) and group B (LEGE06155 fraction B) (B 1–4) with a significance level of $p \leq 0,05$.

Final SSP	Ctr(1)	Ctr(2)	Ctr(3)	Ctr(4)	B(1)	B(2)	B(3)	B(4)
2214					152,9	194,6	249,2	192,6
2505	115,2	128,3	134,1	63,1	147,5	358,1	205,6	359,8
2604	386,3	79,9	156,3	81,3	537	471,8	578,9	959,8
3604	1472,2	1111,5	1268	722,5	3400,3	1978,3	1816,2	4352,9
3607	3776,9	1800	1370,6	751,2	4756,7	4500,5	4666,8	5707,7
5104	443,6	296,3	393,3	166,4	536,3	469,2	680,7	501,4
5602	960,1	955,7	780,8	206,7	1872,4	1669,4	2697,7	1049,3
6506					569,9	277,1	499,1	370,7
7603					294,5	349,2	246	202,6

Table 6 – Normalized intensity values of proteins (SSP numbers) differentially expressed between group A (LEGE06113) (A 1–4) and group B (LEGE06155) (B 1–4) with a significance level of $p \leq 0,05$.

Final SSP	A(1)	A(2)	A(3)	A(4)	B(1)	B(2)	B(3)	B(4)
1202	2559,5	2751,9	2597,6	2850,9	2035,6	1878,7	2511,7	1808,6
6506					569,9	277,1	499,1	370,7
6203	296,2	221,9	247,9	205,4	151,2	147	162,3	88,6
7204	791,8	711,4	777,5	813,1	357	480,6	692,8	263,3
7505	3504,5	3417,4	3672,7	3613,5	1655,9	3279,8	2858,8	2032
7606	21023,9	12123,8	6662,5	15419	5497,1	4546,9	1989,7	3379,5
8228	242,4	327,4	319,1	332,5	118	109,2	218,3	67,2
8503	1106,2	1047	1029,4	1017	655,3	205,6	539,8	444,4