Universidade do Minho
Escola de Ciências

Gabriela Alves Moreira

Discovery of new cyanobacterial natural products with bioactivity

Julho 2021

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Discovery of new cyanobacterial natural products with bioactivity

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Mestrado em Genética Molecular

Trabalho efetuado sob a orientação do
Doutora Sandra Figueiredo
e da
Professora Doutora Maria João Sousa

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Statement of Integrity

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

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Descoberta de novos produtos naturais de cianobactérias com bioatividade

Resumo

O cancro e a resistência antimicrobiana são dois dos principais problemas de saúde na sociedade atual, sendo por isso imprescindível a procura de novos tratamentos mais eficazes nestas áreas terapêuticas. Os produtos naturais têm sido um importante recurso para obtenção de compostos com atividade biológica, incluindo compostos com propriedades anticancerígenas e antimicrobianas. Neste contexto, as cianobactérias têm demonstrado uma elevada taxa de descoberta de novos produtos naturais, alguns dos quais com relevante atividade farmacológica.

Este trabalho foi realizado no CIIMAR, em Matosinhos, Portugal, especificamente no grupo de investigação CNP. Diferentes metodologias foram aplicadas para encontrar novos compostos produzidos por cianobactérias com atividade biológica. A atividade citotóxica e antimicrobiana foi avaliada por métodos de referência.

No primeiro capítulo uma nova estratégia para a descoberta de produtos naturais produzidos por cianobactérias, desenvolvida recentemente no CNP, foi usada em seis estirpes de cianobactérias. Adicionalmente, três novos compostos previamente descobertos no laboratório, os nocuolactilatos A, B e C, foram avaliados quanto à sua atividade citotóxica e antimicrobiana. Os nocuolactiliatos A e B demonstraram ter atividade citotóxica em 5 linhas celulares (valores de GR_{50} entre 1,73 e 5,90 μM) e adicionalmente o nocuolactilato A revelou ter atividade contra *Bacillus subtilis* (halo difuso com 8 mm).

No segundo capítulo uma amostra ambiental foi fracionada e as frações resultantes foram avaliadas quanto à sua atividade citotóxica e antimicrobiana. Posteriormente, as frações bioativas escolhidas foram novamente fracionadas e duas vias distintas foram seguidas de forma a isolar os compostos responsáveis pela atividade citotóxica e antimicrobiana. Vários fracionamentos se seguiram até que um composto quase puro foi obtido.

Palavras-chave: Bioatividade; Cianobactérias; Produtos Naturais.

Discovery of new cyanobacterial natural products with bioactivity

Abstract

Cancer and antimicrobial resistance are two of the main health problems in today's society, which is why it is essential to seek new and more effective treatments in these therapeutic areas. Natural products have been an important resource for obtaining compounds with biological activity, including compounds with anti-cancer and anti-microbial properties. In this context, cyanobacteria have shown a high rate of discovery of new natural products, some of which have relevant pharmacological activity.

This work was carried out at the Interdisciplinary Center for Marine and Environmental Research (CIIMAR), Matosinhos, Portugal, specifically in the research group Cyanobacterial Natural Products (CNP). Different methodologies were applied to find new compounds produced by cyanobacteria with biological activity. Cytotoxic and antimicrobial activity was evaluated by reference methods.

In the first chapter, a new strategy for the discovery of natural products produced by cyanobacteria, recently developed at CNP, was used in six strains of cyanobacteria. In addition, three new compounds previously discovered in the laboratory, nocuolactylates A, B and C, were evaluated for their cytotoxic and antimicrobial activity. Nocuolactylates A and B have been shown to have cytotoxic activity against 5 cell lines (Half Maximal Growth Rate Inhibition (GR_{50}) values between 1.73 and 5.90 μM) and in addition nocuolactylate A has been shown to have activity against Bacillus subtilis (diffuse halo with 8 mm).In the second chapter, an environmental sample was fractionated by Vacuum Liquid Chromatography (VLC) and the resulting fractions were evaluated for their cytotoxic and antimicrobial activity. Subsequently, the chosen bioactive fractions were fractionated by Flash Chromatography (FC) and two distinct pathways were followed in order to isolate the compounds responsible for cytotoxic and antimicrobial activity. Several High Pressure Liquid Chromatography (HPLC) fractionations followed until an almost pure compound was obtained.

Key words: Bioactivity; Cyanobacteria; Natural products.

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Abbreviations' list

ACN – Acetonitrile

AmpB - Amphotericin B

ATCC – American Type Culture Collection

BBE - Blue Biotechnology and Ecotoxicology

BGC - Biosynthetic Gene Cluster

BYT – Blue Young Talent

C_6:O-control - Non-labelled Hexanoic Acid

C_6:O-d_{11} - Deuterated Hexanoic -d11

CIIMAR – Interdisciplinary Center for Marine and Environmental Research

CNP – Cyanobacterial Natural Products

CO_2 – Carbon Dioxide

DCM – Dichloromethane

DMEM - Dulbecco's Modified Eagle Medium

DMSO - Dimethylsulphoxide

ERDF - European Regional Development Fund

EtOAc – Ethyl Acetate

EUCAST – European Committee on Antimicrobial Susceptibility Testing

FA – Fatty Acid

FBS - Fetal Bovine Serum

FC – Flash Chromatography

FCT - Science and Technology Foundation

GR_{50} – Half Maximal Growth Rate Inhibition

H_2O - Water

hCMEC/D3 – Human brain capillary endothelial cell line

HCT-116 – Human colorectal carcinoma cell line

HepG2 – Human hepatocellular carcinoma cell line

Hex – Hexane

HPLC - High Pressure Liquid Chromatography

HRESIMS/MS - High Resolution Electrospray Ionization Mass Spectrometry / Mass Spectrometry

HTS – High Throughput Screening

IC_{50} – Half Maximal Inhibitory Concentration

IPA – Isopropanol

L:D - Light : Dark

LC-HRESIMS – Liquid Chromatography – High Resolution Electrospray Ionization Mass Spectrometry

LEGE cc – Ecotoxicology, Genomics and Evolution Laboratory culture collection

MCF7 – Human breast adenocarcinoma cell line

MeOH – Methanol

MG-63 – Human osteosarcoma cell line

min – Minutes

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide

NIES – National Institute of Environmental Studies

NMR – Nuclear Magnetic Resonance

NP – Natural Product

NRPS – Non-ribosomal Peptide Synthetases

PCC – Pasteur Culture Collection

Pen/Strep – Penicillin and Streptomycin

PKS – Polyketide Synthases

RP – Reverse Phase

RP-SPE - Reverse Phase - Solid Phase Extraction

SPE – Solid Phase Extraction

TLC - Thin Layer Chromatography

Triplex – Triple Express

VLC – Vacuum Liquid Chromatography

WHO – World Health Organization

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1. Introduction

This introduction will focus on the problematics of cancer and antimicrobial resistance, the importance of natural products in finding solutions for these health issues and on cyanobacteria as a source of bioactive natural products. Two different methodologies used to discovery natural products in this dissertation and the biological assays performed will also be addressed briefly.

1.1. Cancer

Cancer is often described as a group of diseases with origin in any part of the organism when normal cells lose their ability to control proliferation, start to divide uncontrollably and avoid death. This process is designated as carcinogenesis and lead to the formation of tumors that can be benign, when the tumor cells do not spread to other organs or tissues, or malignant, when the cells invade the adjacent tissues and form metastases (National Cancer Institute, 2015; World Health Organization, 2020b).

For normal cells to evolve into tumor cells there are certain distinguishing capabilities – hallmarks – that are usually present and are important for the tumor growth and metastatic dissemination. Tumors comprise different types of cells that interact with each other which contributes to their complexity. In addition, the contributions of the surrounding environment and the tumor microenvironment are also important in the biology of tumors (Hanahan & Weinberg, 2011).

In 2000 Hanahan and Weinberg described the importance of six hallmarks of cancer and in 2011, with a growing knowledge about cancer these were updated into eight hallmarks and two enabling characteristics (Hanahan & Weinberg, 2011). The original six hallmarks are related to the cell's proliferation and cell death (sustaining proliferative signaling, evading growth suppressors, resisting cell death and enabling replicative immortality) and to the sustainability and metastasis (inducing angiogenesis and activating invasion and metastasis). The more recent hallmarks lay in the metabolism and immune system (reprograming energy metabolism and evading immune destruction) and the enabling characteristics are the genome instability and mutation and the tumor 4promoting inflammation (Hanahan & Weinberg, 2011).

Cancer is considered a genetic disease since it is caused by alterations in the genes that are responsible for controlling cell functionality. These genetic changes can emerge at a certain point of life as a result of errors that occur during normal cell division or they can be hereditary when

exists an increase in the risk to develop cancer within the family. Hereditary cancers represent approximately 10% of all cancers and the higher risk is due to heritable mutations in specific genes, such as mutations in BRCA1 and/or BRCA2 that increase the risk of developing breast and ovarian cancers (AlHarthi et al., 2020). Types of cancer are usually named for the organs or tissues where they start, for instance lung cancer is a cancer that originated in the lung; and for this reason, there are more than 100 types of cancers. There are other classifications, as the one that rely on the type of tissue affected, and differentiates from carcinoma (the most common, begin in epithelial cells), sarcoma (origin in the bone and soft tissues), leukemia (start in the blood-forming tissue), lymphoma and multiple myeloma (initiate on immune cells) and central nervous system (arise in brain or spinal cord) (National Cancer Institute, 2015).

Although this disease can affect everyone there are some aspects that can increase the risk of developing cancer. Age, poor dietary choices, sedentarism, obesity and the use of tobacco and alcohol are some of this risk factors (Gakidou et al., 2017). There are biological carcinogens as human papillomavirus that can also lead to the formation of tumors. Additionally, there are environmental chemical and physical carcinogens that can promote the formation of tumors (World Health Organization, 2020b).

1.1.1. Cancer statistics

In 2018, cancer was responsible for approximately 9.6 million deaths worldwide, which represents one sixth of all fatalities, and according to the World Health Organization (WHO) it was the second cause of death around the world (World Health Organization, 2020b). The number of new cases of cancer in 2018 was approximately 18 million, with lung and breast cancer being the most common, both representing 11.6% of cancer cases. Regarding mortality, lung (18.4%) and colorectum (9.2%) cancers were the most represented types. The incidence and mortality of cancer were bigger in the Asia continent and men have higher risk of developing cancer than women in about 20%. (International Agency for Research on Cancer & World Health Organization, 2020; World Health Organization, 2020b).

In Portugal, in the year of 2018 there were over fifty-eight thousand new cases, with the most recurrent type being colorectal cancer (17.6%) and almost twenty-nine thousand deaths related to cancer, with lung cancer as the most mortal (16.1%) (World Health Organization, 2020b).

With the increment in life expectancy the incidence of cancer tends to also rise, since age is one of the risk factors in the incidence of cancer. This happens given that cells lose the capability

of regenerating and eliminating abnormal cells and that is one of the reasons why it is expected that one in three persons will have at least one type of cancer in their lifetime. Even with the increment of the incidence, it is expected that the rate of survival will also increase due to the methods for diagnosis and therapeutics that are becoming more developed and advanced (World Health Organization, 2020b).

1.1.2. Cancer treatment strategies

As mentioned before there are several different types of cancer which hampers the finding of a universal cure. Currently, the most common options to treat cancer are surgery, radiotherapy and chemotherapy. However radiation and chemotherapeutic agents also produce some toxicity to normal cells which leads to major secundary effects and limitations on the dosages. Lately, hormone and immunotherapy have been combined with improved radiotherapy and chemotherapy methods, in order to increase the rate of success of the treatment and diminuished side effects (World Health Organization, 2020b).

Nevertheless, cancer cells have the hability to overcome some of these treatments and, for that reason, it is important to develop new treatment strategies with higher specificity, more efficient and less toxic. The increasing in knowledge about this group of diseases, and mainly about the hallmarks that contibutes to the formation, growth and metastasis of tumors has been important and new therapeutics are in development to target specific molecules involved in the potencialization of these hallmarks. (Hanahan & Weinberg, 2011).

1.2. Antimicrobial resistance

When microorganisms change the way they behave when expose to an antimicrobial drug, resulting in a decrease or complete elimination of the antimicrobial activity, it is called antimicrobial resistance (World Health Organization, 2020a). This process is an expected result of evolution and interactions between microbes and antimicrobial producers in nature, given that most of these type of drugs used in clinical are of natural origin or derived of natural products (Gil-Gil et al., 2019; Munita; & Arias, 2016). There are four main mechanisms of action for the gain of resistance: i) modification of the antimicrobial molecule – the cells inactivate or destroy the molecule; ii) decrease antimicrobial penetration or efflux – increase the difficulties of the antimicrobial agent to enter the cell or, in case it gets inside, it is send outside by efflux pumps; iii) changes in the target

sites – increase the protection to the target site or it is modified; iv) resistance due to global cell adaptive processes (Munita; & Arias, 2016).

The discovery of antimicrobials and their use in clinic was one of the most successful advances in modern medicine (Munita; & Arias, 2016) but the history in the antimicrobial field tends to repeat itself, having cycles of antimicrobial discovery and cycles of increasing antimicrobial resistance, and nowadays there is a lack of new antimicrobials (Gil-Gil et al., 2019; Waglechner & Wright, 2017).

Microbes with resistance to several antimicrobials are present in soil, waterbodies and other environments, and can infect plants, animals and humans (Gil-Gil et al., 2019). This is a global health concern, given that the ability to treat common diseases caused by microbial infections is lost, resulting on a slower process of recovery, in the best-case scenario, or in death, on the worst-case scenario. In fact, antimicrobial resistance is related to an increase in mortality and it is expected to cause around 300 million deaths by 2050 (Munita; & Arias, 2016; World Health Organization, 2020a).

To develop new antimicrobials, it is important to consider all the knowledge regarding antimicrobial resistance, such as the fact that when an antimicrobial has multiple target sites it is harder for the microbial strain to become resistant (Waglechner & Wright, 2017). Also, the gain of resistance to an antimicrobial can increase or diminished the susceptibility to other, depending on the target (Gil-Gil et al., 2019).

1.3. Natural products

Natural products (NPs) are chemical compounds produced by living organisms, including the metabolites from primary metabolism – conserved compounds directly related to the growth and development of the organism – and secondary metabolism – compounds that are not essential for the growth or reproduction of the organism, but confer a selective advantage. These compounds present a great diversity in respect to the size, composition, complexity and chemical properties, even within the same species, and for many centuries the base for pharmacological preparations has been NPs, especially the ones coming from eukaryotes (Harvey et al., 2015). In fact the discovery of several new NPs had a significantly impact on the history of biology and medicine (Shen, 2015). The biodiversity and capacity of adaption are higher in microorganisms then eukaryotes, and for that they are now considered the most promising producers of compounds with biological activity. In fact, it is believed that less than 1% of all this biodiversity has been studied

due to difficulties in cultivating these microorganisms in the laboratory (Harvey et al., 2015) which increases the importance of the study overlooked microorganisms just as the study of already known strains through different approaches.

Despite the tradition of NPs to play an important role in drug discovery and development, the screening of these type of products decreased in the nineties since many pharmaceuticals substituted their programs for natural compounds discovery for new programs using high-throughput screening (HTS) and combinatorial synthesis originating libraries with a big amount of compounds (Butler et al., 2014; Shen, 2015). These new libraries present a great amount of compounds but the overall chemical and structural diversity and complexity of NPs are higher than in the synthetic compounds library, and the abandon of NPs discovery can even be correlated with a decrease in the number of new drugs approved (Atanasov et al., 2021; Shen, 2015). Also NPs, due to their role in nature, have been "perfected" through natural evolution and interactions with the environment and other living organisms (Atanasov et al., 2021). Even with the decrease in the discovery rate, from 2008 to 2011, 5 new NPs and 20 natural product-derived drugs were approved for medical usage, and from 2000 to 2013, 25% of drugs approved were derived from NPs (Butler et al., 2014). The are several recent technological advances that are contributing to a new golden age in the discovery of NPs (Shen, 2015). For example, advances in microbial genomics and metagenomics – that revealed the biodiversity yet to be explored; in microbial cultivation techniques and synthetic biology – allowed to discover new compounds produced by previously uncultivated strains; in understanding the biosynthesis of NPs – enable the manipulation to produce these compounds or their derivatives; in bioinformatics – permit rapid discovery and isolation of new natural products and can help in the structural prediction.

Although NPs can derive from the primary or secondary metabolism, the secondary metabolites are more often in drug discovery due to their higher structural diversity and their biological functions in the original organisms that lead to more active compounds. These metabolites are often related to the species and can be produced as a chemical defense mechanism (Altmann, 2017). Secondary metabolites can be found in different ranges of concentrations which sometimes can be an obstacle to the isolation of those compounds, when the concentrations are low (Harvey et al., 2015).

The study of marine organisms was overlooked for a long time mainly on the account of difficulties in the collection and isolation of samples. However, the competition and diversity on this

environment resulted on the production of several unique molecules as secondary metabolites and for that the marine environment is considered an interesting source of bioactive natural compounds. Until 2017 more than twenty-nine thousand NPs coming from marine organisms have been identified with more than 40% discovered in the last decade and the major bioactivity described was cytotoxicity and anticancer activity (Carroll et al., 2019; Jiménez, 2018). As of October 2020, fourteen compounds related to marine NPs have already been approved for clinical use and twenty-three are in clinical test phases (Midwestern University, 2020). In fact, the total number of NPs with marine origin that were discovered and approved is higher than the one referring to synthetic compounds, which shows the relevance of this research field (Jiménez, 2018).

There are several strategies to discover new natural products, and they can be divided in two categories: top-down – methods that begin in the organism level where there is no prior knowledge of the gene responsible for the natural product; and bottom-up – approaches where the gene cluster (group of genes that code for the biosynthetic pathway responsible for the production of natural products) is first identified and then the compound is obtain through gene manipulation techniques (Luo et al., 2014).

The top-down strategies are traditionally the methods of choice to discover NPs given that they do not require many advanced techniques. Essentially a biological sample is collected (it can be from the environment or from a lab culture), the biomass is extracted, the bioactivity is checked and posteriorly the extract is fractionated until a compound is isolated and structurally characterized. New methods include diverse sampling and culturing conditions, since different culture conditions can lead to the production of different compounds by the microorganism, for instance microbial strains in stress will produce more secondary metabolites to protect them from those stress conditions, and HTS that allows large-scale acquisition and metabolomic analysis to process complex samples and possibly find new NPs that would potentially be overlooked due to low concentrations or masked by more active compounds (Luo et al., 2014).

Bottom-up genetic approaches can also be used to detect new NPs undetected in normal conditions. Some methods include the use of bioinformatics tools, with the biosynthetic gene clusters (BGCs) being identified and characterized; techniques of native host expression, where it is possible to make a connection between gene clusters and known products or discover new NPs; or the use of heterologous host expression, when the genetic manipulation is difficult in the native

species, a single gene or a BGC can be expressed in another organism to confirm the end product (Luo et al., 2014).

1.4. Cyanobacteria

Cyanobacteria, previously known as blue-green algae, are ancient prokaryotic microorganisms, specifically gram-negative bacteria that perform photosynthesis. These microorganisms are morphologically diverse, and can be found as unicellular, filamentous or forming colonies (Chlipala et al., 2011; R. K. Singh et al., 2011). Their cells are organized like other prokaryotes but have a system of internal membranes responsible for respiration and photosynthesis (R. K. Singh et al., 2011).

These microorganisms are ubiquitously spread on Earth and found in different kinds of environments, from soil, rocks, aquatic and marine environments, including some very extreme environments, which reveals their great capacity of adaptation. Although the majority is found as free living organisms, sometimes symbioses with plants or animals are discovered (Chlipala et al., 2011; Costa et al., 2014; R. K. Singh et al., 2011).

Cyanobacteria are known producers of different types of compounds and that may be due to their abundant occurrence and long evolutionary history. They are also known for the blooms they form in waterbodies around the globe, especially by *Microcystis spp.*, the most studied cyanobacteria. Many strains produce toxic compounds called cyanotoxins that can be hepatotoxic, nephrotoxic, neurotoxic or dermatoxic and microcystin is the most known cyanotoxin (Harke et al., 2016).

A single cyanobacterial strain is capable of producing several secondary metabolites with different chemical characteristics and biological functions (Costa et al., 2014; R. K. Singh et al., 2011), which makes them a good source for drug discovery. In fact, it is believed that almost every cyanobacterial strain produces some unique secondary metabolite (Nunnery et al., 2010). In addition, their rich phylogenetical diversity, the reduced cost of their cultivation in the laboratory (only require simple inorganic nutrients) (R. K. Singh et al., 2011) and the higher discovery rate of new compounds compared to traditional microbial sources (Uzair et al., 2012), increases the importance of these microorganisms in the research of bioactive natural products.

Until recently the study of these microorganisms and their secondary metabolites have been concentrated in Japan, the Pacific region and the Caribbean (Carroll et al., 2019). Since this is a promising phylum for the discovery of natural products, their study should be extended to other regions either tropical or not.

The advances in technology and study of bacterial genomes provided information regarding the potential of several well-known strains to produce more secondary metabolites than it was thought. Given this, the concept of genome mining emerges as refereeing to the prediction and isolation of NPs based on the genetic information. After observing the capability of these approach to find possible new compounds in strains already studied, this technique was applied to other microbes, such as cyanobacteria, contributing to increase the knowledge about these microorganisms (Ziemert et al., 2016). The genetic information on cyanobacteria reveals that the number of known NPs are much lower than what is still to be discovered (Dittmann et al., 2015). Normally, BGCs in cyanobacteria code for multidomain enzymes such as nonribossomal peptide synthetases (NRPSs) and polyketide synthases (PKSs), transporters and tailoring enzymes, and their expression is regulated by genes usually found flanking the BGC (Welker & Von Döhren, 2006).

As the information about the molecules produced by cyanobacteria increases, including the knowledge about their biosynthetic pathways, the impact of these metabolites in drug discovery also increases (Tan, 2013).

1.4.1. Bioactive compounds from cyanobacteria

The chemical diversity of cyanobacteria compounds may lead to differences in the targets and their mechanisms of action. In fact, some cyanobacterial secondary metabolites are already known due to their activity against fungi, protozoans, crustacean, mollusks, fish and mammals, acting on different target cells, such as kidney, lung, muscle and bone cells (Huang & Zimba, 2019).

A recent review on beneficial products from cyanobacteria showed that in 670 publications from 1970 to April 2019, 1630 unique molecules from these microorganisms were reported and clustered in 260 families of metabolites (Demay et al., 2019). The organization of these compounds into metabolite families and chemical classes does not guarantee the activity to be the same within each group. In fact, there is no relation between the chemical class and the type of activity, with metabolites from the same family sometimes presenting different activities (Demay et al., 2019). The majority of these compounds were isolated from marine cyanobacteria (53%), the most studied group within these microorganisms (Demay et al., 2019).

The bioactivities found include antibacterial, antifungal, cytotoxic, namely anti-cancerous, anti-inflammatory, immunosuppressive, lethality, neurotoxicity, dermaltoxicity, antiviral, antioxidant, antiprotozoal, anti-algal, anti-tuberculosis activities, and protease or enzyme inhibition. From those, cytotoxicity is the most frequent type of bioactivity detected, but also the most tested, which may

influence the results. Overall, the potential of cyanobacterial secondary metabolites is huge, and they can be used in several fields such as pharmacology, cosmetology, agriculture and food industry (Demay et al., 2019; R. Singh et al., 2017).

1.4.2. Anticancer compounds from cyanobacteria

The study of cyanobacteria as a source of new compounds with anticancer activity was started in the 1990´s at Richard Moore and William Gerwick´s laboratories (R. K. Singh et al., 2011). Regarding the anticancer activity of these compounds, it is also important to note that their rich chemical diversity has the potential to trigger different cytotoxic modes of action in cancer cell lines (Costa et al., 2014). The cellular targets may vary and include the cytoskeleton, enzymes, proteasome and histone deacetylases (Tan, 2013).

The best example of anticancer products derived from cyanobacteria is the dolastatin derivatives. Three synthetic analogues of dolastatin are already approved for the treatment of Hodgkin's lymphoma, urothelial cancer and tumors expressing the epidermal growth factor receptor, that has an important role in the pathogenesis of some carcinomas (Demay et al., 2019; Mittapalli et al., 2019; Morrison et al., 2016). In fact, one of the last anticancer drug approved related to a marine natural product is the antibody-drug conjugate brentuximab vedotin, or Adcetris, constituted by a tumor-specific antibody and a derivative of dolastatin 10 (Jiménez, 2018). Other families also showed to have cytotoxic compounds on *in vitro* assays, and some have reached clinic assays (Demay et al., 2019).

The results of one large-scale study of several strains from LEGE cc, a culture collection located at CIIMAR and a source of strains to this work, revealed cytotoxic activity in some strains. LEGE cc comprises more than 1000 microalgae and cyanobacteria strains from all continents but the majority (84%) is from Portugal, being an important resource for bioactivity studies (Ramos et al., 2018). The strains were previously screened for their cytotoxicity by bioassay-guided assays in human cancer cells, using only crude extracts and simple fractions. The crude extracts from each strain, as well as fractions obtained with hexane (fraction A), ethyl acetate (fraction B) and methanol (fraction C), were used against several cell lines and the cytotoxicity was evaluated through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Analyzing all the results, the crude extracts were the ones that showed higher bioactivity which can be explain given that the extracts harbors all the compounds produced by the cyanobacteria that posteriorly are divided in fractions A, B and C (Costa et al., 2014).

1.4.3. Antimicrobial compounds from cyanobacteria

Antimicrobial compounds are important not only for the treatment of microbial infections in humans, but also for the food industry and preservation (Demay et al., 2019).

There are over 100 compounds from cyanobacteria that revealed to have antimicrobial activity but few proceeded to clinical trials and until 2017 no cyanobacterial natural product was approved in this field (Swain et al., 2017). This antimicrobial activity comprises antibacterial, antialgal, antiviral, antifungal and antiprotozoal activity. Although some compounds present a wide range of activity, some appear to be specific to a certain type of microorganism or even specie (Demay et al., 2019). Natural products from marine cyanobacteria have been studied as lead compounds for the treatment of malaria (Linington et al., 2009), tuberculosis (Demay et al., 2019), fungal infections (Shishido et al., 2015), among others.

1.5. New strategy for NP discovery in cyanobacteria

A new method to find new cyanobacterial natural products was designed and validated in our laboratory and consists in the supplementation of cyanobacteria with isotopically labelled fatty acids (FA) (Figueiredo et al., 2021). FA derived moieties are commonly find in cyanobacterial NPs (Martins et al., 2019). Even if they do not present high activity on their own, they are sometimes incorporated in the biosynthesis of more complex cyanobacterial NPs (Tidgewell et al., 2010). Enzymes related to the incorporation of FA are quite abundant in cyanobacterial genomes and the majority of them are find in orphan BGCs (BGCs that are not related to the production of a known natural product), is a major opportunity for the discovery of new NP (Martins et al., 2019).

The metabolism of FA in cyanobacteria is quite particular since they seem to lack an operational beta-oxidation pathway which leaves the incorporation of FA into lipids as the main destination for imported FA. When FA is in excess in the organism they can also be secreted to the surrounding medium (von Berlepsch et al., 2012). Even with the absence of proteins of the beta-oxidation pathway, there has been some reports of catabolism of FA, suggesting there is an alternative pathway for lipid degradation in cyanobacteria that is not still characterized (Mills et al., 2020).

Given all this previous knowledge, it is believed that when cyanobacteria strains with the capacity of producing complex natural products with fatty acid incorporation are supplemented with labelled fatty acids, they will produce the original compound as well as the corresponding labelled one - isotopologues. The labelled compounds, which present an increment of mass

relatively to the non labelled compound, can then be easily detected by liquid chromatography – high resolution electrospray ionization mass spectrometry (LC-HRESIMS) analysis. The next step is dereplication, i.e., the search of the exact molecular masses of the non-labelled natural product in order to verify if the compound is already known, to avoid re-isolation. If the compound was not previously described it is considered new and its structure is elucidated and the biological activity is studied, in this case by cytotoxic and antimicrobial assays. This was the new methodology developed in the CNP laboratory, using deuterated FA (FA where the hydrogens were replaced by deuterium - ^{2}H, an heavier isotope of hydrogen) as the labeled FA (Figueiredo et al., 2021).

This approach, that is part of the compound guided approaches, has the advantage that the compound discovered is assured to be new and its chemical characteristics and novelty can be established by the preliminary data, although it has the disadvantage of not guaranteeing a promising bioactivity once the compound is isolated and tested (Welker et al., 2012).

Cyanobacteria from different culture collections were used for this method, including NIES (National Institute of Environmental Studies) culture collection, PCC (Pasteur Culture Collection) but mainly LEGE cc, given its accessibility.

1.6. Environmental biomass evaluation

One classic methodology to search for new compounds consists of having a biomass of known organisms or environmental samples and perform an organic extraction. The extract is then fractionated, and the fractions are tested for their bioactivity. If any fraction shows activity, a sub-fractionation is performed and the resulting fractions should be tested again, and if any shows some activity, the process should be continuing until the compound responsible for the activity is isolated. This strategy is part of the bioactivity-guided approaches.

An advantage of these strategy is the assumption that the pure compound will show bioactivity, while one of the disadvantages is the risk that after all the assays performed, the pure compound isolated is already known (Welker et al., 2012).

In this case, it was used an environmental sample collected in Cape Verde in April of 2018 more precisely in Baía das Gatas on São Vicente Island (Figure 1.1). The sample consisted in tufts growing in the bay and the sample was originally described as a cyanobacterial filamentous strain.

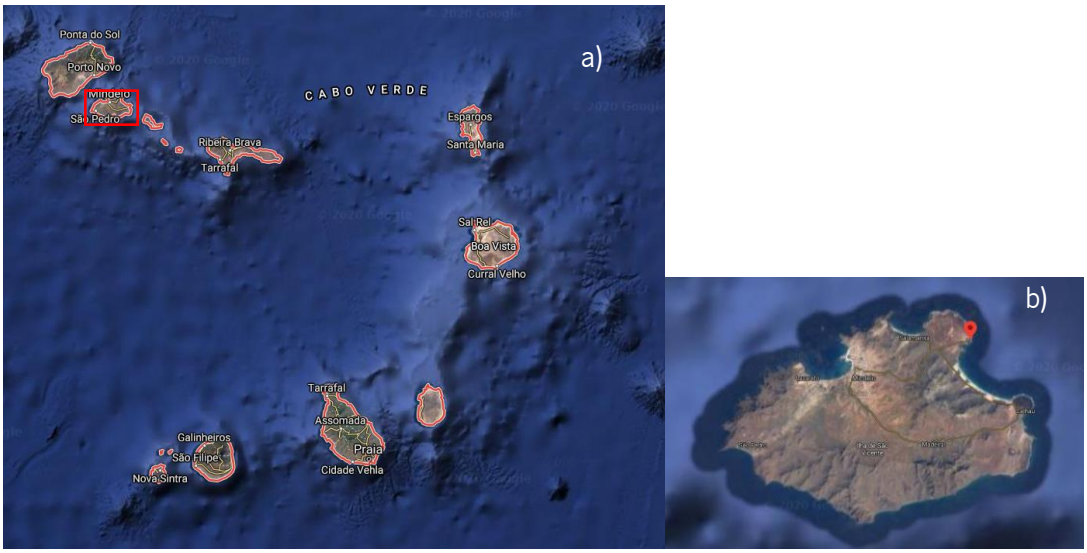

Figure 1.1. a) Cape Verde archipelago, with São Vicente Island marked b)São Vicente Island, with Baía das Gatas marked.

1.7. Biological assays

The choice of bioactivity assays in the evaluation process is crucial for the discovery of new bioactive natural products, since different compounds present different types of bioactivities, and promising compounds can be overlooked if not tested in their field of activity. In this dissertation, the compounds and fractions obtained were evaluated for their cytotoxic and antimicrobial activity.

1.7.1. Cytotoxic assays

Cell-based assays can be used to study physiological, pathophysiological and differentiation processes of specific cells and also to screen various compounds to determine their effect on the cells proliferation, in particular to establish therapeutic or toxic concentrations of compounds, drugs or extracts (T. L. Riss et al., 2004; Ulrich & Pour, 2001).

The measurement of the cell viability often relies on the incubation of the cells with a substrate that will be converted to another product by the viable cells and generate a signal proportional to the number of viable cells (T. L. Riss et al., 2004) and that is the basis of the assay that was used in this work, the MTT assay.

Two different metrics were used to evaluate the cytotoxicity of the compounds: the half-maximal inhibitory concentration (IC_{50}) and GR_{50}.

IC_{50} is currently the most common way to evaluate the efficacy of a drug, indicating the amount of a drug or compound needed to inhibit in half a biological process *in vitro*. It is widely used in

pharmacology and generally rely in whole cells systems, providing important information to evaluate potency therapeutics (Aykul & Martinez-hackert, 2016).

Hafner and co-workers developed a new method to determine the cellular response of a certain drug that takes in consideration the growth rate in the presence or absence of the drug, the GR_{50} (Hafner et al., 2016). It quantifies the response of the drug considering the number of divisions of the cells and so it ensures that cells with different times of growth but with similar biochemical responses to the drug obtain a similar score. In practice, GR_{50} takes in consideration the initial cell number (measured at the same time as the compounds are exposed, time 0) and compares it to the final number of cells of the untreated cells (control) and cells treated with different concentrations of the compound, both at 48 hours of exposure (Hafner et al., 2016).

1.7.1.1. MTT assay

This colorimetric assay is based on the reduction assay of the dye MTT, it was the first cell viability assay developed for 96-well plates compatible with HTS (Mosmann, 1983) and it is still largely implemented in research (T. L. Riss et al., 2004).

The MTT is positively charged which facilitates its entrance in viable eukaryotic cells that convert MTT (yellow dye) into a purple-colored formazan product through the mitochondrial enzymes. Metabolic inactive cells are not able to perform this reduction and for that the purple-color formation is a useful way to mark only the viable cells (Rani et al., 2018; T. L. Riss et al., 2004). The formazan accumulates as an insoluble product and after its solubilization with dimethylsulphoxide (DMSO), for instance, the absorbance is read at 550 nm, and the quantity of formazan is likely to be directly proportional to the amount of viable cells (T. L. Riss et al., 2004).

1.7.2. Antimicrobial assays

Antimicrobial susceptibility tests are commonly used in laboratory as a starting point in the identification of possible antimicrobial compounds. The disk diffusion assay is one of the oldest method and still is widely used in specialized research facilities around the world due to its high rate of reproducibility and accuracy (Matuschek et al., 2014). The microbial strain is cultivated, the disk is implemented in the plate and the compound or fraction is placed in the disk at 1 mg/mL. In the next day, the plate is visual analyzed to verify the presence of inhibition halos and if that is the case, the halos are measured.

2. Objectives

The main goal of this work was to discover new NPs produced by cyanobacteria that present bioactivity, namely cytotoxic and antimicrobial activity. Cancer and antimicrobial resistance are two of the biggest global health issues and because of that have been two of the main focuses of researchers worldwide. The discovery of new therapeutic compounds with higher efficacy rates for the treatment of cancer and to treat microbial infections is, for that, quite important.

To achieve the main goal, we will focus on two main strategies used to discover bioactive NPs. One focuses on a new molecule previously found by a comparative metabolomic screening that was posteriorly isolated, chemically characterized and finally tested for bioactivity – the compound-guided approach (Welker et al., 2012). This strategy was chosen given that a new method to discover new cyanobacterial natural products was design in CNP (Figueiredo et al., 2021) and these master's dissertation was part of that project. The second method is the bioactivity-guided approach, where the isolation is guided through the results of the bioactivity assays, until a single compound is obtained (Welker et al., 2012).

In both approaches, several chromatographic techniques were used, from VLC, FC to HPLC, among others, in order to fractionate the biomass and isolate the compounds. The evaluation of the bioactivity was performed with the use of the MTT assay to evaluate the cytotoxicity in different cell lines and the use of the disk diffusion assay to evaluate the antimicrobial activity in some bacteria and fungus.

There are some specific goals necessary to achieve the main goal, such as the maintenance of several strains of cyanobacteria in culture, which is essential to perform the supplementation assays and to harvest enough biomass to isolate the new compounds discovered, and the maintenance of several cell lines in culture and microbial strains, to perform the bioactivity assays. The bioactivity of the initial fractions and the pure compounds was evaluated through exposure to several cell lines (for the cytotoxicity assay), and microbes (for the antimicrobial assay).

3. Material and Methods

3.1. Culture of cyanobacteria strains

3.1.1. Maintenance of cultures

Cyanobacteria strains were maintained at 25°C ± 2°C with a photoperiod of L:D 14:10 (14 hours of light and 10 hours of dark), in Z8 medium as described in (Kotai, 1972). For marine strains, 25 g/L of tropical marine salt was added to the medium. The initial culture for each strain is about 20 mL and it can be scaled up to 80 L, in plastic sleeve bags, as Figure 3.1 shows.

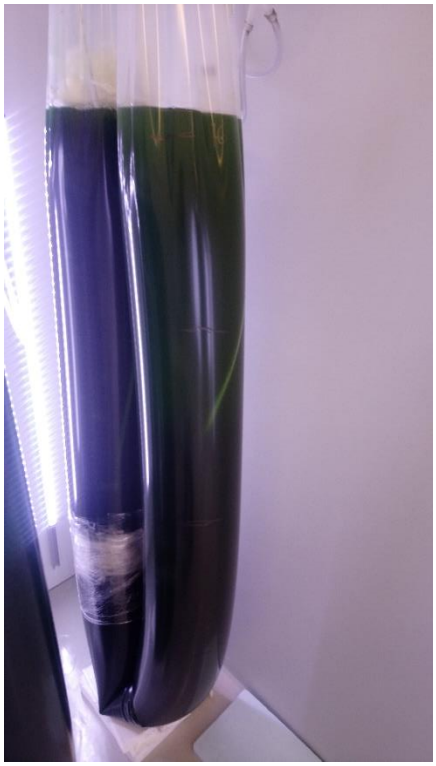

Figure 3.1. Sleeve bag (80 L) of Nodularia sp. LEGE 06071 culture.

The cyanobacteria strains in culture can display different behaviors according to the culture conditions and the strain itself. The material in which the culture is maintained (plastic or glass), the amount of light received, the volume of the culture or the aeration, which is recommended in volumes bigger than 500 mL, are all factors that can affect the way the culture behaves (Welker et al., 2012).

In total, for this dissertation work, six strains were subjected to these stable-isotope labeled fatty-acid incorporation experiments: *Nodularia sp.* LEGE 04288, *Scytonema hofmannii* PCC 7110, *Calothrix brevissima* NIES 22, *Microcystis aeruginosa* LEGE 91094, *Stanieria sp.* NIES 3757 and unidentified filamentous *Synechococcales* LEGE 06144. These six cyanobacterial strains were maintained in culture up to 200 mL to perform the supplementation assays, as explain next.

LEGE 04288 belongs to the *Nostocales* order and it was collected in Marco de Canaveses, Portugal in 2004 (Ramos et al., 2018). PCC 7110 was isolated in 1971, is a filamentous strain originated from Bermuda and it is available in PCC (Pasteur, 2020). NIES 22 is a filamentous strain that belongs to the NIES culture collection since 1941 when it was isolated from Palau Island (NIES, 2020). LEGE 91094 belongs to the order *Chroococcales* order and it was first collected in Lagoa de Mira, Portugal in 1991 and is responsible for the production of toxins (Ramos et al., 2018). NIES 3757 was first collected in 2013 in Japan (NIES, 2020). LEGE 06144 is part of the *Synechococcales* order, it was collected in Praia do Burgau, Portugal in 2006 (Ramos et al., 2018).

The strain *Nodularia sp.* LEGE 06071 was cultivated in different scales, from 20 mL until 80 L on a sleeve bag, to obtain enough biomass to isolate the compounds of interest, previously discovered through this methodology.

3.1.2. Harvesting of biomass

Different cyanobacteria strains have different morphologies which leads to different methods to separate the biomass from the culture medium. When the strain forms biofilms or aggregates is used a process of filtration with a nylon net (different pore sizes exist) where the biomass is collected from the net and the medium is discarded. For the strains that cannot be harvested by this method, the culture is centrifuged, and the biomass is collected as a pellet that results from the centrifugation, being the supernatant (medium) discarded. Afterwards, the collected biomass is lyophilizate and stored at -20 °C until the organic extraction.

3.2. New strategy for NP discovery in cyanobacteria

The six strains previously described were subjected to the new assay that consists in supplementing the cultures of selected cyanobacteria with a stable-isotope labelled fatty acid (in the experimental situation) and then search by LC-HRESIMS for compounds that have incorporated the labelling. After the identification of the referred compounds, it is time to verify if they are new or already known. In the first case we proceed to the isolation of the new compounds discovered by this methodology.

3.2.1. Supplementation assay

On day 01 of the experiment, a cyanobacterial culture of 200 mL of a strain is prepared with a small inoculum and this culture is let to grow for two days in the general conditions previously

described of light and temperature, with agitation (200 rpm). At day 04 the culture is split into 6 erlenmeyers' with 25 mL of culture each for two conditions (control and experimental conditions), with triplicates for each condition. In days 04, 06 and 08 the cultures are supplemented by pulse-feeding with deuterated hexanoic-d_{11} acid (C6:0-d_{11}) (CDN Isotopes Inc) in the experimental condition and with non-labelled hexanoic acid (C6:0-control) in the control condition to a final concentration of 0.1 mM from a 1000x concentrated solution of each acid in DMSO. This way it is possible to detect the compounds that are incorporating the labelled substrate, by comparing the data from the labelled and control conditions by LC-HRESIMS, using specific software, such as MzMine2 (Pluskal et al., 2010).

3.2.2. Chlorophyll *a* assay

This assay is used to quantify the chlorophyll *a* in the strains subjected to the feeding assays and serves as an indirect measure of the amount of culture. This way, if the assay needs to be repeated, the amount of culture should be approximately the same, and can be controlled by the amount of chlorophyll *a*.

At day 04, 3 mL of culture is collected to measure the concentration of chlorophyll *a* (1 mL/eppendorf for each triplicate). After two centrifugations (3 minutes, 17000 g) and a washing step the pellet is collected, and the chlorophyll *a* is extracted with 90% methanol in water, overnight at 300 rpm.

In day 05, another centrifugation (3 minutes, 17000 g) is done, and the supernatant is collected to measure the absorbance at 663 nm. The mean of the triplicates is calculated and the chlorophyll *a* concentration value is obtained by applying the formula in Equation 3.1.

Equation 3.1.Formula to calculate chlorophyll a concentration.

$$\textbf{Chlorophyll } \boldsymbol{a} \textbf{ = Abs 663 nm * 12,7 (µg/mL)}$$

3.2.3. Harvesting biomass and organic extraction

At day 11 the biomass from each erlenmeyer from the supplementation experiment is collected, and an organic extraction is performed. The protocol for harvesting the biomass is the same as previously described for standard culturing of cyanobacteria and can vary from strain to strain.

The organic extraction consists in the extraction of the organic compounds produced by the microorganisms using organic solvents.

Afterwards, the biomass is incubated with approximately 40 mL of dichloromethane (DCM)/methanol (MeOH) (2:1) for at least 30 minutes (min) with continuous gentle agitation. Next, the organic extract is filtered, the solvents are removed in the rotavapor, and the extracts are stored at -20 °C in glass vials.

3.2.4. LC-HRESIMS analysis

The LC-HRESIMS analysis was performed on an UltiMate 3000 HPLC (Thermo Fisher Scientific), which consists of the following components: LPG-3400RS pump, WPS-3000RS autosampler, TCC-3x00RS column compartment, and MWD-3000RS UV/visible detector coupled to a Q Exactive Focus Hybrid Quadrupole Orbitrap Mass Spectrometer (Thermo Fisher Scientific), controlled by Q Exactive Focus (Exactive Series) 2.9 and Thermo Scientific Xcalibur 4.1.31.9 software. The crude extracts were prepared in 2 mg/mL concentration and analyzed in full scan positive and negative modes in a C18 column, ACE® UltraCoreTM SuperC18.

The gradient program (Figure 3.2) used to separate the compounds from the organic extract was achieved with a flow rate of at 0.4 mL/min over a gradient of 90 % solvent A (water (H_2O):MeOH 1:1 v/v with 0.1% formic acid) to reach 70 % solvent B (isopropanol (IPA) with 0.1% formic acid) during 5 min, followed by isocratic 70% solvent B for 12 min, and a gradient of 30 % solvent A to reach 80 % solvent B during 2 min, followed by isocratic 85% solvent B for 9 min and return to the initial conditions followed by isocratic 90 % solvent A for 2 min.

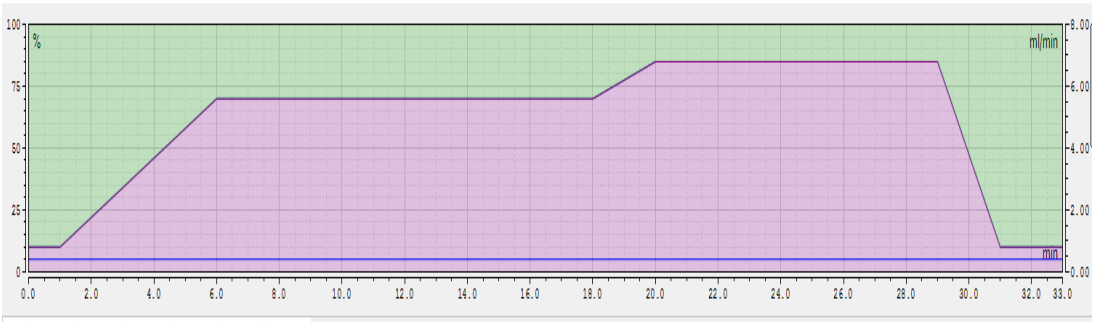

Figure 3.2. Flow gradient of solvent A (green) and solvent B (purple) during the LC-HRESIMS analysis.

3.3. Isolation of nocuolactylates A, B and C

3.3.1. Organic Extraction

The extraction was conducted according to the protocol of BBE laboratory at CIIMAR (LEGE, 2004). The technique is similar to what was previous described in the supplementation assays, but in a bigger scale.

The dry biomass is submerged in a solution of organic solvents, in this case DCM/MeOH 2:1, and stirring until the entire biomass was in contact with the solution. This step should last at least 10 minutes, and the organic extract was filtered by vacuum through a paper filter, cloth and cotton into a round flask, as represented in Figure 3.3. These steps should be repeated until the solvents become colorless. Ultra-sound bath can be used to increase the efficacy of the extraction.

In the case of the extraction of *Nodularia sp.* LEGE 06071, approximately 6 L of the mixture DCM/MeOH 2:1 was used in the process that lasted 3 workdays.

Figure 3.3. Extraction apparatus for organic extraction of Nodularia sp. LEGE 06071.

The organic solvents were then evaporated in the rotavapor and the resulting crude extract was transferred to a glass vial previously weighted, so the amount of crude extract could be measure and the yield of the extraction calculated.

The isolation of the nocuolactylates was guided by LC-HRESIMS, with the same conditions as mentioned before. This way, after each chromatography, the fractions were analyzed by LC-HRESIMS to search for the compounds and decide the fractions to proceed the isolation.

3.3.2. Vacuum Liquid Chromatography (VLC)

The crude extract was prepared for the VLC by dissolving it in DCM, adding the same amount of silica gel 60 (0.015-0.040 mm, Merck) as the extract and dried in the rotavapor until a loose powder was obtained for the dry loading. The VLC apparatus, represented in Figure 3.4, was filled from bottom to top with sea sand (Merck), approximately 2 cm, silica gel 60 (0.015-0.040 mm,

Merck), approximately 35 cm, the dry loading of the sample, more sea sand, approximately 2 cm, and cotton to protect the sample of the addition of the eluents. The column was then connected to a vacuum pump.

The eluent gradient is performed from the least polar to the most polar in order to separate the crude extract into fractions. In this case, since the goal was to obtain three specific compounds, the gradient used was to ensure the best separation, and it started with 10% ethyl acetate (EtOAc) in hexane (Hex) and ended with 100% MeOH. The resulting fractions, collected by volume (around 250 mL each, except the last ones) were then collected in round flasks, dried and transferred to previously weighted vials in order to calculate the mass of each fraction.

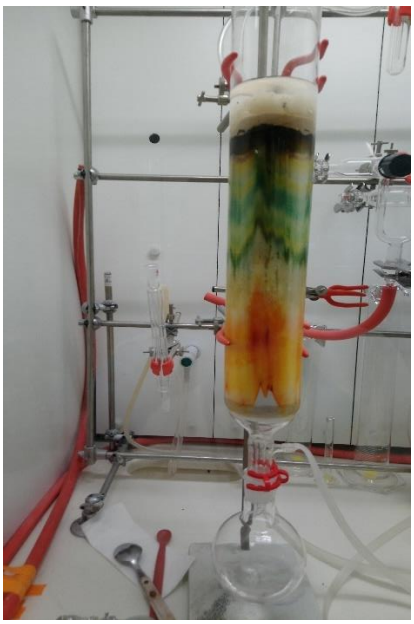

Figure 3.4. VLC apparatus from the VLC of crude extract of Nodularia sp. LEGE 06071.

3.3.3. Flash Chromatography (FC)

The preparation of the sample and the general procedure occurred in the same way as described in 3.3.2. Vacuum Liquid Chromatography (VLC), with the main difference being the size of the FC apparatus, represented in Figure 3.5, and that instead of creating vacuum at the bottom of the column, for the FC it was applied pressure on top to force the mobile phase to pass through the stationary phase.

Once again, the solvent gradient goes from the least polar to the most polar, starting in hexane, going to EtOAc and then to MeOH. The collected fractions were pooled based on Thin Layer Chromatography (TLC) analysis, a form of separation of compounds or complex mixtures where the samples are placed in the stationary phase (aluminum silica gel F254 TLS plates from Merck)

and then the stationary phase is submersed in the eluent (different mixtures can be used), causing the samples to migrate according to their polarity in the stationary phase, leaving different profiles.

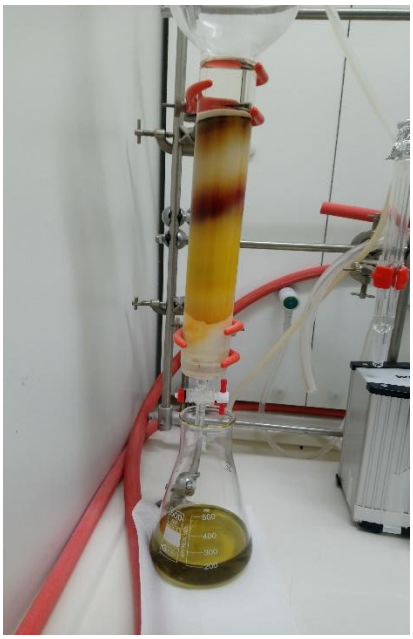

Figure 3.5. FC apparatus of FC of VLC fractions (GH) from Nodularia sp. LEGE 06071.

3.3.4. Solid Phase Extraction (SPE)

The fractions of interest resulted from FC were pooled together and fractionated by RP-SPE (reverse phase-SPE) (Strata ® C18-E, 55 μm, 70 Ă, 70g / 60 mL, Phenomenex). It was used a gradient with decreasing polarity from MeOH:H_2O 1:1 to IPA to DCM for the elution.

3.3.5. High Pressure Liquid Chromatography (HPLC)

To obtain the pure compounds, SPE fractions were further fractionated by RP analytical HPLC (ACE 5 C18, 250 × 4.6 mm, 5 μm, 100 Å, ACE), using MeOH:H_2O 1:1 (v/v) (eluent A) and IPA (eluent B). The sample was prepared in the minimum amount of solvent (MeOH) necessary to completely dissolve it.

The gradient program, in Figure 3.6, started with 10% of eluent B (green), held for 1 min and increased linearly to 60% of eluent B over 2 min, ant it was held for 13 min, followed by a linear to reach 85% of eluent B over 1 min, and held at for 5 min, before returning to initial conditions for 3 min.

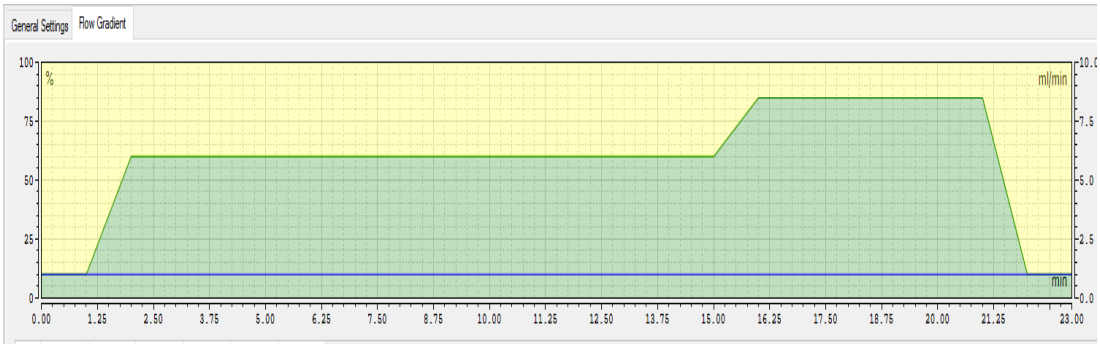

Figure 3.6. Conditions of the HPLC run for the isolations of the nocuolactylates.

3.4. Environmental biomass evaluation

3.4.1. Organic extraction

The organic extraction was once again performed according to the BBE protocol, adapted from (Edwards et al., 2004) as described previously. This Cape Verde biomass, named CV1, was extracted for 10 days, first with approximately 6 L of MeOH (first three days) and after with approximately 10 L of DCM/MeOH 2:1 for 7 days.

3.4.2. Fractionation

In this case, the fractionation was guided according to the biological assays (cytotoxic and antimicrobial) results, except for the VLC which is the first step of fractionation.

The VLC and FC followed the same method as previously described in the isolation of the nocuolactylates.

Several consecutive HPLC separations were conducted next, using as a mobile phase H_2O (eluent A) and acetonitrile (ACN) (eluent B) in the LC-4000 series HPLC (Jasco), coupled with a photometric diode array detector. The samples were prepared in the minimum amount of solvent (MeOH) necessary to completely dissolve them. The different columns and gradient programs are described below.

3.4.2.1. HPLC 01

FC fractions (GH09-11) were further fractionated by an analytical column RP analytical HPLC (ACE 5 C18, 250 × 4.6 mm, 5 µm, 100 Å, ACE).

The gradient program (Figure 3.7) started with 5% of ACN, held for 5 min and increased linearly to 99% of ACN over 35 min and it was held for 30 min. Next a decrease of the percentage of ACN

was conducted to return to the initial conditions, for 3 min, and held for 2 min at this initial conditions (5% ACN + 95% H_2O).

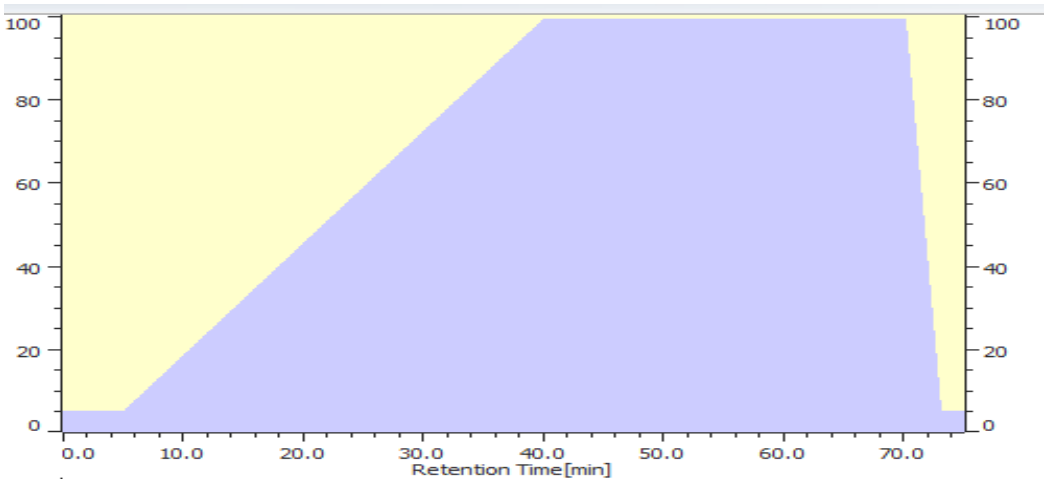

Figure 3.7. Conditions of the HPLC 01 run.

3.4.2.2. HPLC 02

FC fractions (GH03-06) were further fractionated using a semi-preparative column RP analytical HPLC (ACE 5 C18, 250 × 10 mm, 10 µm, ACE).

As Figure 3.8 shows, the initial conditions were 45% H_2O plus 55% ACN and the gradient increased to 90% of ACN, over 20 min and continue to increase linearly to 98% of ACN over 5 min and these conditions were held for 35 min. Next ACN percentage was linearly decreased to return to the initial conditions (55% ACN + 45% H_2O), for 3 min an held for 7 min at these conditions.

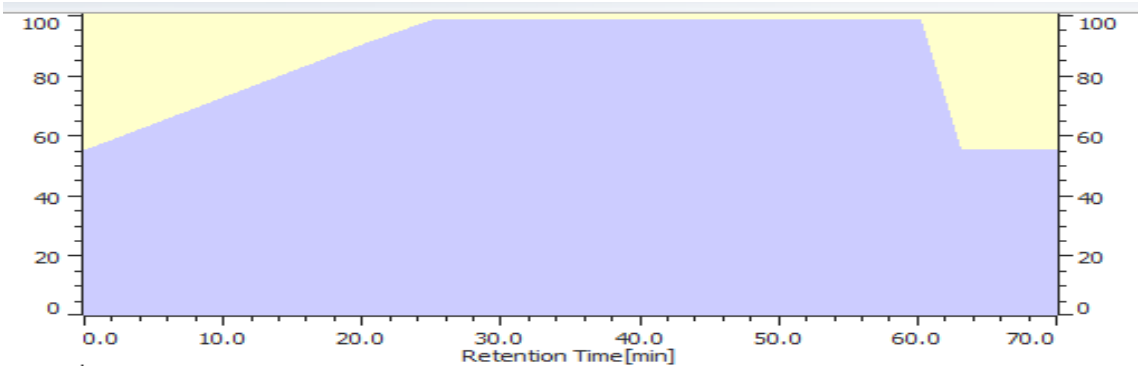

Figure 3.8. Conditions of the HPLC 02 run.

3.4.2.3. HPLC 03

HPLC 02 fractions were further fractionated using an analytical column RP analytical HPLC (Surf C18, 250 × 4.6 mm, 5 µm, 100 Å, imChem).

As represented in Figure 3.9 the initial conditions were 50% H_2O and 50% ACN and the HPLC run started with this mixture and it was held for 10 min followed by an increase linearly to 90% of ACN over 5 min and these conditions were held for 10 min followed by a decreasing of the ACN percentage to return to the initial conditions that were held for 7 min.

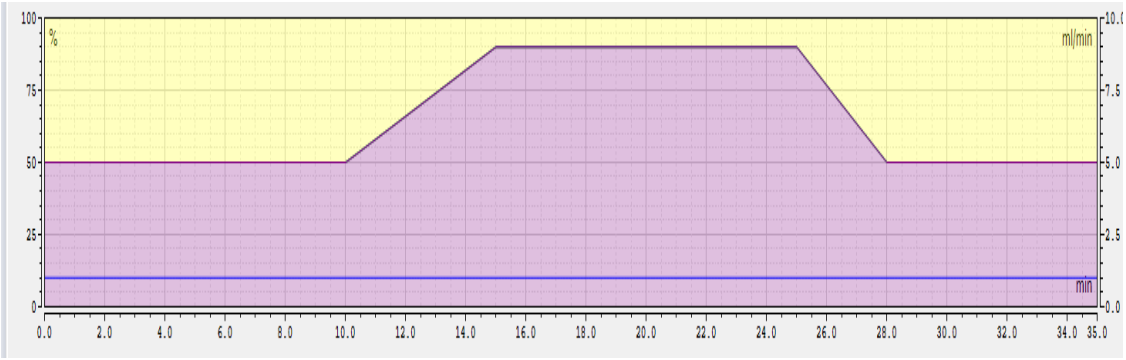

Figure 3.9. Conditions of the HPLC 03 run.

3.4.3. Nuclear Magnetic Resonance (NMR)

The samples were prepared in deuterated solvents (Cambridge Isotope Laboratories) and analyzed in NMR 400 or 600 MHz, depending on the complexity of the samples. The analysis was performed in the Materials Center of the University of Porto (CEMUP) on BRUKER AVANCE III 400 MHz, 9.4 Tesla or BRUKER AVANCE III HD 600 MHz, 14.1 Tesla, in liquid samples on appropriated tubes (diameter of 5 mm) compatible with the frequency of the equipment. The data was analyzed in MestReNova 12.0.3 (MestrelabResearch).

3.5. Screening of biological activity

3.5.1. Maintenance of cell lines

In order to evaluate the anticancer activity of the fractions and pure compounds, several tumor and non-tumor cell lines should be maintained. The cell lines used are cultured in 75 cm^2 culture flasks in complete medium that can be Dulbecco's Modified Eagle Medium (DMEM) or McCoy's 5A modified medium, depending on the cell line. The complete medium is prepared by adding to the medium 10% (v/v) of Fetal Bovine Serum (FBS) (from Biochrom) which provides all the nutrients that the cells need to growth, 1% (v/v) of a solution with Penicillin and Streptomycin (Pen/Strep) (from Biochrom) that prevents contaminations from bacteria and 0.1% (v/v) of amphotericin B (AmpB), a fungicide to avoid fungal contaminations. The cells are maintained in an incubator at 37 °C with 5% carbon dioxide (CO_2).

The cells maintenance implies their passage at least once a week, where the cells are removed from the medium, with Triple Express (Triplex), from Gibco. Triplex is a group of recombinant cell-dissociation enzymes, used to detach the cells from the flask. The next step is adding medium and centrifugate for 5 min at 1500 rpm and part of the cells are cultured again in new medium, to renew the source of nutrients and diminish the confluence in the flask.

Four different cell lines were used, four tumoral – human osteosarcoma cell line (MG-63), human breast adenocarcinoma cell line (MCF7), human colorectal carcinoma cell line (HCT116) and human hepatocellular carcinoma cell line (HepG2), obtained from American Type Culture Collection (ATCC) – and one non-tumor cell line – human brain capillary endothelial cell line (hCMEC/D3), kindly donated by Dr. P. O. Courad (INSERM, France). The cell lines MG-63, MCF7, HepG2 and hCMEC/D3 were maintained in DMEM (from Gibco) while the cell line HCT-116 was maintained in McCoy's 5A (from Roth).

3.5.2. Cell density assay

The different cell lines are plated in 96-well plates (1 cell line per plate) in different cell densities to determine the number of cells to plate in the exposure assay. Cells have an exponential growth, characterized by a growth curve as demonstrated in Figure 3.10. In order to guarantee reproducibility, the number of cells plated in each assay should be in the middle of the linear part of the curve (marked with the red box in Figure 3.10), to assure that the cells are actually growing in the same scale in the different assays.

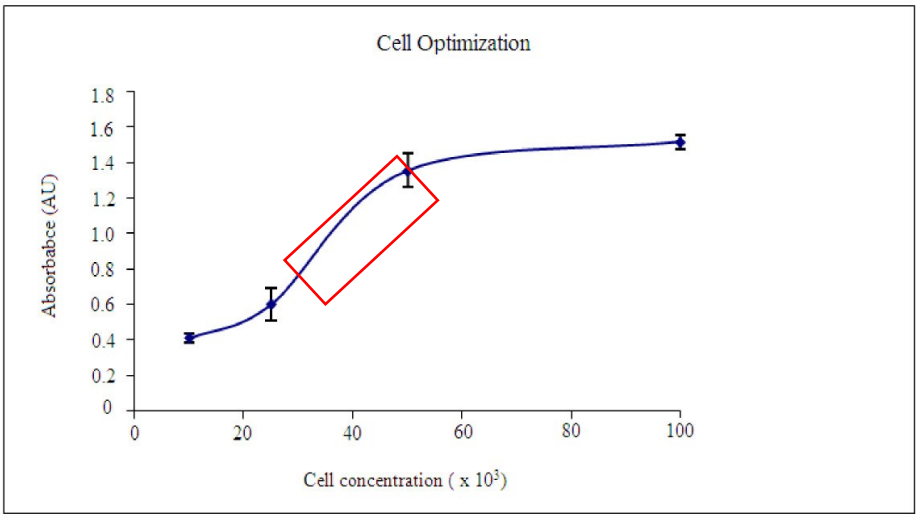

Figure 3.10. Graphic representation of a cell growth curve. Adapted from (Osibote et al., 2011).

3.5.3. Cytotoxic assay

The cell lines MCF7, MG-63, HepG2 and HCT116 were seeded at 9 x 10^4 cells per mL while hCMEC/D3 was seeded at 5 x 10^4 cells per mL in 96 wells plates, at 100 µL per well. These cell concentrations were previously calculated by the cell density assays. To count the cells, in order to plate them in the correct concentrations, 10 µL of a 1:10 dilution of the cells and 10 µL of trypan blue were mixed, with 10 µL of this mixture being counted in an automated cell counter, *Countess* (from Invitrogen).

Trypan blue is a dye that only penetrates dead cells with damages on the plasma membrane and for that it can be used to verify the cells viability. The use of the automated cell counter is well-known in the step of seeding microwell plates (T. Riss et al., 2019).

The cells were plated and 24 hours later the medium was removed and new medium with the fractions or pure compounds in different concentrations (tested in triplicates) was added to the wells. The solvent used to dissolve the compounds, DMSO, was also tested in the maximal concentration (0.5%) and it works as the negative control. After 48 hours of exposure, the cytotoxicity was evaluated by the MTT assay.

To confirm the results for the nocuolactylates, a positive control of anticancer activity, Paclitaxel, was used to compare the results. This drug is a NP obtained from a tree (*Taxus spp.*) and belongs to the group of compounds called taxanes that are mitosis inhibitors. This therapeutic is currently used in the treatment of breast, ovarian, non-small cell lung cancer and Kaposi's sarcoma (Lichota & Gwozdzinski, 2018). Additionally Nocuolin A was also tested in the cytotoxic assays since it is a nocuolactylate-related compound produced by *Nodularia sp.* LEGE 06071 with cytotoxic activity already described (Sousa et al., 2019). The compounds were evaluated in different concentrations.

The evaluation of cytotoxicity of the fractions from the environmental biomass was assessed in a different way, testing only one concentration of each fraction (10 µg/mL). With these assays, it was verified if the cells viability was affected by the fractions, through the formula represented in Equation 3.2.

Equation 3.2. Percentage of cells viability through MTT assay.

$$\% \text{ viability} = (\text{Abs Fraction} / \text{Abs DMSO 0.5\%}) * 100$$

For each cell line two independent assays were done (in different weeks) and three replicates in each assay. A fraction was considered active if it had a cell viability reduction of more than 20%, in at least one assay, when compared with the negative control (DMSO at 0.5%).

3.5.3.1. MTT assay

The MTT dye (20 µL at 1 mg/mL) was added to the medium to each well and the plate was left in the incubator for two and half hours. The conversion of MTT to formazan by cells in culture is time dependent, so it is important to incubate the cells on the different assays for the same time (T. L. Riss et al., 2004).

Then the solution was removed, and 100 µL of DMSO (from VWR) was used to dissolve the formazan crystals formed by the reduction of MTT by the mitochondrial enzymes and allow the absorbance measure at 550 nm on the microplate reader *Synergy HT* (from BioTek).

Subsequently, IC_{50} and GR_{50} values for the nocuolactylates, nocuolin A and paclitaxel were determined using the online GR Calculator (http://www.grcalculator.org/) (Hafner et al., 2016), as described by Hafner and co-workers. To calculate de GR_{50} it was necessary to use the MTT assay in non-treated cells at time 0 and at the end of exposure.

3.5.4. Antimicrobial assay

The antimicrobial activity was evaluated through the antimicrobial susceptibility disks assay, which followed the recommendations of European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Matuschek et al., 2014) in two gram-negative bacteria - *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 25241 - two positive-bacteria - *Staphylococcus aureus* ATCC 29213, *Bacillus subtilis* ATCC 6633 - and a fungi - *Candida albicans* ATCC 10231.

The five microbial strains were plated from glycerol stocks in agar plates and left in the incubator overnight at 37 °C and then storage in the fridge at 4 °C. On the day before the assay, a new plate of each strain was done, by pricking some culture from the original plates, and left to grow for 24 hours in the incubator at 37 °C. On the assay day, a small loop of each microbe in agar plates was transferred to 2 mL of liquid medium (Mueller-Hinton, from Alliance Bio Expertise, for bacteria and Sabourad Dextrose, from Liofilchem, for fungi) and the absorbance was read at 600 nm in a bench spectrophotometer, *Ultrospec 10*, from Biochrom, and adjusted to 0.1 for bacteria and 0.5 for *Candida albicans* through the addition of medium or inoculum.

The inoculation was done transferring 100 µL to new agar plates (medium according to the bacteria/fungi), to guarantee reproducibility, and with a swab, the inoculum was spread through the entire plate. It is very important to guarantee the fulfilment of the plate to easily detect and measure the possible inhibition halos. Next, the blank disks (6 mm), from Oxoid, are placed in the plates and 15 µL of each compound and controls were applied to the respective disk, in a 1 mg/mL concentration. The negative control was DMSO, from Fisher, since it was the solvent used to dissolve the compounds, and it was applied in a 100% concentration, while the positive control was Kanamycin (1 mg/mL) for bacteria and Nystatin (1 mg/mL) for fungus.

After applying the compounds or fractions in the disks, the plates were allowed to dry for at least 30 minutes. Next, the plates were stored in the incubator at 37 °C, upside down, overnight. On the next day, the diameter of the inhibition halos was measured.

The halos produced by the compounds or fractions are then compared to the halo produced by the negative control. The difference between the halo from the negative control (when there is one) and the size of the disk (6 mm) is than calculated and removed from the measurements of the halos produced by the samples.

4. Results

4.1. New strategy for NP discovery in cyanobacteria

At the beginning of this project, six strains were subjected to the supplementation assays, chlorophyll *a* assay, organic extraction and LC-HRESIMS analysis (Table 4.1) which is the first part of the new methodology to discover new natural products from cyanobacteria.

Table 4.1. Cyanobacterial strains subjected to supplementation assays, indicating the mean of chlorophyll a and the variation of masses obtained from the extraction.

Strain	Chlorophyll *a* (µg/mL)	Extracts (control condition) (mg)	Extracts (experimental condition) (mg)
LEGE 04288	2.862	1.03 – 1.42	1.79 – 2.20
PCC 7110	5.744	1.80 – 2.62	2.68 – 3.24
NIES 22	5.723	3.85 – 7.56	3.74 – 9.27
LEGE 91094	3.607	1.90 – 8.01	1.83 – 2.26
NIES 3757	1.338	3.18 – 4.19	0.63 – 2.11
LEGE 06144	1.016	2.67 – 5.03	2.64 – 4.40

Some of these cyanobacterial strains grow homogeneously but others are filamentous which can explain the differences in the chlorophyll *a* and the wide ranges within the masses extracted in each strain.

These results were not yet further studied to search new natural products given that three new compounds from other cyanobacterial strain were previously discovered in the lab through this methodology and the study of those compounds was prioritized. The new compounds, called nocuolactylates A, B and C, are produced by *Nodularia sp.* LEGE 06071, that belongs to the order *Nostocales* and it is available in the LEGE cc. It was originally collected from Vouga estuary in Aveiro, Portugal, in 2006 (Ramos et al., 2018).

4.2. Isolation of nocuolactylates A-C

Nodularia sp. LEGE 06071 was growth in large scale up to 80 L in a sleeve bag in order to obtain enough biomass to isolate the nocuolactylates A, B and C, whose characteristics obtained by LC-HRESIMS, namely the hypothetical molecular formula and molecular weight, are shown in Table 4.2. These compounds differ from each other in the number of chlorine atoms:

nocuolactylate C does not have any chlorine atom, while nocuolactylates A and B have 1 and 2 chlorine atoms, respectively, as it is possible to see in Figure 4.1.

Table 4.2. Chemical characteristics of nocuolactylates A, B and C from Nodularia sp. LEGE 06071 obtained from LC-HRESIMS analysis.

Compound	Hypothetical Molecular Formula	[M] (m/z)	$[M+H]^+$ (m/z)
A	$C_{31}H_{53}O_6N_2Cl$	584.3590	585.3663
B	$C_{31}H_{52}O_6N_2Cl_2$	618.3201	619.32737
C	$C_{31}H_{54}O_6N_2$	550.3980	551.40526

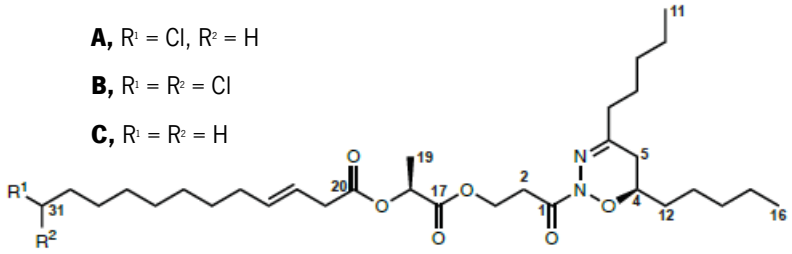

Figure 4.1. Chemical structure of Nocuolactylates A, B and C.

4.2.1. Organic extraction

From 140 L of culture (one sleeve bag and three 20 L nalgenes) it was retrieved approximately 77.7 g of biomass, which corresponds to an average of 0.55 g of biomass per L of culture. From the biomass it was retrieve 7.8 g of crude extract which corresponds to a 10% yield.

4.2.2. VLC

The VLC of the crude extract of *Nodularia sp.* LEGE 06071 (7.8 g) resulted in 11 fractions (A to K), represented in Table 4.3. In total, the fractions account for 8.3 g, which indicates a yield of 106%. This yield can be justified with the presence of silica in the last fractions.

Table 4.3. VLC fractions from the crude extract of Nodularia sp. LEGE 06071. Higlighted in orange are the fractions that presented the nocuolactylates compounds.

	Mobile phase	Fraction (mg)
A	10% EtOAc (hex)	49.6
B	20% EtOAc (hex)	33.6
C	30% EtOAc (hex)	220.1
D	40% EtOAc (hex)	296.6
E	50% EtOAc (hex)	536.7
F	60% EtOAc (hex)	492.7
G	70% EtOAc (hex)	179.6
H	80% EtOAc (hex)	150.8
I	90% EtOAc (hex)	73.9
J	100% EtOAc	3087.1
K	100% MeOH	3160.3

The VLC fractions were then analyzed by LC-HRESIMS to search for the nocuolactylates that were found in fractions D, E and F. This way, these fractions were pooled together and further fractionated through FC.

4.2.3. FC

The FC of fractions D-F (1.326 g) from VLC resulted in 101 test tubes that after TLC analysis were pooled into 15 fractions, represented in Table 4.4. In total, these fractions have 1.103 g, which represents a chromatography yield of 83%.

The FC fractions were also analyzed by LC-HRESIMS to search for nocuolactylates A, B and C, that were found mainly in fractions 08 and 09. These fractions were further fractionated through an SPE.

Table 4.4. FC fractions from the VLC of the crude extract of Nodularia sp. LEGE 06071.

Fraction	Mobile phase	Volume (mL)	Flasks or test tubes	fraction (mg)
01	10% AcOEt (Hex.)	400	1	115.3
02	20% AcOEt (Hex.)	45	02-04	16.9
03	20% AcOEt (Hex.)	60	05-08	18.1
04	20% AcOEt (Hex.)	45	09-11	11.3
05	20% AcOEt (Hex.)	45	12-14	24.7
06	20% AcOEt (Hex.)	60	15-18	29.1
07	20% AcOEt (Hex.)	120	19-26	16.2
08	30% AcOEt (Hex.)	135	27-35	68.7
09	30% AcOEt (Hex.)	120	36-43	60.9
10	30% AcOEt (Hex.)	105	44-50	66.3
11	40% AcOEt (Hex.)	225	51-65	502.7
12	50% AcOEt (Hex.)	300	66-85	68.1
13	70% AcOEt (Hex.)	240	86-101	58.8
14	AcOEt	100	penultimate	17.6
15	20% MeOH (AcOEt) MeOH	100	final	28.1

4.2.4. SPE

Fractions 08 and 09 (129.6 mg) were subjected to a first SPE resulting in four fractions, represented in Table 4.5, with an 83% yield.

Table 4.5. SPE01 fractions from fractions 08 and 09 from FC of Nodularia sp. LEGE 06071.

Fraction	Mobile phase	Volume (mL)	Fraction (mg)
01	40 % IPA (MeOH:H_2O 1:1)	100	8.6
02	50 % IPA (MeOH:H_2O 1:1) 60 % IPA (MeOH:H_2O 1:1)	50 200	10.3
03	80 % IPA (MeOH:H_2O 1:1)	100	5.7
04	100 % IPA 100% DCM	50 100	82.7

In the search for nocuolactylates A-C on the LC-HRESIMS it was verified that fraction 02 was the one with higher concentration of these compounds even though fractions 03 and 04 also

presented the compounds of interest. Considering that fraction 04 has a much higher total mass than the other fractions, it was decided to do another fractionation (SPE02) to separate the compounds from the pigments present in fraction 04. This second fractionation of SPE01 fraction 04 (82.7 mg) resulted in two fractions, as Table 4.6 shows and had a yield of 98%.

Table 4.6. SPE02 fractions resulted from fraction 04 of SPE01 of Nodularia sp. LEGE 06071.

Fraction	Mobile phase	Volume (mL)	fraction (mg)
01	55 % IPA (MeOH:H_2O 1:1)	500	1.4
02	100% IPA 100% DCM	100 100	79.8

The next step was to pool together fraction 02 and 03 from SPE01 with fraction 01 from SPE02 for another fractionation (SPE03) (17.4 mg). Six fractions resulted from this fractionation, as Table 4.7 shows, that had a yield of 105%.

Table 4.7. SPE03 fractions originated of fractions 02 and 03 of SPE01 and 02 of SPE02 of Nodularia sp. LEGE 06071.

Fraction	Mobile phase	Volume (mL)	fraction (mg)
01	55 % IPA (MeOH:H_2O 1:1)	100	12.0
02	55 % IPA (MeOH:H_2O 1:1)	100	3.4
03	55 % IPA (MeOH:H_2O 1:1)	75	0.8
04	55 % IPA (MeOH:H_2O 1:1)	150	3.1
05	55 % IPA (MeOH:H_2O 1:1)	100	3.3
06	100% IPA 100% DCM	50 50	3.9

The LC-HRESIMS search for nocuolactylates A, B and C revealed they were present with higher intensity on fraction 01. To pursue the isolation, fractions 01, 02 and 03 were chosen, and were grouped and subjected to an HPLC.

4.2.5. HPLC

The HPLC fractionation afforded 4 subfractions as it is possible to see in Figure 4.2. HPLC fraction 1 corresponds to nocuolactylate A while HPLC fraction 2 corresponds to nocuolactylate B.

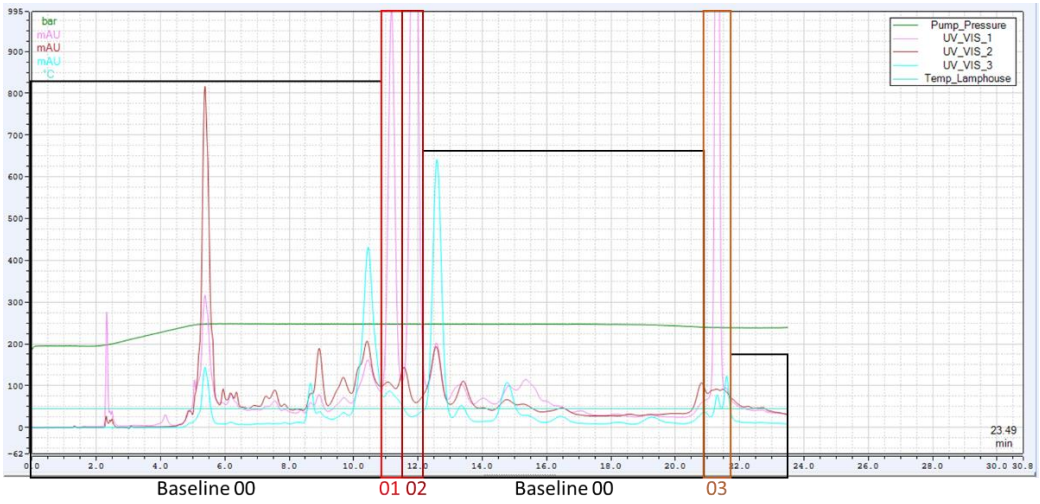

Figure 4.2. HPLC chromatogram and fractions division.

4.2.6. NMR of Nocuolactylates

Nocuolactylates A and B, isolated by HPLC, were subjected to NMR analysis, and considered to be approximately 95% pure in proton NMR. Compound A, NMR spectrum in Figure 4.3, was considered to be purer and for that was the one used to pursue the characterization steps and structural elucidation. A combination of 1D and 2D NMR was used to identify several substructures of nocuolactylate A and High Resolution Electrospray Ionization Mass Spectrometry - Mass Spectrometry (HRESIMS/MS) data helped established the correlation between the substructures and reaching the structure of this compound. Nocuolactylate B was also subjected to 1D and 2D NMR and HRESIMS/MS and revealed the presence of an extra chlorine atom when compared to nocuolactylate A. The nocuolactylate C was obtained only partially purified but the HRESIMS/MS data revealed this compound shared the structure with A and B but without chlorine atoms (Figueiredo et al., 2021).

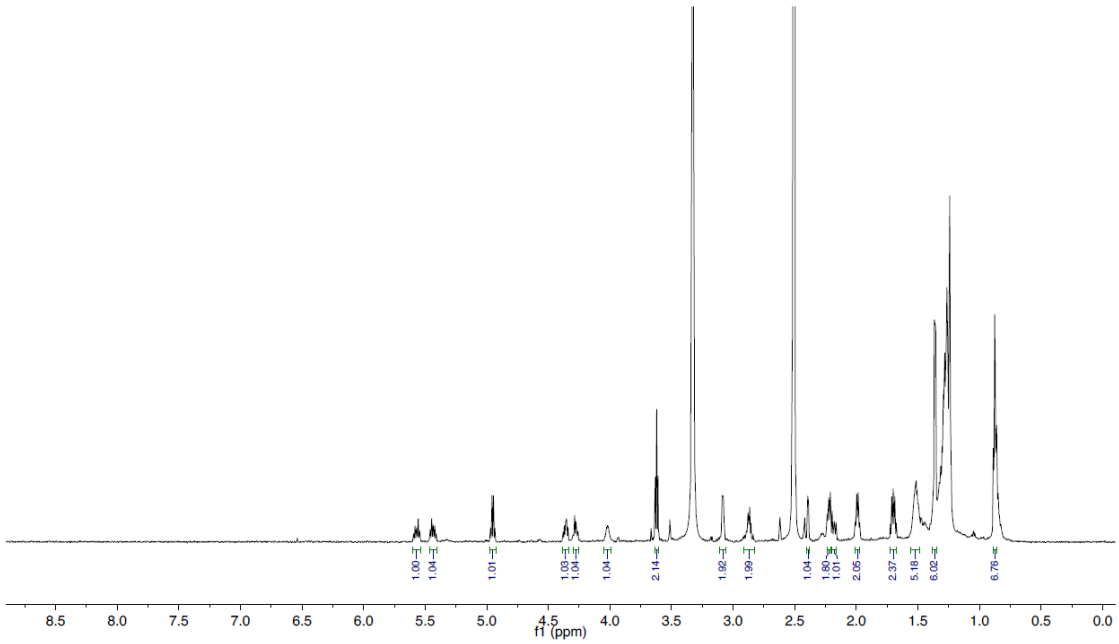

Figure 4.3. Proton NMR (DMSO deuterated, 600 MHz) of nocuolactylate A.

In Figure 4.4 is shown the fractionation scheme that resumes the several chromatographic steps that led to the isolation of nocuolactylates.

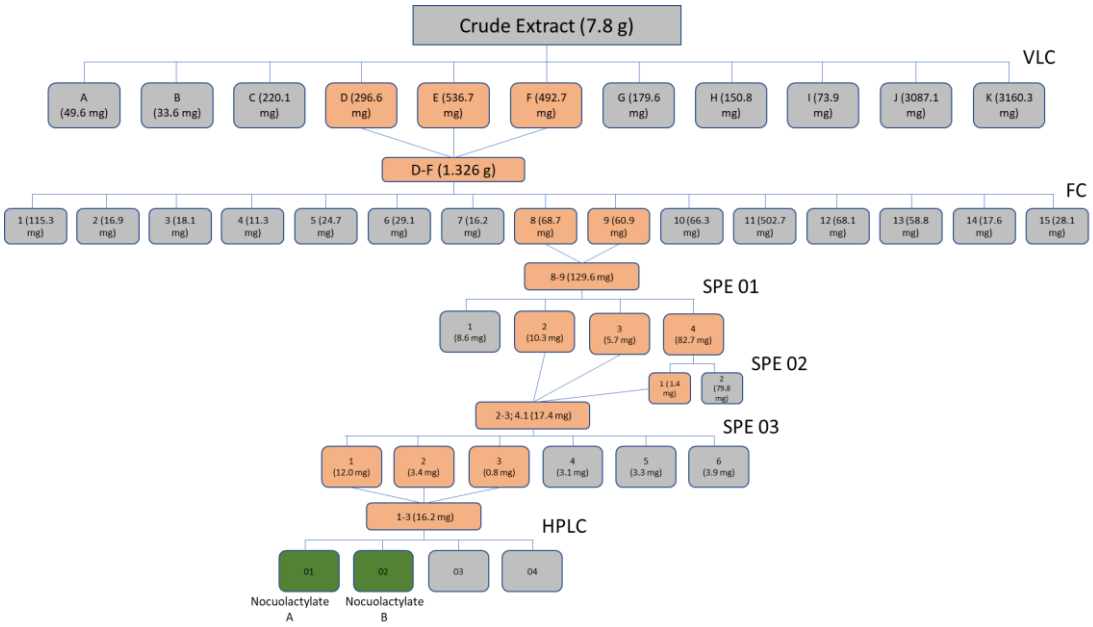

Figure 4.4. Resume of the fractionation to obtain nocuolactylates A and B.

4.3. Biological assays for nocuolactylates A-C

4.3.1. Cytotoxic assays

Nocuolactylates A, B and Nocuolin A were tested in concentrations up to 25 µM while paclitaxel was tested in concentrations up to 4 µM.

In Figure 4.5, Figure 4.6, Figure 4.7 and Figure 4.8 it is possible to see the media of IC_{50} and GR_{50} values for HCT-116, MG-63, MCF7 and hCMEC/D3 cell lines, respectively. In the Appendix are the tables (Table 9.1, Table 9.2, Table 9.3 and Table 9.4) with the values of the independent assays for each cell line and compound.

On a general analysis, it is possible to verify that the positive control, paclitaxel, showed to be the compound with higher activity in the four cell lines tested, presenting values in the nanomolar range. Nocuolin A demonstrated to be more cytotoxic then the nocuolactylates A and B in all cell lines. The IC_{50} and GR_{50} values of these three compounds are in the micromolar range. The nocuolactylates presented IC_{50} values that vary from 3.67 to 15.7 µM and GR_{50} values between 1.73 to 5.90 µM. Nocuolin A showed IC_{50} values between 0.76 to 1.72 µM and GR_{50} values from 0.31 to 0.84 µM. Paclitaxel revealed IC_{50} values from 12.7 to 95.6 nM and the GR_{50} values vary between 4.97 and 37.1 nM.

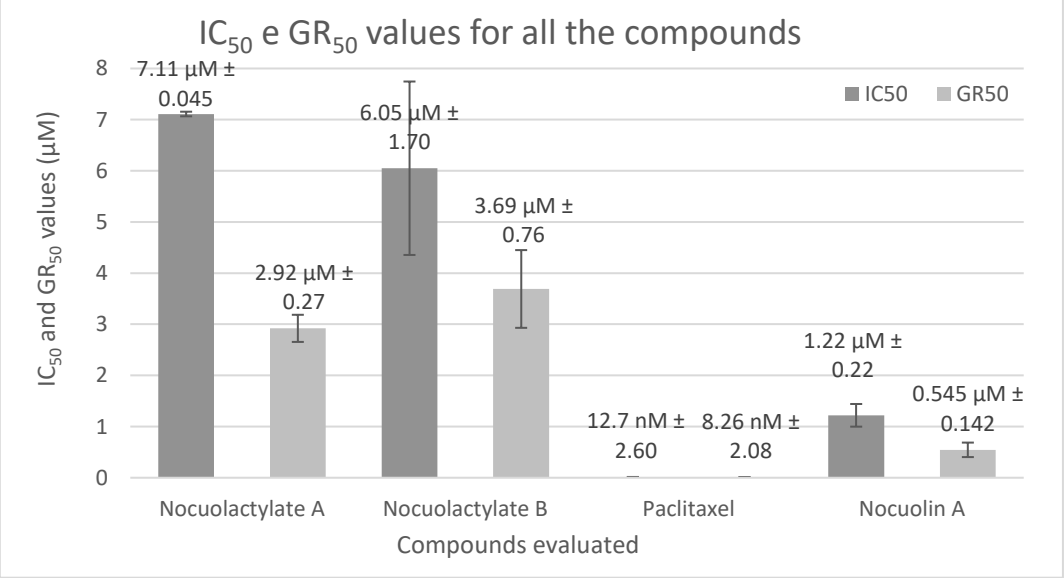

Figure 4.5. IC_{50} and GR_{50} values for nocuolactylates A and B, paclitaxel and nocuolin A on the cell line HCT-116.

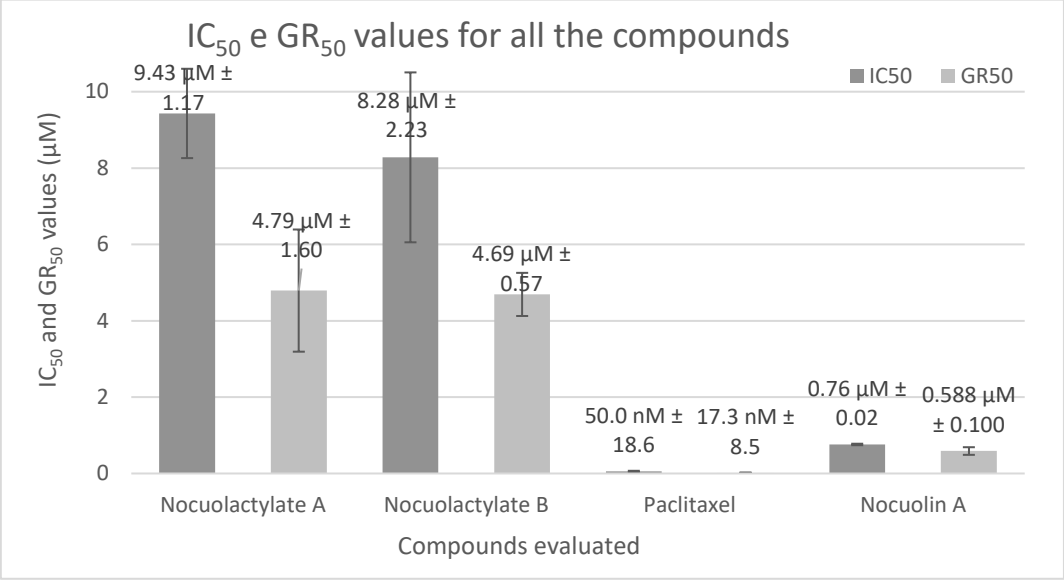

Figure 4.6. IC_{50} and GR_{50} values for nocuolactylates A, B, paclitaxel and nocuolin A on the cell line MG-63.

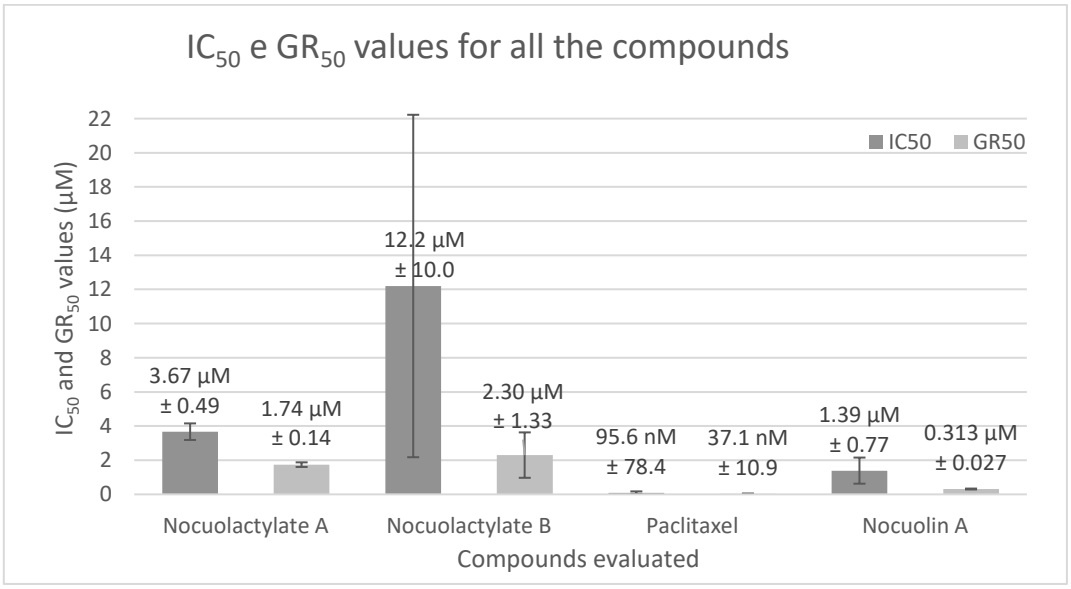

Figure 4.7. IC_{50} and GR_{50} values for nocuolactylates A and B, paclitaxel and nocuolin A on the cell line MCF7.

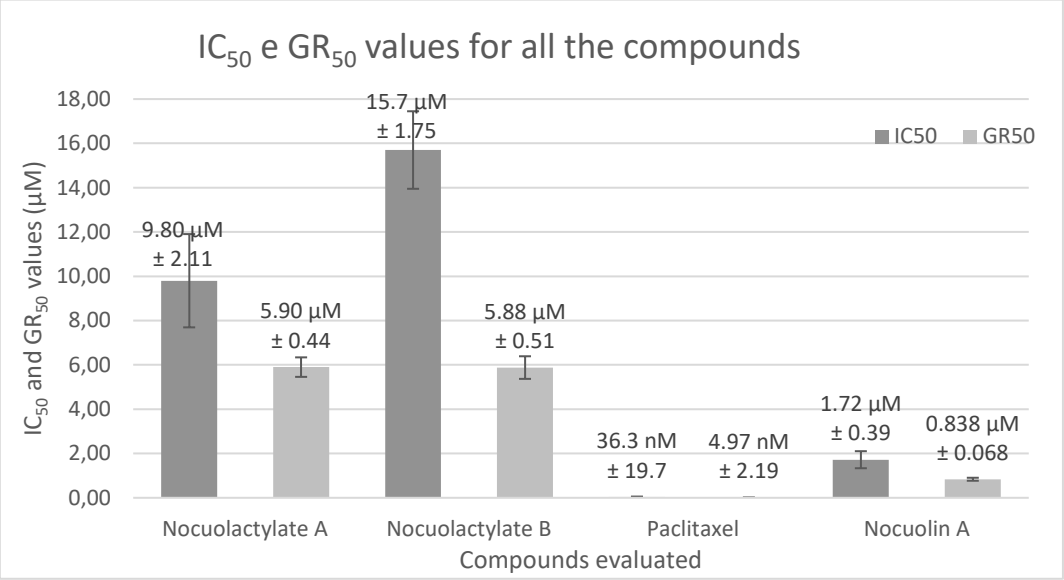

Figure 4.8. IC_{50} and GR_{50} values for compounds 02, 03, paclitaxel and nocuolin A on the cell line hCMEC/D3.

4.3.2. Antimicrobial assays

Table 4.8 presents the inhibition halos of nocuolactylates A and B in each microbial strain. Only a diffuse halo was found for nocuolactylate A against *Bacillus subtilis*, as it shows in Figure 4.9. The cases where the negative control also presented an inhibition halo are marked with a *.

Table 4.8. Halos of inhibition for nocuolactylates A and B.

Strain / Compound	*Escherichia coli*	*Salmonella typhimurium*	*Staphylococcus aureus*	*Bacillus subtilis*	*Candida albicans*
Nocuolactylate A	No halo	No halo	No halo	No clear halo; Diffuse: 8 mm	No halo*
Nocuolactylate B	No halo	No halo	No halo	No halo	No halo*

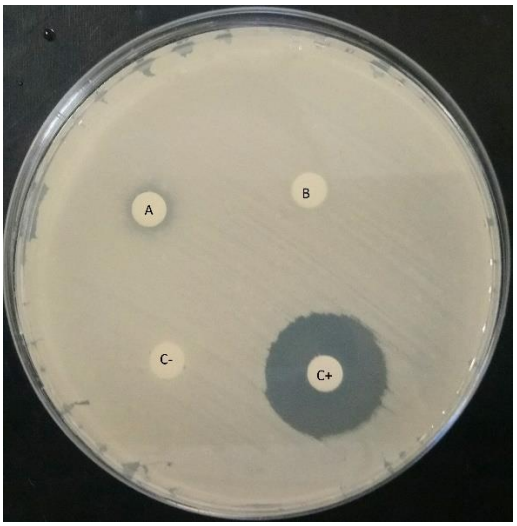

Figure 4.9. Antimicrobial activity of nocuolactylates A and B in Bacillus subtilis. A- Nocuolactylate A; B- Nocuolactylate B; C– Negative control (DMSO); C+- Positive control (Kanamycin).

4.4. Organic extraction of CV1 environmental biomass

The lyophilized environmental sample CV1 (Figure 4.10) was weighed prior to the extraction and had 461.7 g. The crude extract (result of the organic extraction) weighed 14.3 g, which represents an extraction yield of only 3.1%.

Figure 4.10. Environmental sample CV1 after lyophilization.

4.5. VLC of CV1 crude extract

The crude extract subjected to the VLC resulted in nine fractions (A to I), represented in Table 4.9. In total, the VLC fractions had a weight of 5.1 g, which indicates a chromatography yield of 36%.

Table 4.9. Fractions A to I from the VLC of the Cape Verde biomass.

	Mobile phase	fraction (mg)
A	10% EtOAc (hex)	219.9
B	20% EtOAc (hex)	138.9
C	30% EtOAc (hex)	168.7
D	40% EtOAc (hex)	249.7
E	60% EtOAc (hex)	140.6
F	80% EtOAc (hex)	224.1
G	100% EtOAc	230.8
H	25% MeOH (EtOAc)	147.6
I	100% MeOH	3590.1

The nine fractions were posteriorly evaluated in their bioactivity in cytotoxic and antimicrobial assays, with the results presented next.

4.5.1. Cytotoxic assays

Globally, fractions G, H and I revealed to be cytotoxic against all the cell lines tested.

In the HCT-116 cell line, fraction F also demonstrated to have some cytotoxicity but fraction I was the one considered to be more active, as Figure 4.11 shows. Regarding the cell line HepG2, as it is possible to see in Figure 4.12, fraction F also showed to have cytotoxic activity and fraction H was the most active fraction. The cell line MG-63 also presented more than 20% reduction of viability in the fractions E and F, as it is represented in Figure 4.13. In this cell line, fraction I was the one considered to be more active. In the cell line MCF7, fraction H was the one that affected more the cell viability, as Figure 4.14 demonstrates. Finally, considering the non-tumor cell line hCMEC/D3, fractions D and F also reduced the cells viability in more than 20%, as it is possible to see in Figure 4.15, and fraction H was the most active fraction.

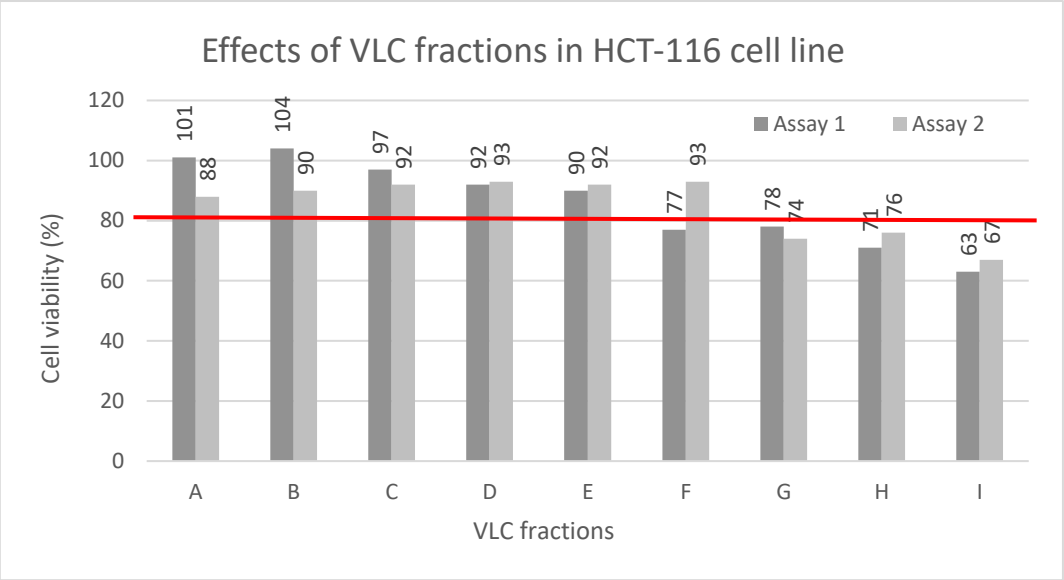

Figure 4.11. Viability percentages for HCT-116 cell line exposed to fractions A to I from VLC 01 of Cape Verde Biomass.

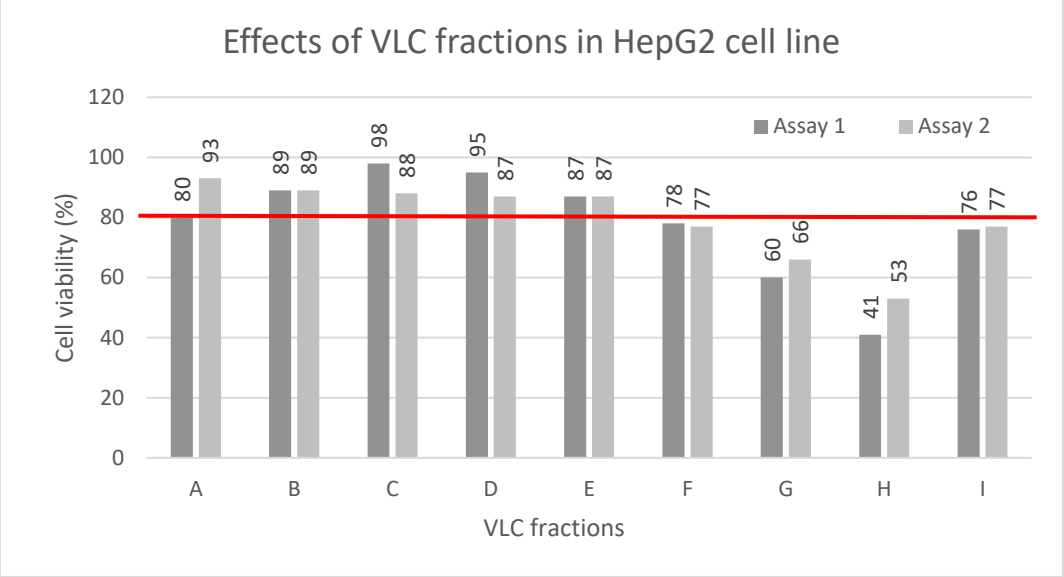

Figure 4.12. Viability percentages for HepG2 cell line exposed to fractions A to I from VLC 01 of Cape Verde Biomass.

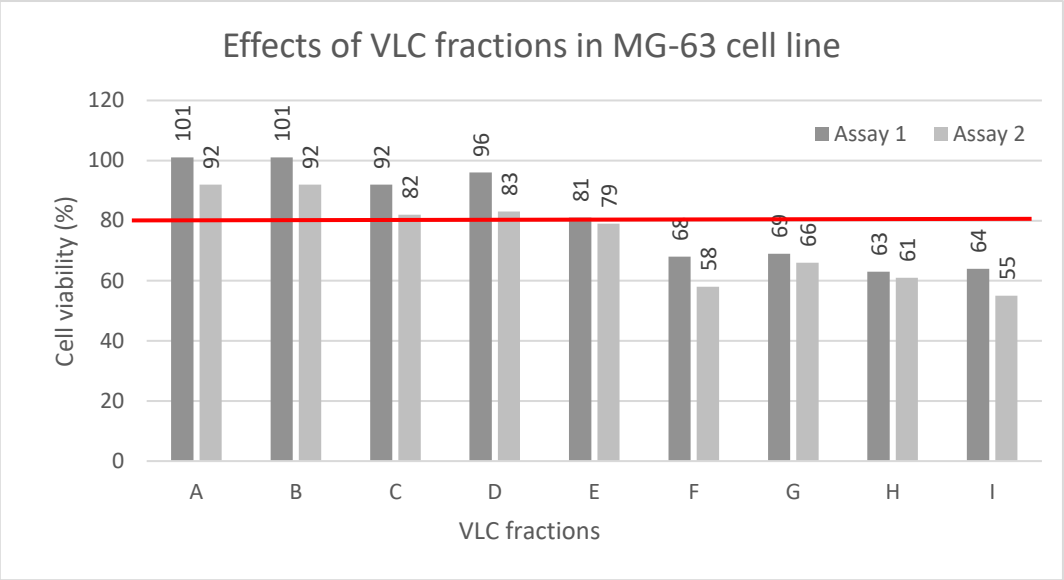

Figure 4.13. Viability percentages for MG-63 cell line exposed to fractions A to I from VLC 01 of Cape Verde Biomass.

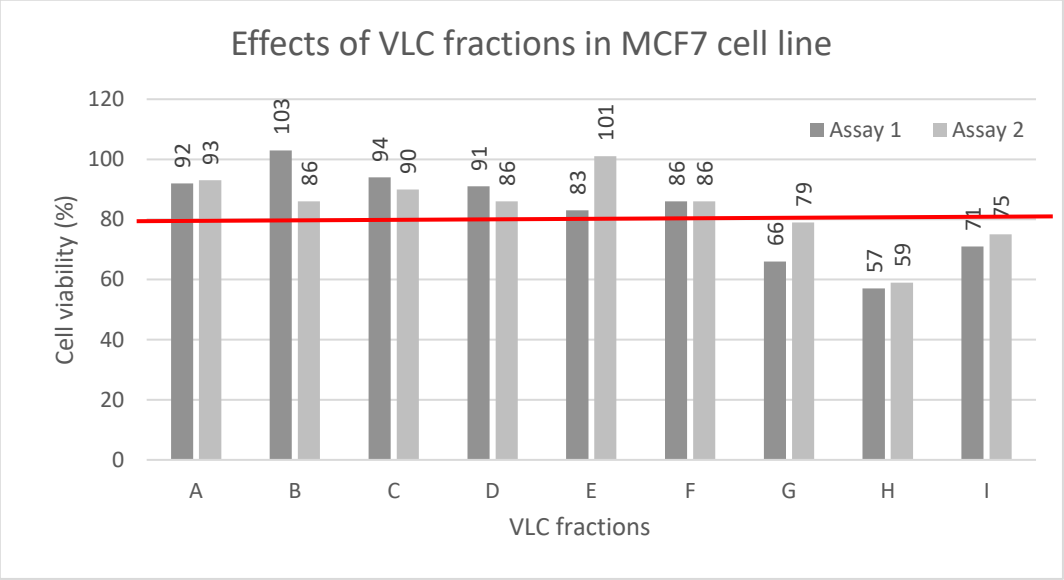

Figure 4.14. Viability percentages for MCF7 cell line exposed to fractions A to I from VLC 01 of Cape Verde Biomass.

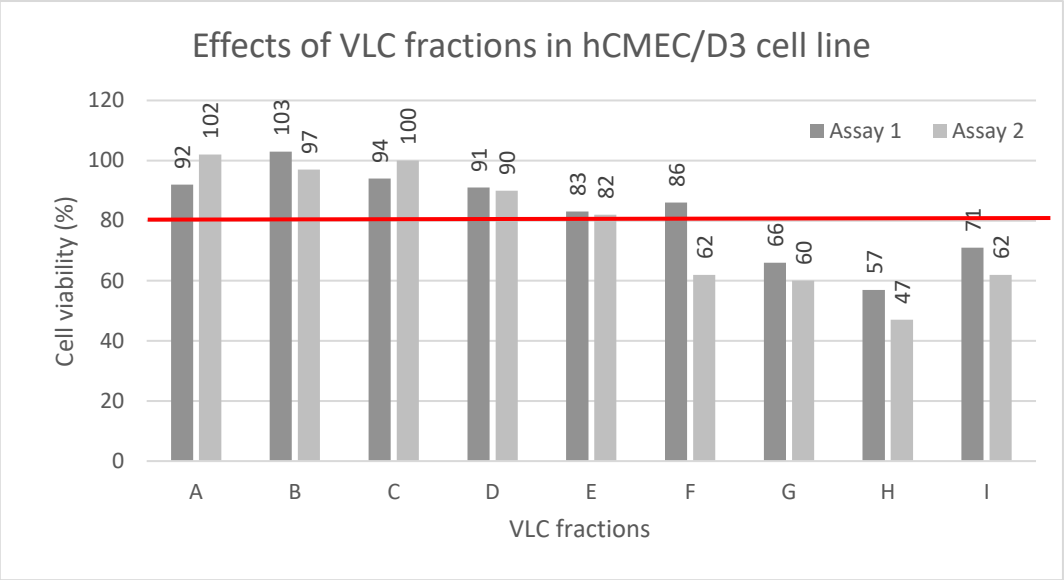

Figure 4.15. Viability percentages for hCMEC/D3 cell line exposed to fractions A to I from VLC 01 of CV1 Biomass

4.5.2. Antimicrobial assays

The clear and diffuse inhibition halos of each fraction in each microbial strain are represented in Table 4.10. The cases where the negative control also presented an inhibition halo are marked with a *.

Table 4.10. Halos of inhibition of the VLC fractions from the Cape Verde biomass.

Strain / Fraction	*Escherichia coli*	*Salmonella typhimurium*	*Staphylococcus aureus*	*Bacillus subtilis*	*Candida albicans*
A	No halo	No halo	No halo	8 mm	7 mm*
B	No halo	No halo	No halo	No halo	No halo*
C	No halo	No halo	No halo	No halo	No halo*
D	No halo	No halo	No halo	No halo	No halo*
E	No halo	No halo	No halo	No halo	No halo*
F	No clear halo; Diffuse: 10 mm	No halo	No halo	No halo	No halo*
G	Clear: 8 mm Diffuse: 12 mm	No halo	No halo	6.5 mm	No halo
H	Clear: 8 mm Diffuse: 15 mm	No halo	No halo	6.5 mm	7 mm
I	No clear halo; Diffuse: 10 mm	No halo	No halo	No halo	No halo

In Figure 4.16 it is possible to see the inhibition halos of fractions G and H (clear halos and bigger diffuse halos) and fractions F and I (diffuse halos) against *Escherichia coli*. In this case, fraction H was considered the most active.

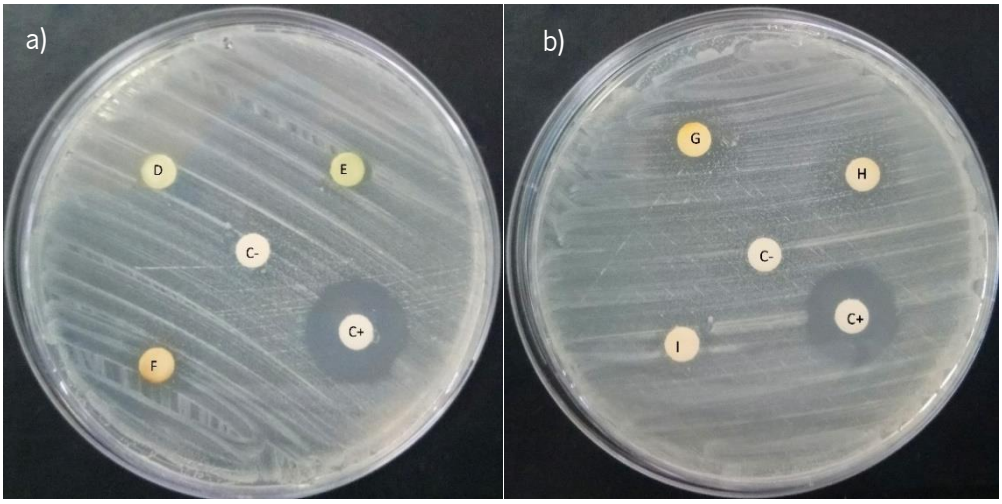

Figure 4.16. Halos of inhibition in Escherichia coli caused by VLC fractions. a) Fraction F; b) Fractions G, H and I; C- - Negative Control (DMSO); C+ - Positive Control (Kanamycin).

Regarding *Bacillus subtilis*, in Figure 4.17 it was possible to observe the three inhibition halos caused by fractions A, G and H, with fraction A producing the bigger halo, and for that considered the most active fraction.

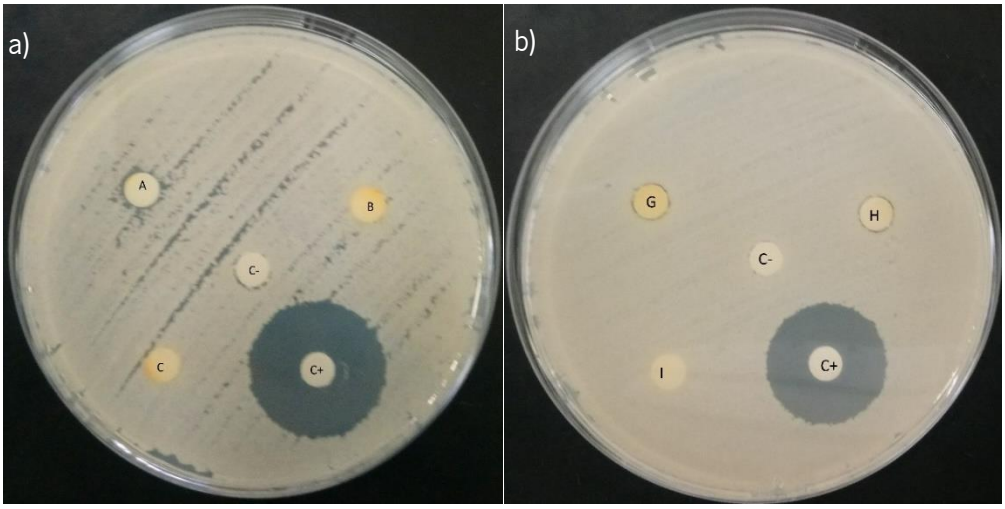

Figure 4.17. Halos of inhibition in Bacillus subtilis caused by VLC fractions. a) Fraction A; b) Fraction G and H; C- - Negative Control (DMSO); C+ - Positive Control (Kanamycin).

Fractions A and H present clear inhibition halos with the same dimensions against *Candida albicans*, as demonstrated in Figure 4.18.

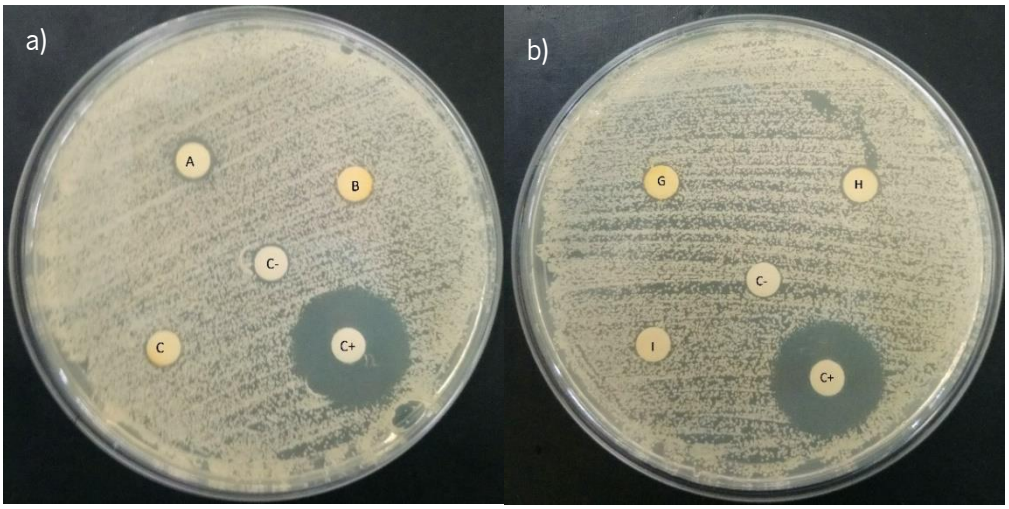

Figure 4.18. Halos of inhibition in Candida albicans caused by VLC fractions. a) Fraction A; b) Fraction H; C- - Negative Control (DMSO); C+ - Positive Control (Nystatin).

4.5.3. NMR analysis

The VLC fractions with biological activity and the fractions that flanked them were subjected to NMR analysis in order to choose the fractions that will follow the fractionation. Fraction H was one of the most active in the cell assays (alongside with fraction I) and it also had activity in the antimicrobial assays. Through the analysis of the NMR spectra, fractions G and H presented a similar profile, represented in Figure 4.19, and so they were pooled together, and further fractionated them by flash chromatography.

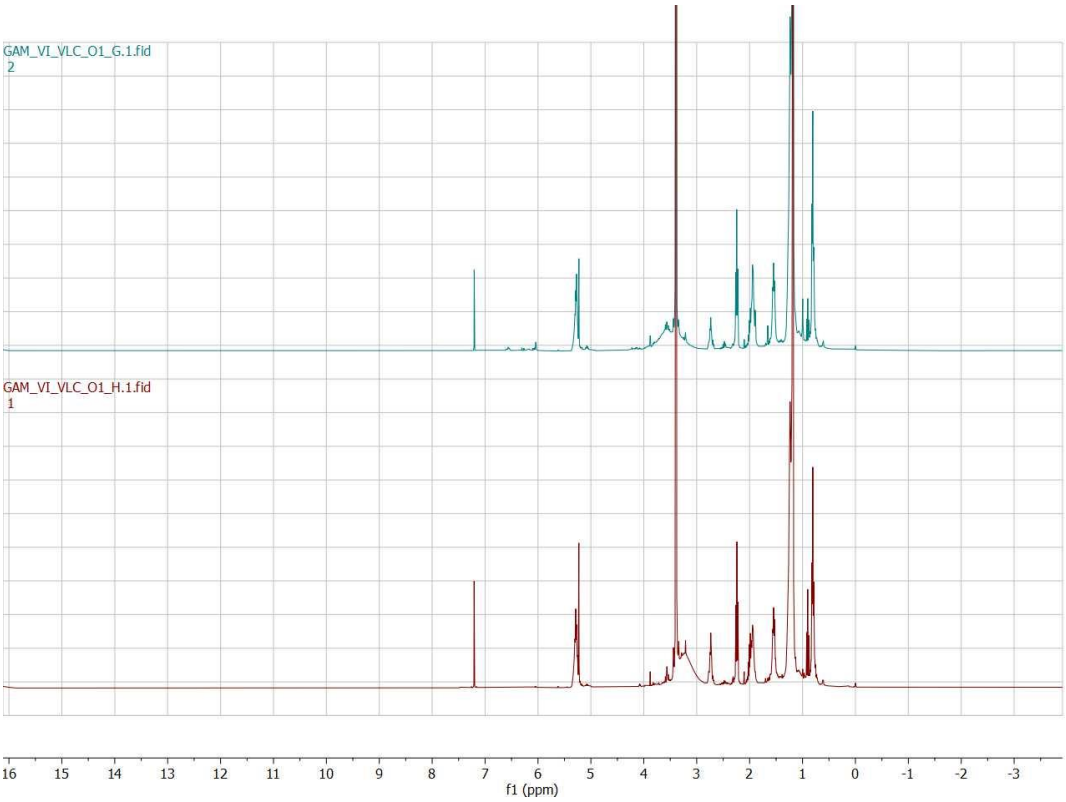

Figure 4.19. Proton NMR (Chloroform deuterated, 400 MHz) spectra of fraction G (top) and H (bottom).

4.6. FC of fractions G and H from CV1

Fractions G and H (378.4 mg) from the VLC of the crude extract of CV1 biomass were pooled together and further fractionated in a flash chromatography. The collected fractions were pooled based on TLC analysis of the test tubes (86 in total), which afforded 13 sub-fractions (GH01-GH13), represented in Table 4.11. In total the FC fractions add up 290.8 mg, which represents a 76.8% yield.

Table 4.11. Distribution of the FC fractions.

	Mobile phase	Volume (mL)	Flasks or test tubes	fraction (mg)
GH01	50% AcOEt (Hex.)	350	0	117.7
GH02	50% AcOEt (Hex.)	120	01-07	37.2
GH03	60% AcOEt (Hex.)	75	08-12	27.0
GH04	60% AcOEt (Hex.)	90	13-18	21.3
GH05	60% AcOEt (Hex.)	90	19-24	13.1
GH06	60% AcOEt (Hex.)	105	25-31	11.0
GH07	60 to 70% AcOEt (Hex.)	105	32-38	4.4
GH08	70 to 80% AcOEt (Hex.)	165	39-49	3.2
GH09	80 to 90% AcOEt (Hex.)	135	50-58	3.5
GH10	90 a 100% AcOEt (Hex.) + 10% MeOH (AcOEt)	150	59-68	2.9
GH11	10 to 20% MeOH (AcOEt)	165	69-79	3.9
GH12	40% MeOH (AcOEt)	105	80-86	13.8
GH13	60 to 100% MeOH (AcOEt)	300	Last	31.8

The resulting 13 fractions had their cytotoxic and antimicrobial activities tested, and the results are presented next.

4.6.1. Cytotoxic assays

Globally, seven fractions (GH06, GH08, GH09, GH10, GH11, GH12 and GH13) revealed to be cytotoxic against all the cell lines they were exposed to.

In Figure 4.20 it is possible to see that fraction GH09 was the most active against HCT-116 cell line. Regarding cell line HepG2, in addition to the seven fractions mentioned before, five more fractions (GH01, GH03, GH04, GH05 and GH07) demonstrated to be cytotoxic, as Figure 4.21 shows, and fraction GH10 was the one considered to be the most active. In cell line MG-63, data represented in Figure 4.22, it is possible to see that fractions GH02, GH03, GH04, GH05 and GH07 also affected the viability of the cells and fraction GH10 was the one that reduced more the cells viability. Additionally, the cell line MCF7 demonstrated to be affected by four more fractions (GH01, GH04, GH05 and GH07), as Figure 4.23 presents, and fraction GH10 was, once again,

the one with higher activity. All the FC fractions exhibit cytotoxicity against the non-tumor hCMEC/D3 cell line in at least one assay, as Figure 4.24 demonstrates and the fraction with bigger cytotoxic activity was fraction GH10.

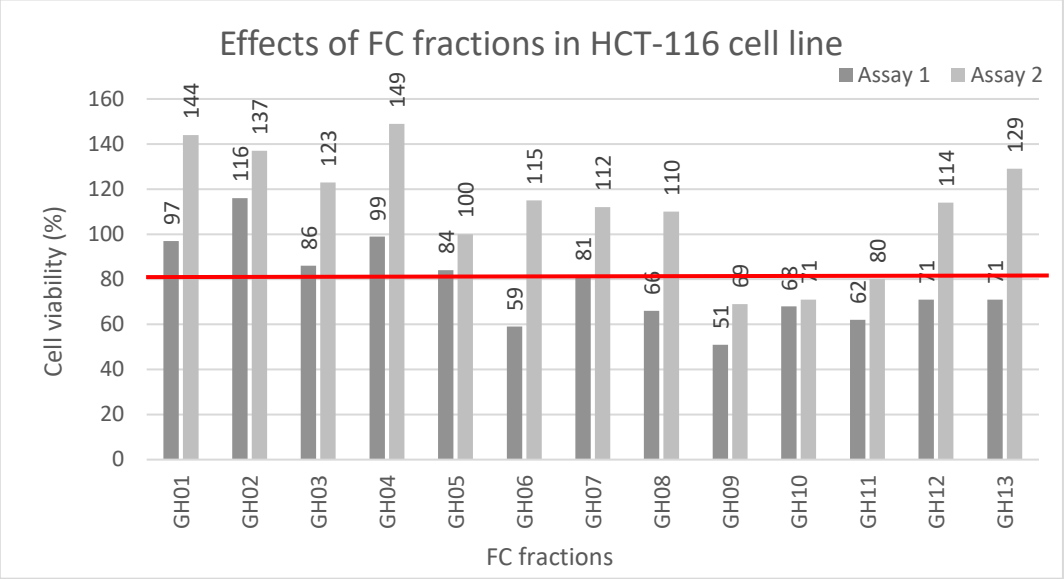

Figure 4.20. Viability percentages for HCT-116 cell line exposed to fractions GH01 to GH13 from FC.

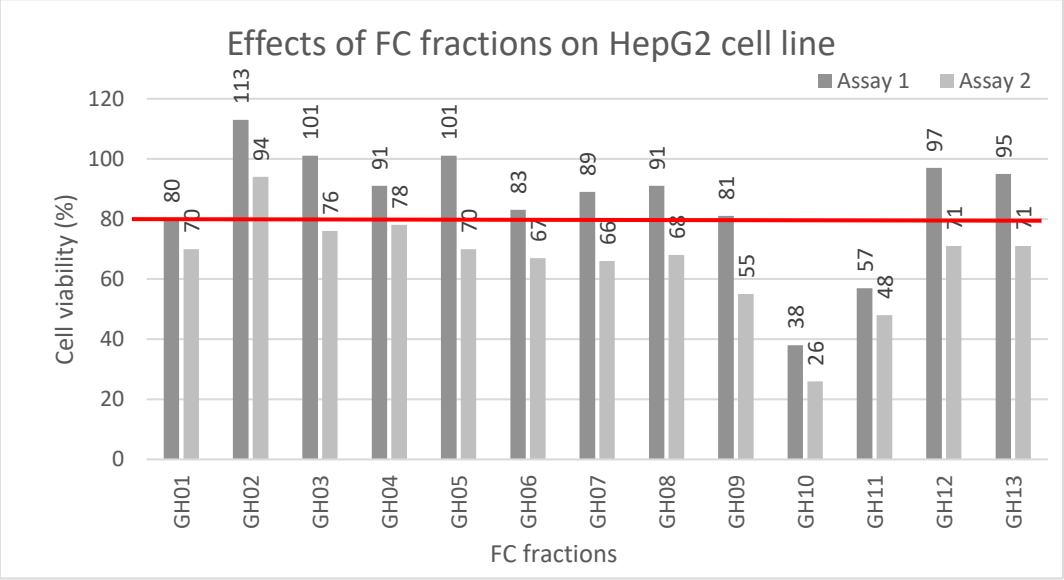

Figure 4.21. Viability percentages for HepG2 cell line exposed to fractions GH01 to GH13 from FC.

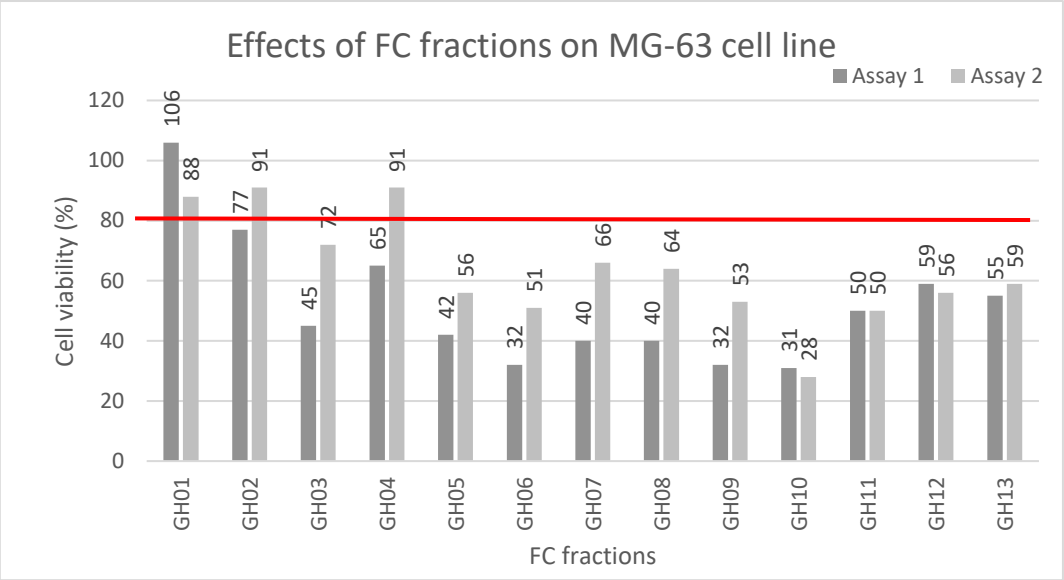

Figure 4.22. Viability percentages for MG-63 cell line exposed to fractions GH01 to GH13 from FC.

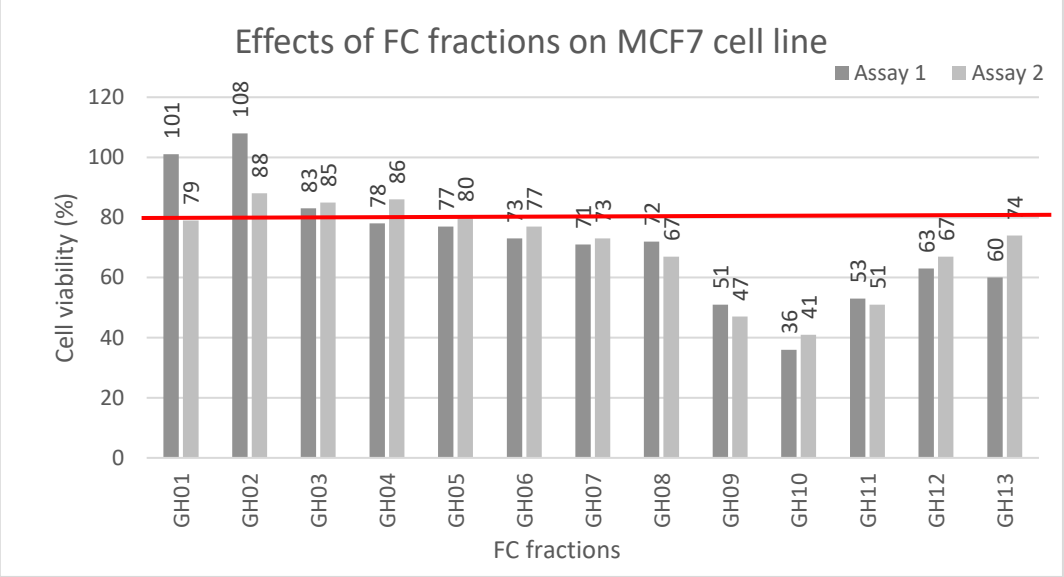

Figure 4.23. Viability percentages for MCF7 cell line exposed to fractions GH01 to GH13 from FC.

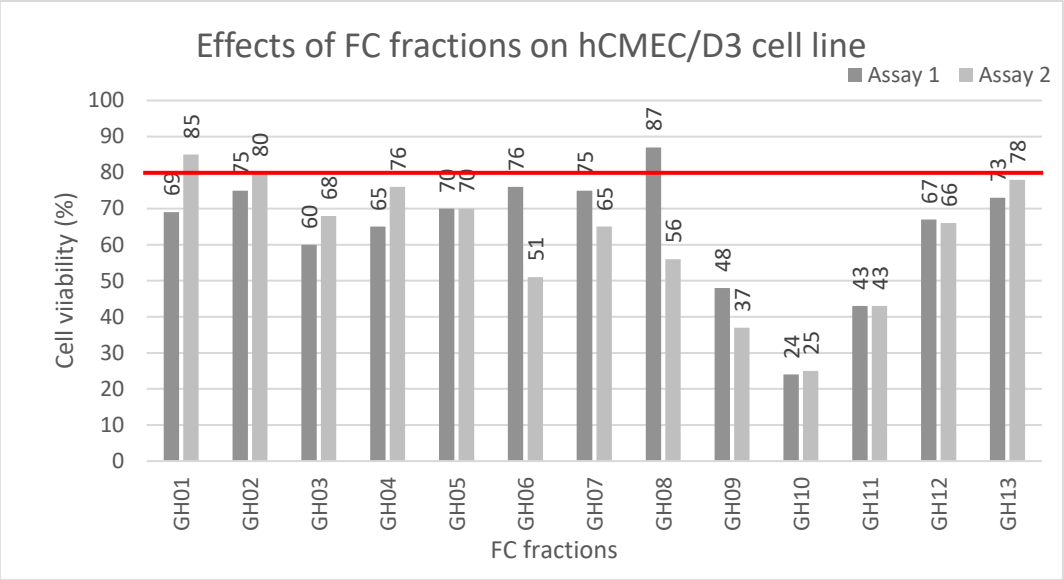

Figure 4.24. Viability percentages for hCMEC/D3 cell line exposed to fractions GH01 to GH13 from FC.

4.6.2. Antimicrobial assays

In Table 4.12 are represented the inhibition halos of each FC fraction in each microbial strain. The cases where the negative control also presented an inhibition halo are marked with a *.

Table 4.12. Halos of inhibition of the FC fractions from the Cape Verde biomass.

Strain / Fraction	*Escherichia coli*	*Salmonella typhimurium*	*Staphylococcus aureus*	*Bacillus subtilis*	*Candida albicans*
GH01	No halo	No halo	7.5 mm	8 mm*	No clear halo; Diffuse: 7 mm
GH02	No clear halo; Diffuse: 8 mm	No halo	8 mm	9 mm*	No halo
GH03	No clear halo; Diffuse: 7 mm	No halo	7 mm	8 mm*	No clear halo; Diffuse: 7 mm
GH04	7 mm	No halo	7 mm*	11 mm*	9 mm
GH05	7 mm	No halo	7.5 mm*	11 mm*	7 mm
GH06	No halo	No halo	7 mm*	9 mm*	No halo
GH07	No halo	No halo	6.5 mm*	8 mm*	No halo
GH08	7 mm	No halo	6.5 mm*	8 mm*	No halo
GH09	No halo	No halo	No halo*	No halo*	No halo
GH10	No halo	No halo	No halo*	No halo*	No halo
GH11	No halo	No halo	No halo*	No halo*	No halo
GH12	No halo	No halo	No halo*	No halo*	No halo
GH13	8 mm	No halo	7 mm	7 mm*	No halo

For *Escherichia coli* six fractions showed to have some type of antibacterial activity, as represented in Figure 4.25. Four of them (04, 05, 08 and 13) presented clear halos while the other two (02 and 03) only presented diffuse halos. Fraction 13 was the one with higher activity, presenting a halo of 8 mm.

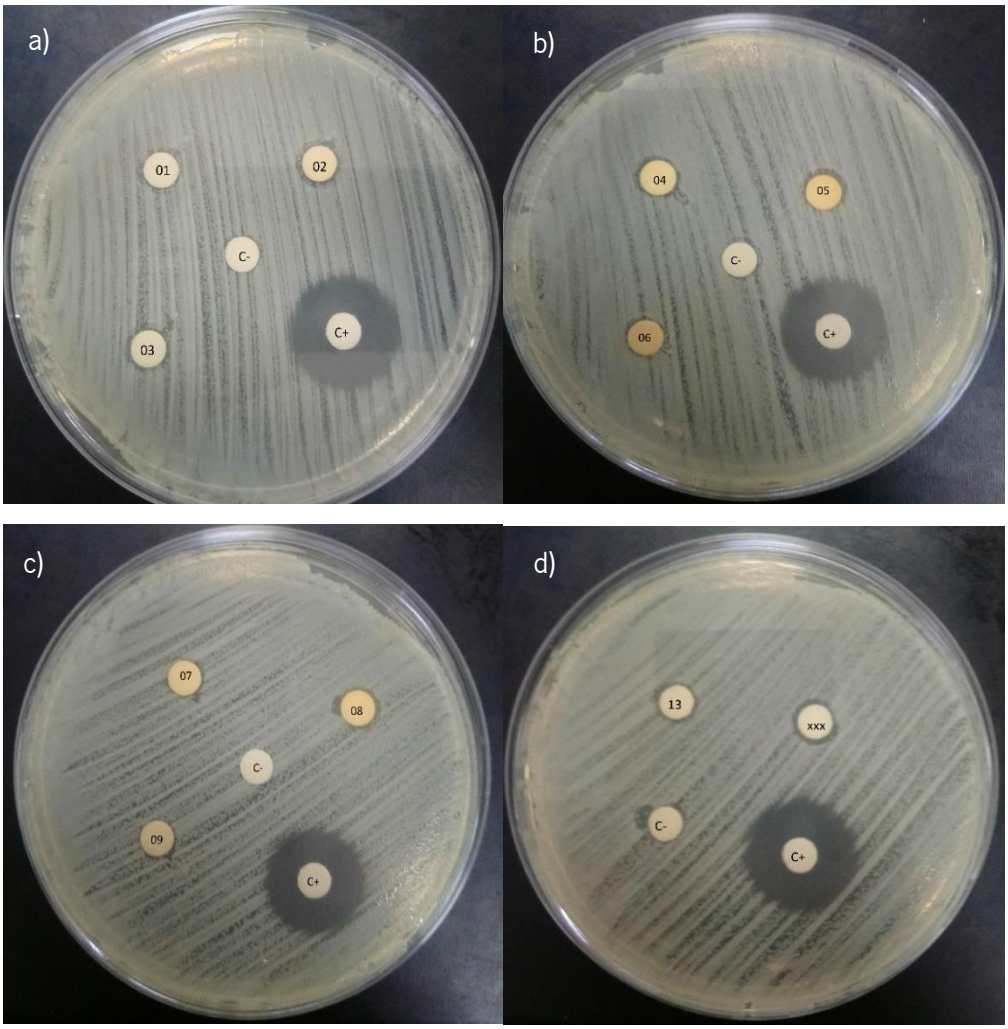

Figure 4.25. Halos of inhibition in Escherichia coli caused by FC fractions. a) Fractions 02 and 03; b) Fractions 04 and 05; c) Fraction 08; d) Fraction 13; C- - Negative Control (DMSO); C+ - Positive Control (Kanamycin).

Nine of these fractions present considerable and clear halos of inhibition against *Staphylococcus aureus* (01, 02, 03, 04, 05, 06, 07, 08 and 13), as it is represented in Figure 4.26. Fraction 02 was the one with bigger halo of inhibition (8 mm).

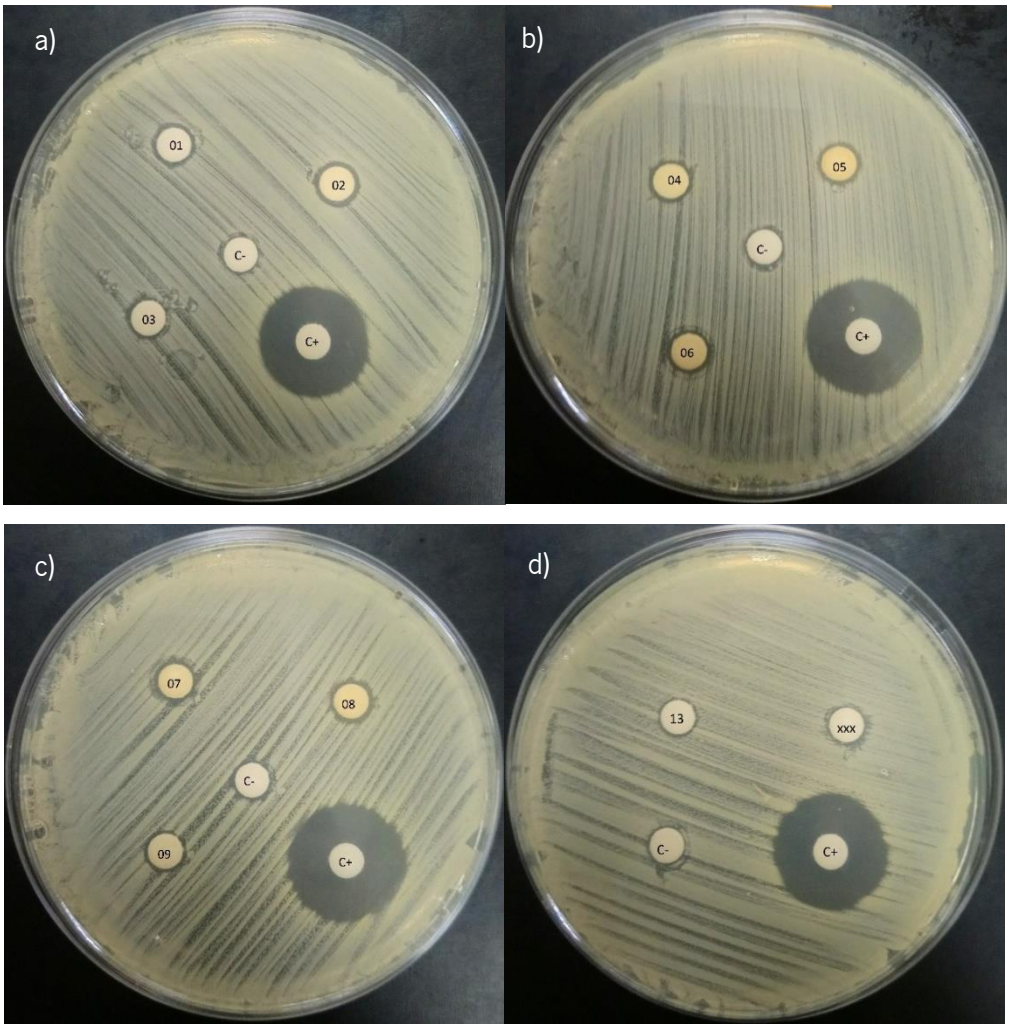

Figure 4.26. Halos of inhibition in Staphylococcus aureus caused by FC fractions. a) Fractions 01, 02 and 03; b) Fractions 04, 05 and 06; c) Fraction 07 and 08; d) Fraction 13; C- - Negative Control (DMSO); C+ - Positive Control (Kanamycin).

Regarding *Bacillus subtilis*, it was possible to observe that nine fractions (01, 02, 03, 04, 05, 06, 07, 08 and 13) have antimicrobial activity, since they exhibit clear and considerable inhibition halos when compared with the negative control, as Figure 4.27 shows. Fractions 04 and 05 were the most active against this strain since both produce halos of 11 mm diameter.

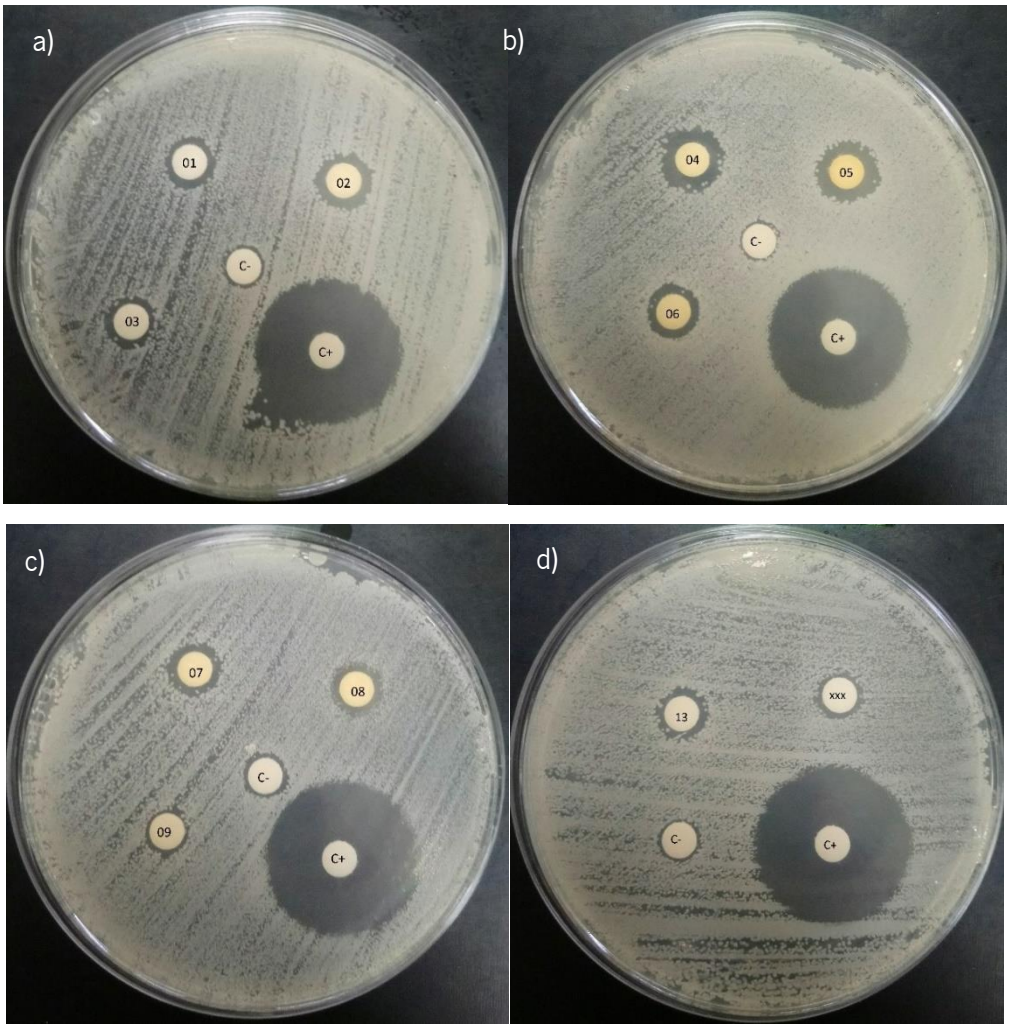

Figure 4.27. Halos of inhibition in Bacillus subtilis caused by FC fractions. a) Fractions 01, 02 and 03; b) Fractions 04, 05 and 06; c) Fraction 07 and 08; d) Fraction 13; C- - Negative Control (DMSO); C+ - Positive Control (Kanamycin).

Two fractions (04 and 05) showed clear halos of inhibition, which means they have antimicrobial activity against *Candida albicans*. Fractions 01 and 03 presented diffuse halos which can also indicate bioactivity, although it is mild. Fraction 04 was the one that presented a bigger halo of growth inhibition, 9 mm. All the halos can be seen in Figure 4.28.

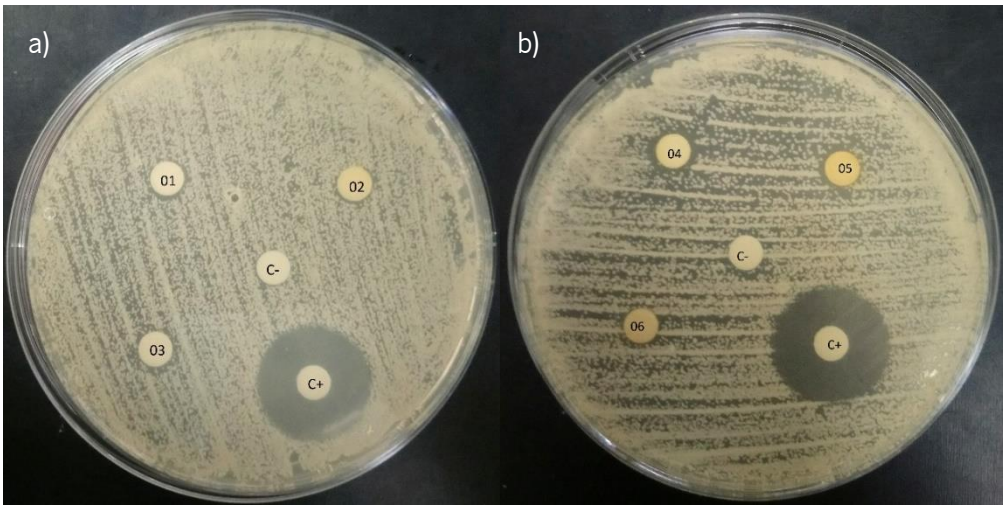

Figure 4.28. Halos of inhibition in Candida albicans caused by FC fractions. a) Fractions 01 and 03; b) Fractions 04 and 05; C- - Negative Control (DMSO); C+ - Positive Control (Nystatin).

4.6.3. LC-HRESIMS analysis

The FC fractions were analyzed in LC/HRESIMS in order to choose the fractions that will follow the fractionation.

Fractions GH09 and GH10 were the most active fractions in the cytotoxic assay. When analyzing the LC/HRESIMS data, Figure 4.29, fraction GH11 showed some similarity with fraction GH10 and given it had a considerable amount of mass, it was decided to pool together fractions GH09, GH10 and GH11 to pursue the isolation of a bioactive compound.

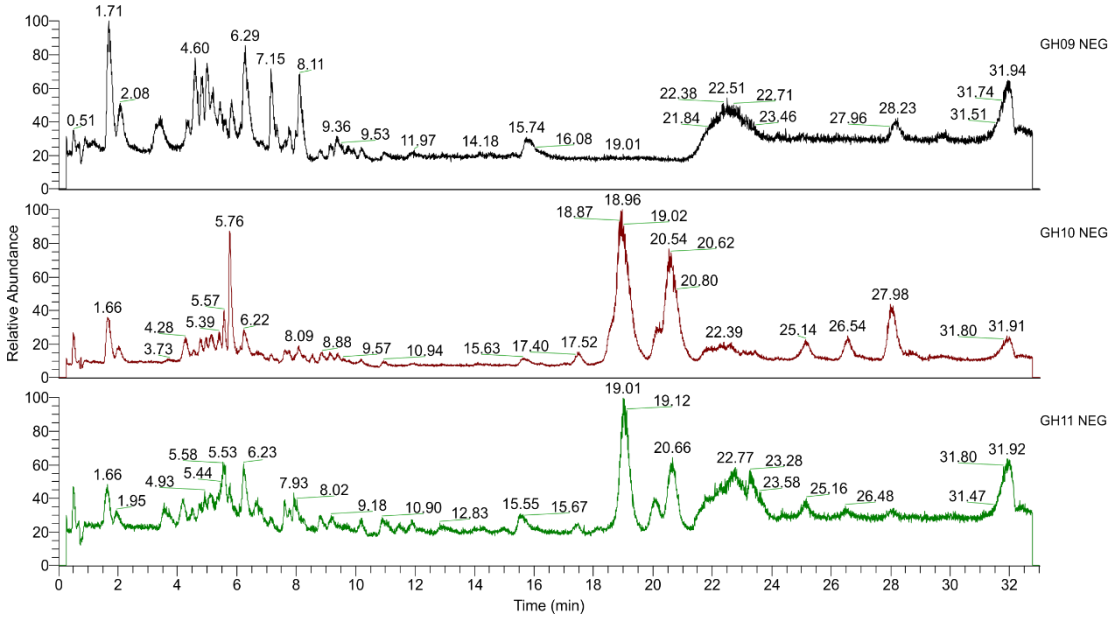

Figure 4.29. LC/HRESIMS chromatogram of fractions GH09, GH10 and GH11 in negative mode.

Fractions GH04 and GH05 were the most active FC fractions in the antimicrobial assay. In the LC/HRESIMS analysis, Figure 4.30, fraction GH03 revealed to have great similarity with fraction GH04, and fraction GH06 showed to have a similar chromatogram profile to the one of fraction GH05. For this reason, fractions GH03, GH04, GH05 and GH06 were pooled together to pursue the isolation of a bioactive compound.

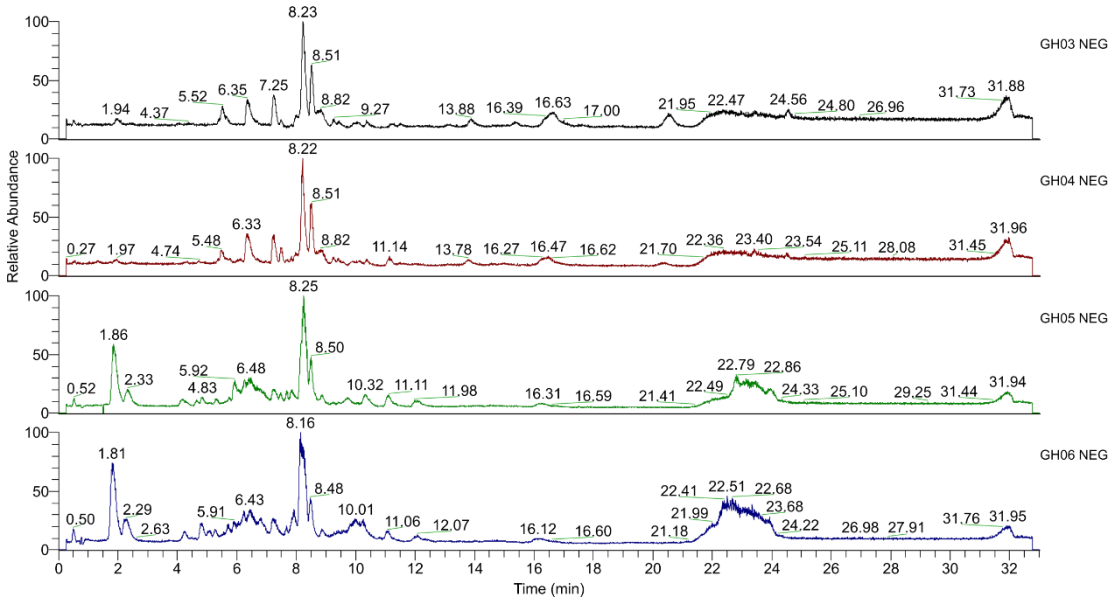

Figure 4.30. LC/HRESIMS chromatogram of fractions GH03, GH04, GH05 and GH06 in negative mode.

4.7. HPLC 01 of GH09-11 from CV1

Fractions GH09, GH10 and GH11 were the ones that presented higher activity in the cytotoxic assays and did not present activity in the antimicrobial assays. Fractions GH09-11 (9.7 mg) were pooled together and dissolved in MeOH to conduct an HPLC, in order to further characterize the compounds responsible for the bioactivity in the cytotoxic assays.

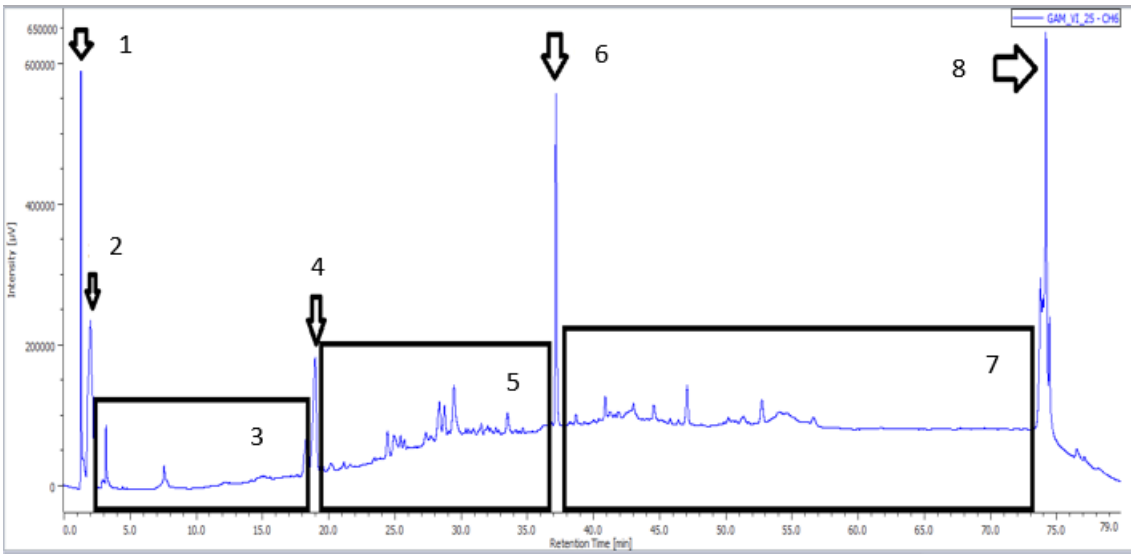

Figure 4.31. Chromatogram of the HPLC 01 run, with the definition of the resulted fractions.

This HPLC resulted in eight fractions (GH09-11_1 to GH09-11_8), represented in Figure 4.31, in a total of 11.7 mg, which reveals a yield of 121%. This yield might be due to some impurities in the flasks.

Given the low amounts of each fraction (Table 4.13), no further cytotoxic assays were conducted. Instead, it was prioritized the antimicrobial-guided fractionations of other fractions (HPLC 02-04).

Table 4.13. Fractions 1 to 8 of the HPLC 01 run.

	Fraction (mg)
GH09-11_1	1.0
GH09-11_2	0.4
GH09-11_3	2.4
GH09-11_4	0.4
GH09-11_5	2.8
GH09-11_6	0.4
GH09-11_7	3.2
GH09-11_8	1.0

In Figure 4.32 is represent the fractionation scheme that was followed in this cytotoxic pathway for the environmental biomass.

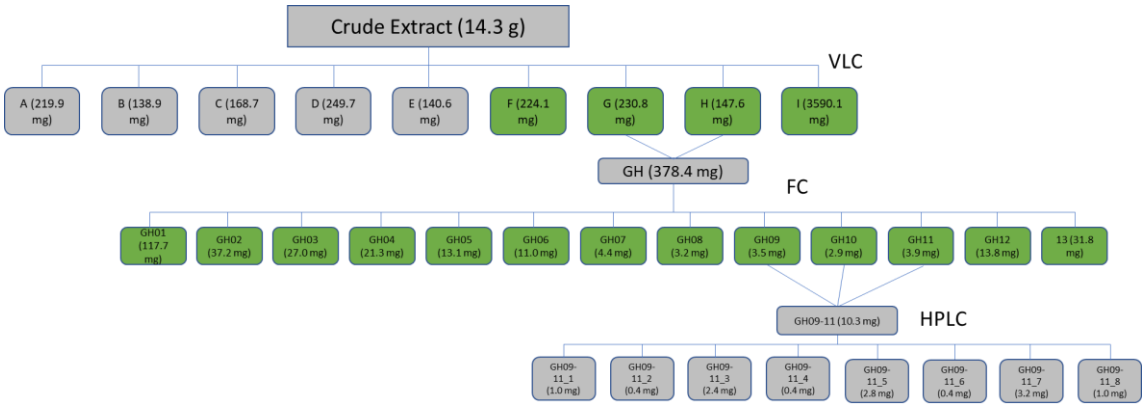

Figure 4.32. Resume of the fractionation of CV1 in the cytotoxic pathway.

4.8. HPLC 02 of GH03-06 from CV1

Fractions GH03, GH04, GH05 and GH06 were the ones that presented higher activity in the antimicrobial assays and accordingly they were chosen to pursue the isolation of a pure compound. Fractions GH03-06 (72.4 mg) were pooled together and dissolved in MeOH and this mixture was injected in the HPLC to perform the chromatography.

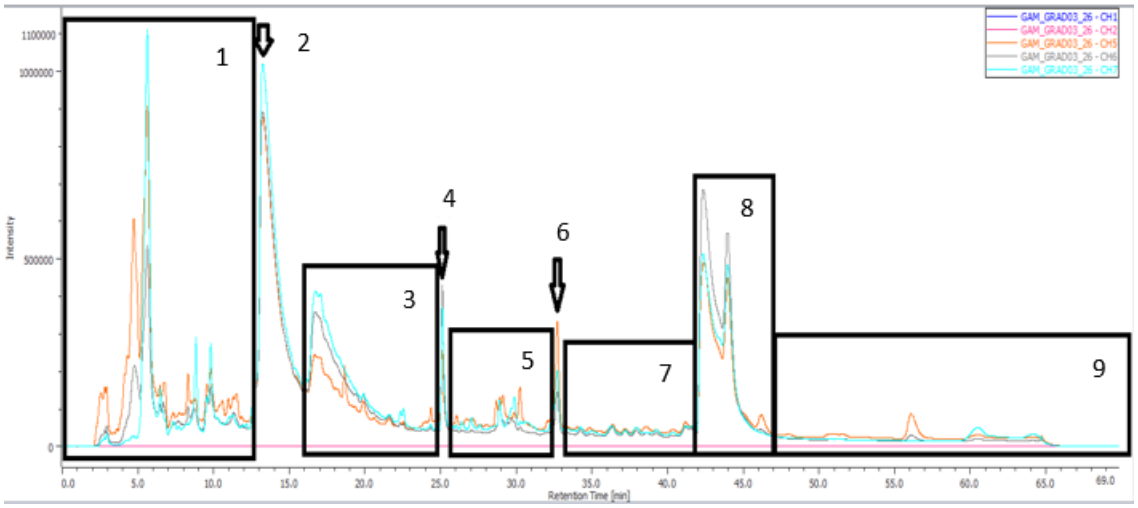

Figure 4.33. Chromatogram of the HPLC 02 run and resulted fractions.

HPLC 02 resulted in nine fractions (GH03-06_1 to GH03-06_9), Figure 4.33, that are represented in Table 4.14, in a total amount of 56.0 mg, revealing a yield of 77%. A part of the original sample (GH03-06) was not run in the HPLC which increases the yield of the chromatography to 87%.

Table 4.14. Fractions 1 to 9 of the HPLC 02 run.

	Fraction (mg)
GH03-06_1	6.1
GH03-06_2	4.7
GH03-06_3	10.9
GH03-06_4	1.3
GH03-06_5	11.7
GH03-06_6	1.9
GH03-06_7	7.3
GH03-06_8	5.1
GH03-06_9	7.0

4.8.1. Antimicrobial assays

In Table 4.15 are represented the inhibition halos of each HPLC 02 fraction in each microbial strain tested. The cases where the negative control also presented an inhibition halo are marked with a *.

Table 4.15. Halos of inhibition of the HPLC 02 fractions from the Cape Verde biomass.

Strain / Fraction	*Escherichia coli*	*Salmonella typhimurium*	*Staphylococcus aureus*	*Bacillus subtilis*	*Candida albicans*
GH03-06_1	No clear halo; Diffuse: 10 mm	No halo	7 mm	9 mm	8 mm
GH03-06_2	No clear halo; Diffuse: 10 mm	No halo	7 mm	10 mm	7 mm
GH03-06_3	No clear halo; Diffuse: 15 mm	No halo	8 mm	9 mm	No halo
GH03-06_4	No halo*	No halo	No halo	No halo	7 mm
GH03-06_5	No halo*	No halo	No halo	No halo	No halo
GH03-06_6	No halo*	No halo	No halo	No halo	No halo
GH03-06_7	No halo	No halo	No halo	No halo	No halo
GH03-06_8	No halo	No halo	No halo	No halo	No halo
GH03-06_9	No halo	No halo	No halo	No halo	No halo

Fractions GH03-06_1, GH03-06_2 and GH03-06_3 revealed to have some type of activity against *Escherichia coli*, as Figure 4.34 shows. The three fractions caused diffuse halos, with fraction GH03-06_03 causing the biggest halo (15 mm diffuse halo), and for that being considered the most active.

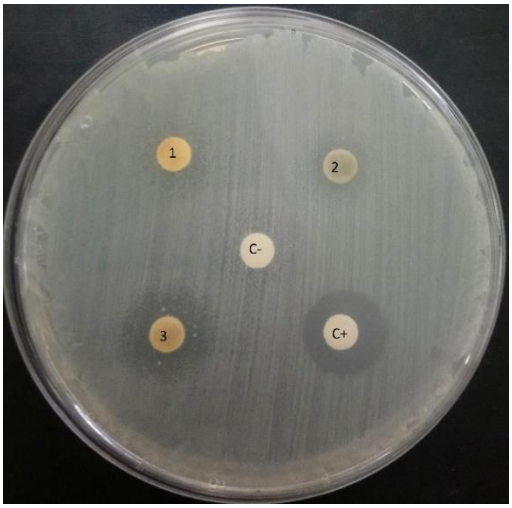

Figure 4.34. Halos of inhibition caused by HPLC 02 fractions GH03-06_1, GH03-06_2 and GH03-06_3 in Escherichia coli. C- - Negative Control (DMSO); C+ - Positive Control (Kanamycin).

Three fractions of the HPLC 02 revealed to have antimicrobial activity against *Staphylococcus aureus* by producing clear inhibition halos, as Figure 4.35 indicates. Fractions GH03-06_3 produced a halo of 8 mm and was considered the most active.

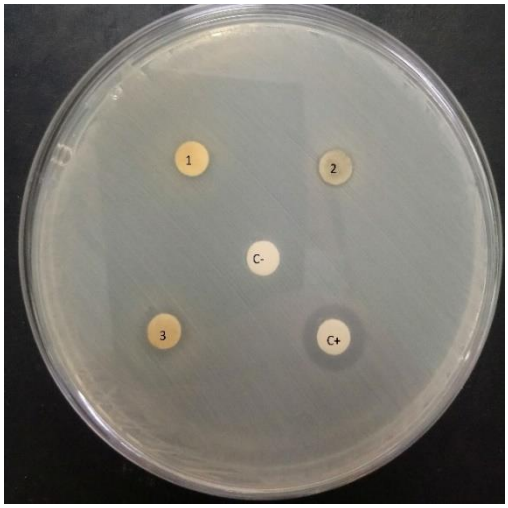

Figure 4.35. Halos of inhibition in Staphylococcus aureus caused by fractions GH03-06_1, GH03-06_2 and GH03-06_3 of HPLC 02. C- - Negative Control (DMSO); C+ - Positive Control (Kanamycin).

Fractions GH03-06_1, GH03-06_2 and GH03-06_3 of HPLC 02 caused inhibition halos in *Bacillus subtilis*, as Figure 4.36 demonstrates and fraction GH03-06_2 was considered the most active with an inhibition halo of 10 mm.

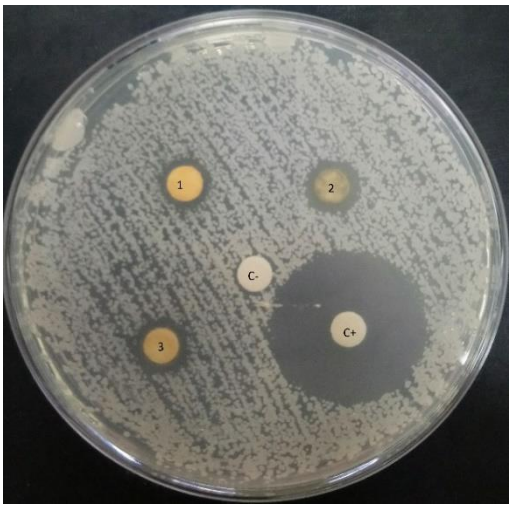

Figure 4.36. Halos of inhibition in Bacillus subtilis caused by fractions GH03-06_1, GH03-06_2 and GH03-06_3. C- - Negative Control (DMSO); C+ - Positive Control (Kanamycin).

Three fractions of the HPLC 02 caused clear inhibition halos in *Candida albicans*, as shown in Figure 4.37. Fraction GH03-06_1 was the most active, with a halo of 8 mm.

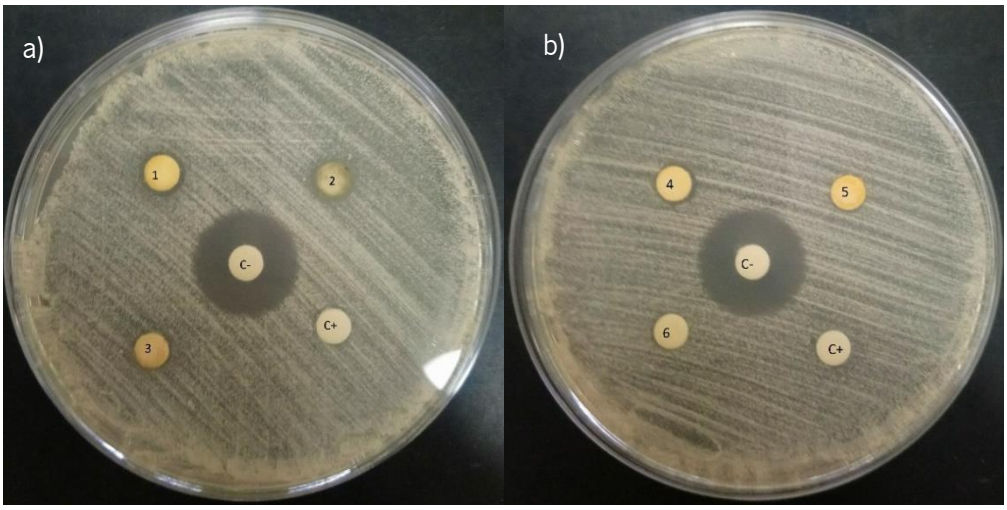

Figure 4.37. Halos of inhibition in Candida albicans caused by HPLC 02 fractions. a) Fractions GH03-06_1 and GH03-06_2; b) Fraction GH03-06_4; C- - Negative Control (DMSO); C+ - Positive Control (Nystatin).

4.8.2. NMR analysis

Fractions from the HPLC 02 with activity (GH03-06_1 to GH03-06_4) were subjected to NMR, Figure 4.38.

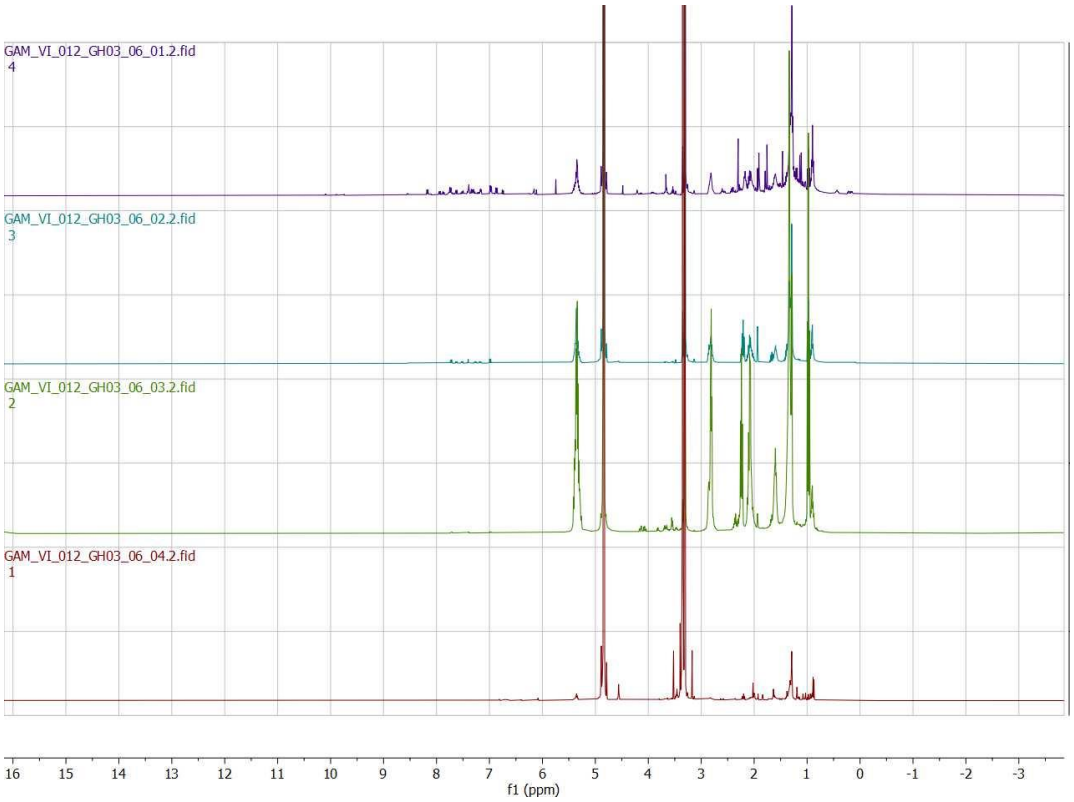

Figure 4.38. Proton NMR (deuterated methanol ,400 MHz) spectra of fractions GH03-06_1-4 of HPLC 02.

In fractions GH03-06_1 and GH03-06_2 the major components seem to be shared, as it is possible to see in Figure 4.39, and for that these two fractions were chosen to pursue the isolation given that both presented activity against almost every strain in the antimicrobial assay.

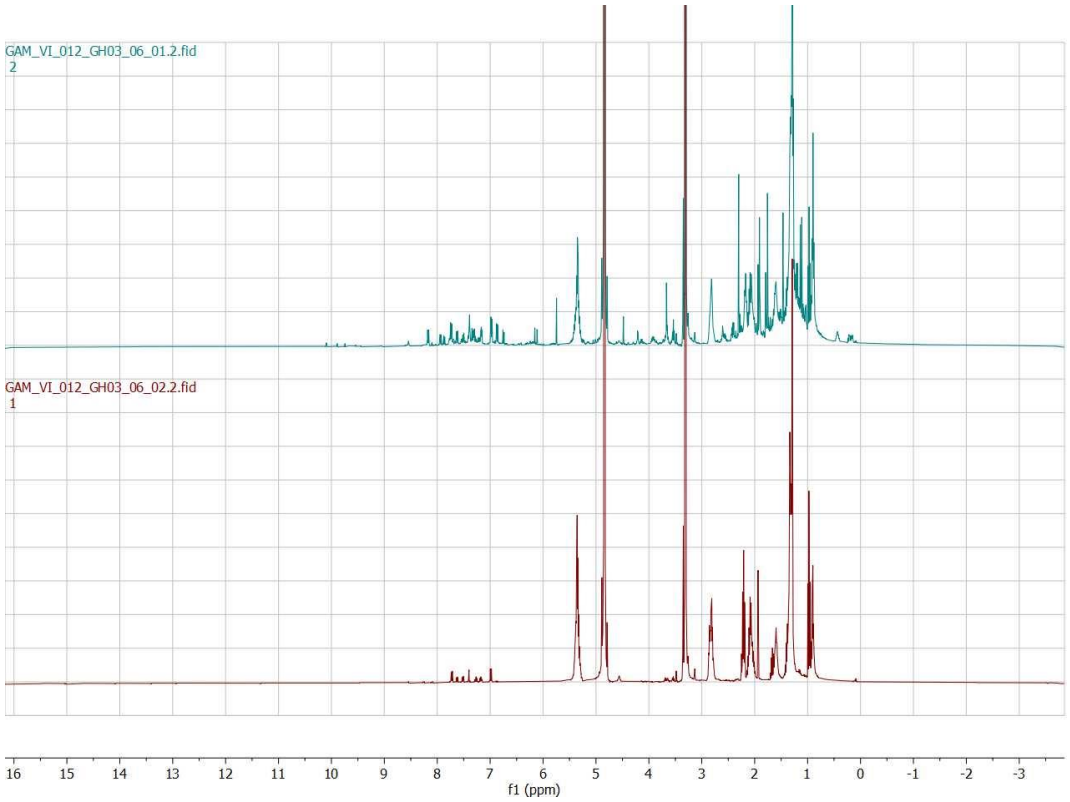

Figure 4.39. Proton NMR (deuterated methanol ,400 MHz) spectra of fractions GH03-06_1-2 of HPLC 02.

4.9. HPLC 03 of GH03-06_1-2 from CV1

Fractions GH03-06_1 and GH03-06_2 (10.8 mg combined) were mixed and dissolved in MeOH in order to pursue the isolation by HPLC.

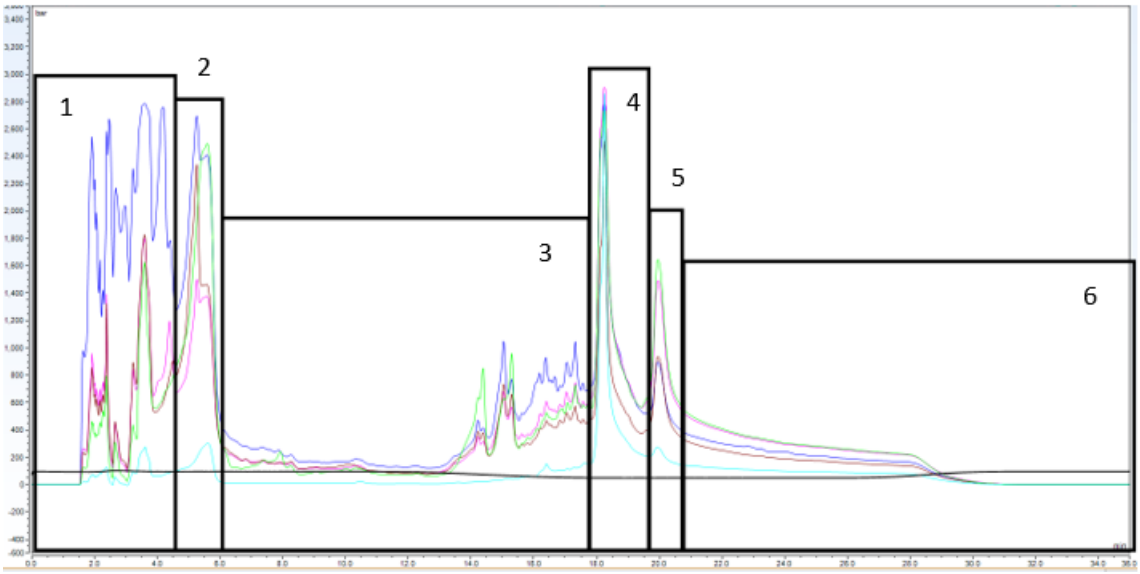

Figure 4.40. Chromatogram of the HPLC 03 run, with the definition of the resulted fractions.

The HPLC resulted in six fractions (GH03-06_1-2_1 to GH03-06_1-2_6), that are represented in Figure 4.40, in a total amount of 9.9 mg distributed by the six fractions as it is possible to see in Table 4.16, revealing a yield of 92%.

Table 4.16. Fractions 1 to 6 of the HPLC 03 run.

	Fraction (mg)
GH03-06_1-2_1	4.4
GH03-06_1-2_2	1.4
GH03-06_1-2_3	2.1
GH03-06_1-2_4	0.6
GH03-06_1-2_5	0.5
GH03-06_1-2_6	0.9

4.9.1. Antimicrobial assays

The inhibition halos of each HPLC 03 fraction in Escherichia coli e Bacillus subtilis are represented in Table 4.17.

Table 4.17. Halos of inhibition of the HPLC 03 fractions from the Cape Verde biomass.

Strain / Fraction	***Bacillus subtilis***	***Escherichia coli***
GH03-06_1-2_1	No halo	No halo
GH03-06_1-2_2	No halo	No halo
GH03-06_1-2_3	0.65 cm	No halo
GH03-06_1-2_4	0.65 cm	No halo
GH03-06_1-2_5	0.65 cm	No halo
GH03-06_1-2_6	0.7 cm	No halo

None of the fractions from HPLC 03 revealed to have antimicrobial activity against *Escherichia coli*. In the other hand, four fractions showed activity against *Bacillus subtilis*, as it is possible to see in Figure 4.41. Fractions GH03-06_1-2_3 to GH03-06_1-2_6 from HPLC 03 lead to halos of inhibition in this microbial strain, with fraction 6 being the most active (halo of 7 mm).

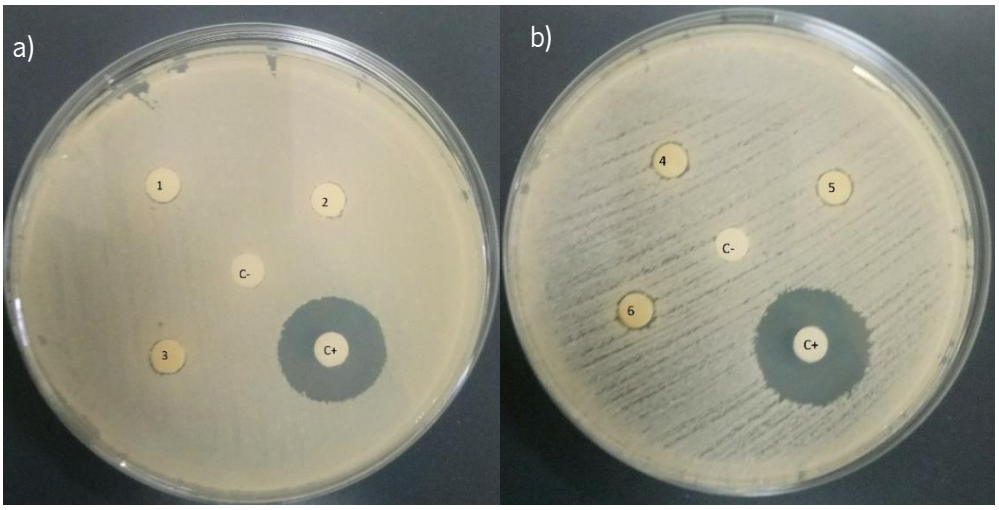

Figure 4.41. Halos of inhibition in Bacillus subtilis caused by HPLC 03 fractions a) Fraction 3; b) Fractions 4, 5 and 6; C- - Negative Control (DMSO); C+ - Positive Control (Kanamycin).

4.9.2. NMR analysis

All the fractions resulting of HPLC 03 were subjected to NMR analysis and after the data analysis, in Figure 4.42, fractions GH03-06_1-2_1 and GH03-06_1-2_2 seemed to be the most promising to isolate a pure compound, given the mass and NMR profile.

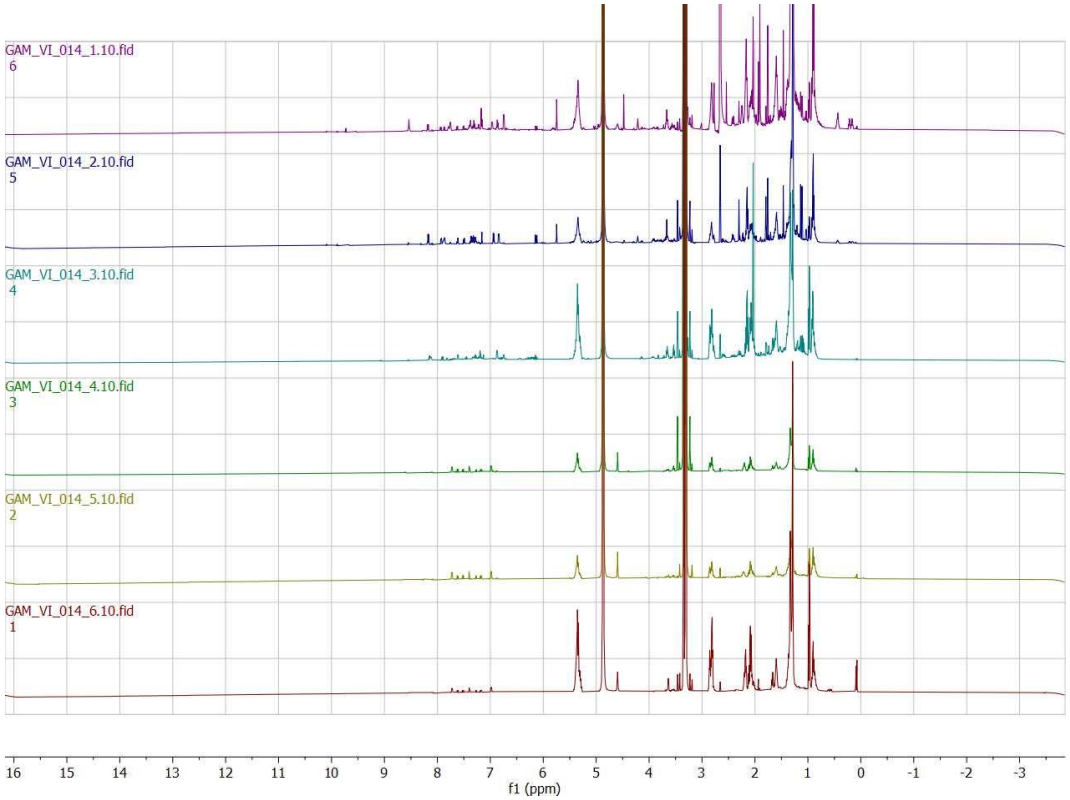

Figure 4.42. Proton NMR (deuterated methanol ,400 MHz) spectra of fractions GH03-06_1-2_1-6 of HPLC 03.

When comparing fractions GH03-06_1-2_1 and GH03-06_1-2_2 in their NMR spectra, Figure 4.43, the second fraction seem to be less complex and harbor only two or three major compounds. For that reason, fraction GH03-06_1-2_2 was chosen to pursue the isolation of a pure compound.

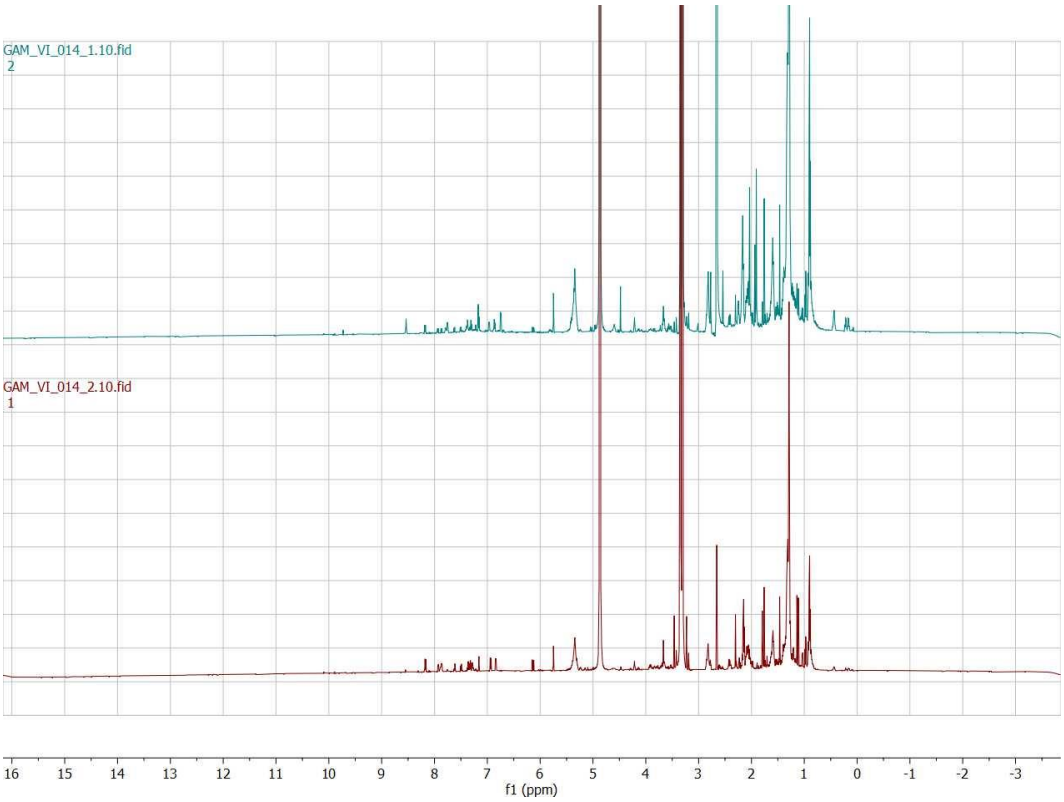

Figure 4.43. Proton NMR (deuterated methanol ,400 MHz) spectra of fractions GH03-06_1-2_1-2 of HPLC 03.

4.10. HPLC 04 of GH03-06_1-2_2 from CV1

Fraction GH03-06_1-2_2 (1.4 mg) was dissolved MeOH and prepared for the HPLC fractionation.

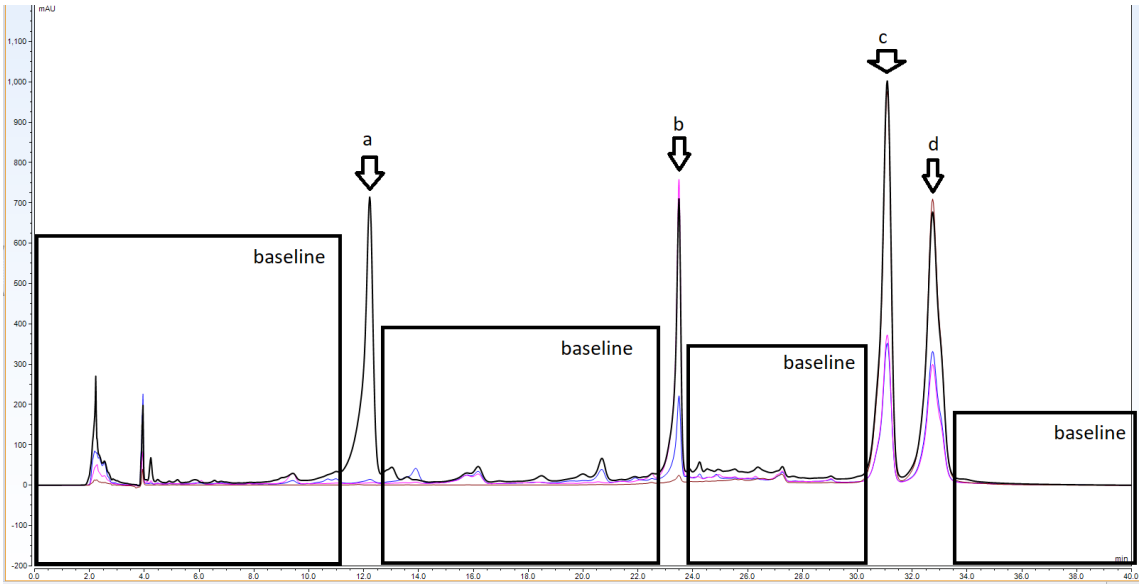

Figure 4.44. Chromatogram of the HPLC 04 run, with the definition of the resulted fractions.

The HPLC resulted in four fractions (peaks) plus baseline that was collected apart (GH03-06_1-2a to GH03-06_1-2_2d), that are represented in Figure 4.44, in a total amount of 1.5 mg distributed by the four fractions and baseline as it is possible to see in Table 4.18, with yield of 109%.

Table 4.18. Fractions a to d plus baseline from HPLC 04 run.

	Fraction (mg)
GH03-06_1-2_2a	0.6
GH03-06_1-2_2b	0.1
GH03-06_1-2_2c	0.2
GH03-06_1-2_2d	0.4
Baseline	0.3
Total	1.6

4.10.1. NMR analysis

Fractions GH03-06_1-2_2a to GH03-06_1-2_2d obtained from HPLC 04 were subjected to NMR analysis, in Figure 4.45.

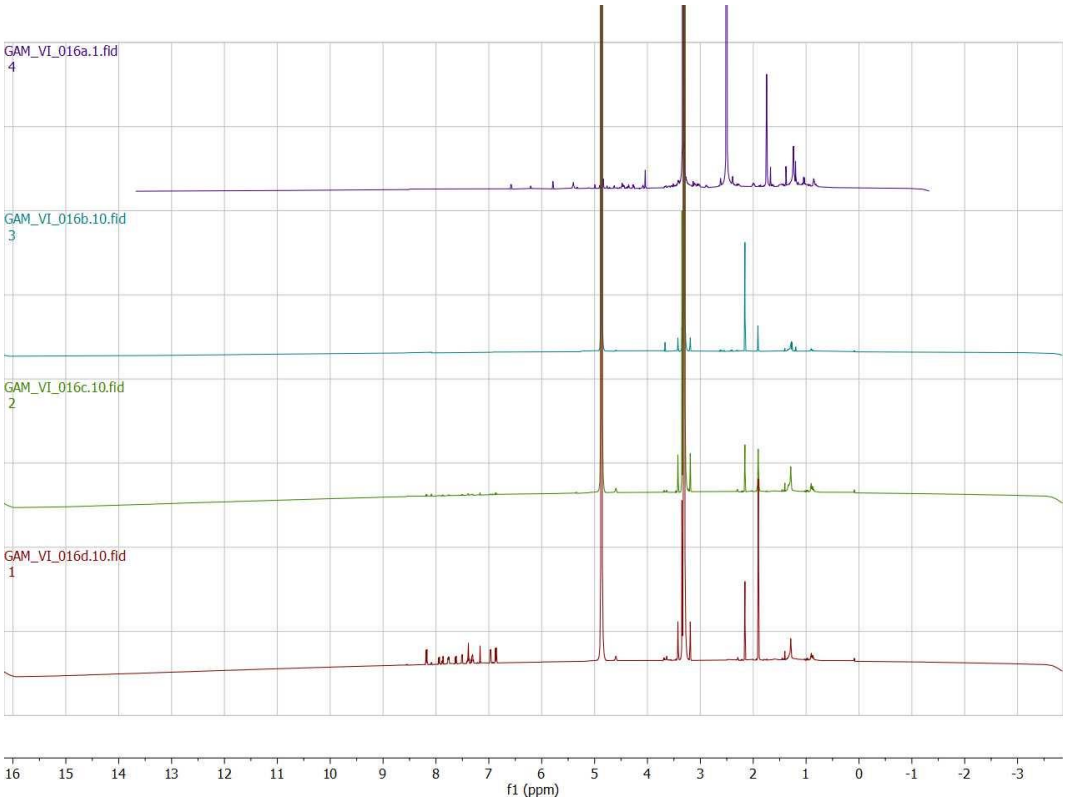

Figure 4.45. Proton NMR (DMSO deuterated, 600 MHz) spectra of fractions GH03-06_1-2_2a-d of HPLC 04.

Fraction GH03-06_1-2_2d appear to include only one major compound, with minor impurities and for that it was subjected to LC/HRESIMS analysis (Figure 4.46) in order to find the m/z of the major compound.

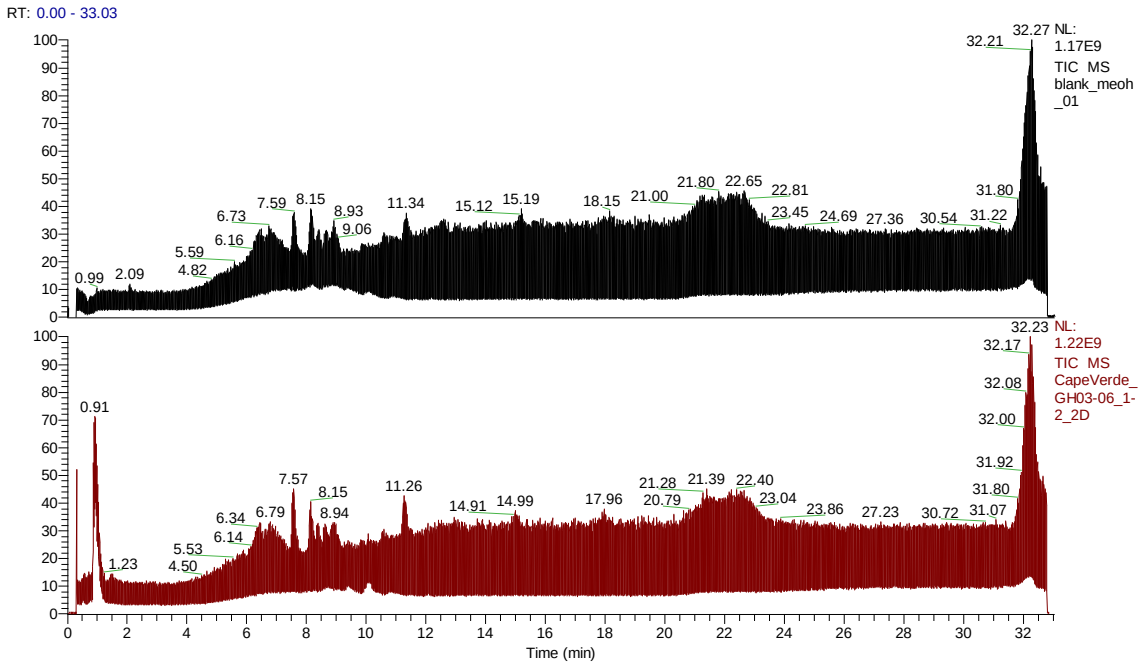

Figure 4.46. LC/HRESIMS chromatogram of fraction GH03-06_1-2_2d (bottom) and blank (top) in switching mode (positive and negative).

In comparison to the blank, the major difference in the chromatogram is at retention time 0.91 min, and the relative abundance of the picks in that retention time is represented in Figure 4.47.

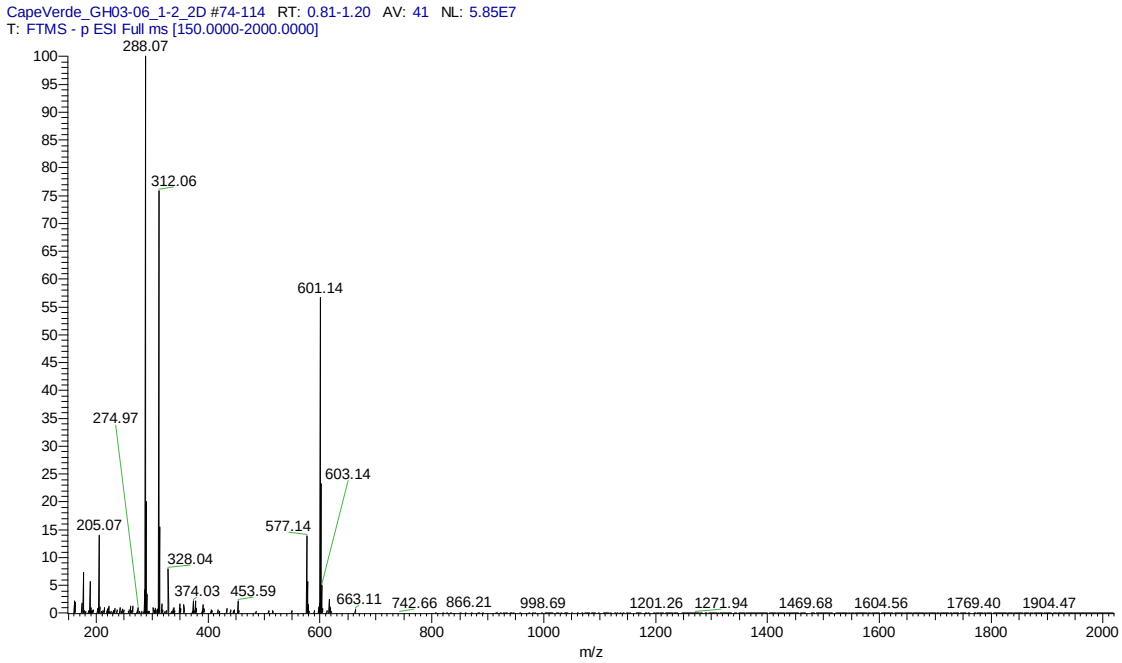

Figure 4.47. Relative abundance at retention time 0.81 - 1.20 min (with pick at 0.91 min).

The m/z retrieved from the LC/HRESIMS analysis was 288.07 in the negative mode, which indicates that the neutral m/z is 289.07. In the dereplication process a compound produced by cyanobacteria was found with that exact mass, Nostodione A (National Center for Biotechnology Information, 2021), which indicates we reach an already know compound.

In Figure 4.48 is represented the fractionation scheme that led to the isolated compounds, in the antimicrobial pathway.

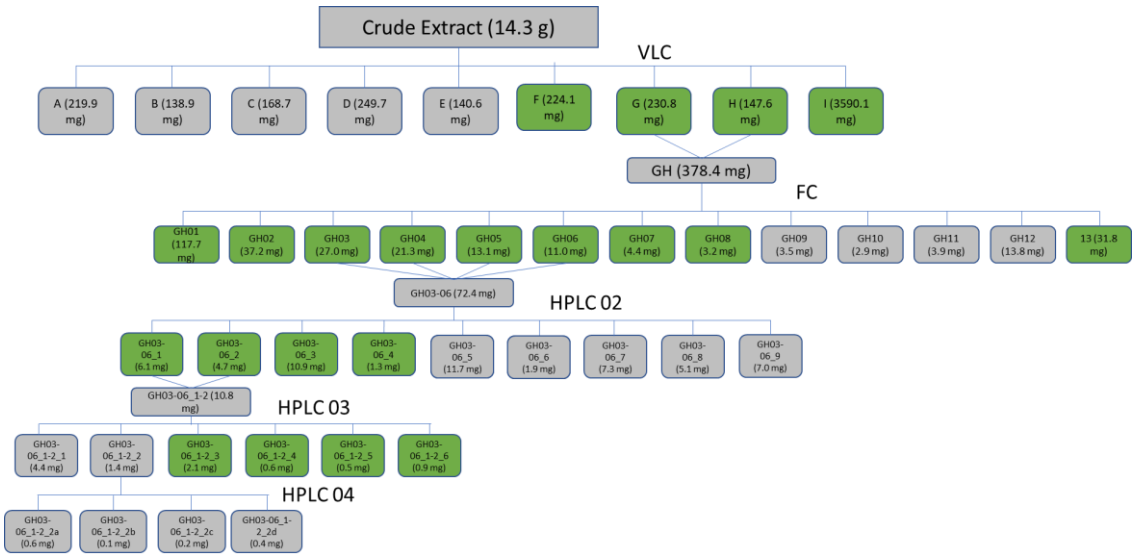

Figure 4.48. Resume of the fractionation of CV1 in the antimicrobial pathway.

5. Discussion

5.1. New strategy for NP discovery in cyanobacteria

In the course of this dissertation work, six strains from different origins and proceeding of different culture collections were subjected to the supplementation assays. The lack of homogeneity in some of the supplementation experiments can lead to difficulties in the final mass of the organic extracts between replicates. However, this did not prove to be a handicap for the analysis of the results. From a preliminary analysis of the LC-HRESIMS data, some molecular features that incorporated the labelled-FA substrates could be detected that could represent new natural products to be isolated in the future. However, the search for these potential new compounds were not further pursued, since three new compounds from *Nodularia sp.* LEGE 06071 had already been discovered in the lab through this methodology, and their study was prioritized. Accordingly, at the end of this study, two out of the three new compounds produced by *Nodularia sp.* LEGE 06071, nocuolactylates A and B, were isolated and their bioactivity in cytotoxic and antimicrobial assays evaluated.

The cyanobacterial strain *Nodularia sp.* LEGE 06071 was grown in culture for months to obtain enough biomass to isolate the new compounds and then the biomass was extracted and further fractionated until nocuolactylates A and B were obtain with a 95% purity. The next step was to study the cytotoxicity, but the number of assays to evaluate the bioactivity of the new compounds was limited due to the low amount of compound previously isolated. Still, we were able to do two independent cytotoxic assays, testing each compound twice in each cell line, in different weeks, and one round of antimicrobial assay, with bioactivity revealed in both.

Nocuolactylates A and B showed similar results in the cytotoxic assays, with the IC_{50} and GR_{50} values being in the same range, in the micromolar range.

Nocuolactylate A revealed to have higher activity in the cell line MCF7 (GR_{50} = 1.74 μM) and lower activity in hCMEC/D3 (GR_{50} = 5.90 μM), which was desirable, since hCMEC/D3 is the non-tumor cell line tested. The other new compound tested also showed to be less active in the non-tumor cell line (GR_{50} = 5.88 μM), while for the highest activity the two assays exhibit different results. The highest activity was found in MCF7 cell line (GR_{50} = 2.30 μM) that seemed to be more affected by nocuolactylate B.

Nocuolin A was overall more active than the two nocuolactylates tested. The non-tumor cell line hCMEC/D3 was the one that revealed to be less sensitive to nocuolin A (GR_{50} = 0.84 μM). This compound showed to be more active in the cell line MCF7 (GR_{50} = 0.31 μM). Paclitaxel was, by far,

the compound that presented the highest bioactivity in all assays against the four cell lines, with the IC_{50} and GR_{50} values falling in the nanomolar range. It showed to be less active in MCF7 (GR_{50} = 0.0371 μM) and more active in in hCMEC/D3 (GR_{50} = 0.00497 μM), which was not foreseen, since this is a non-tumoral cell line, and this compound is already used in clinic.

However, it is important to take into consideration that despite being used as a non-tumoral cell line and, therefore, as a safety representative, hCMEC/D3 cells are immortalized to facilitate the cell culturing. This characteristics *per se*, makes these cells closer to cancer cells by definition. So, non-toxic compounds for non-tumor cells can exhibit *in vitro* activity against non-tumoral cell lines, such as hCMEC/D3, if their target is related to proliferation and/or immortalization.

The differences observed in the IC_{50} and GR_{50} values were expected since GR_{50} is supposed to be more accurate given that overcomes the IC_{50} flaw of not considering the cell growth rate. This way, the GR_{50} values should be more reproducible then the IC_{50} which values can result in multiple false-positive and negative (Hafner et al., 2016). It was also possible to notice that the GR_{50} values were lower than the IC_{50} ones in almost every case, and the variability within the treatment was also lower for the new metric used.

Regarding the antimicrobial activity of the new compounds produced by *Nodularia sp.* LEGE 06071 it was possible to observe that nocuolactylate A was active against the gram-positive bacteria *Bacillus subtilis*, presenting a diffuse halo with 8 mm diameter. The presence of this halo reveals that this compound has some type of activity against this strain, even if it does not completely inhibit the microbial growth. To further evaluate the antimicrobial activity of this compound the minimum inhibitory concentration could be determined to realize the minimum dose required to completely inhibit the growth of *Bacillus subtilis*. This assay was not performed given the low amount of nocuolactylate A available.

Although the activity of these two new cyanobacterial natural products is not as high as the activity of the already tested (nocuolin A) and approved drugs (paclitaxel and kanamycin), these compounds can still be optimized, by semi-synthesis for example, to retrieve more effective compounds. Moreover, these compounds present some structural peculiarities and are structurally related to nocuolin A, which can be used for further structure-activity relationship studies.

5.2. Environmental biomass evaluation

The environmental biomass from Cape Verde, CV1, was subjected to an organic extraction, followed by several steps of fractionation and biological assays in pursuit of a pure bioactive

compound. The organic extraction of this environmental biomass revealed a low yield (3.1%) which can be explained by the composition of the sample, that contained the cyanobacterial biomass but also other impurities, such as inorganic matter as mineral and sediments.

After the organic extract, the resulting crude extract was fractionated through a VLC and 9 fractions were obtained (A to I). Six of those fractions revealed to have activity in the cytotoxic assays (D, E, F, G, H and I) while in the antimicrobial assays five fractions showed activity (A, F, G, H and I). Fraction I was the most active in two cell lines (HCT-116 and MG-63) while fractions H was the most active in the remaining three cell lines (HepG2, MCF7 and hCMEC/D3). In the antimicrobial assay fraction H was the most active against *Escherichia coli*, fraction A was the most active in *Bacillus subtilis* and both fractions (A and H) were equally active against *Candida albicans*. The VLC fractions were subjected to NMR analysis and fractions G and H that presented a similar profile and were two of the most active fractions in both assays were chosen to pursue the fractionation through a flash chromatography.

The FC of the fractions G and H obtained from the VLC originated 13 fractions (GH01 to GH13). All the fractions revealed to have activity in at least one assay in the cytotoxic evaluation, while nine fractions had activity in the antimicrobial assay (GH01, GH02, GH03, GH04, GH05, GH06, GH07, GH08 and GH13). Fraction GH09 was the most active against the HCT-116 cell line while fraction GH10 was the most active in the remaining four cell lines (HepG2, MG-63, MCF7 and hCMEC/D3). In the antimicrobial assays, fraction GH13 was the most active against *Escherichia coli*, fraction GH02 was the most active against *Staphylococcus aureus*, fraction GH04 was the most active against *Candida albicans* while fractions GH04 and GH05 were the most actives against *Bacillus subtilis* with the biggest halos observed in this round of assays (11 mm).

Generally, fractions G and H reduce the cell viability between 21% (MCF7 cell line) and 53% (hCMEC/D3 cell line) and the FC fractions with higher activity (GH09 and GH10) reduced the cell viability between 31 (HCT-116 cell line) and 76% (hCMEC/D3 cell line).

Analyzing the cytotoxic activity in the VLC and FC fractions it is possible to verify that the activity increased with the fractionation – i.e., the FC fractions with higher activity reduced the cells viability more than the VLC fractions that were used in the FC. This increase of the activity with the fractionation was expectable given that the compounds are more concentrated in a more fractionated sample.

When we analyze the results of the antimicrobial assays for the VLC fractions and the FC fractions together, it can be observed that in some cases the VLC fractions presented activity and

that was maintained or increased in the FC fractions, but in other cases the VLC fractions were not active, and the FC fractions demonstrated activity.

In *Salmonella* both sets of fractions (VLC and FC) failed to present inhibition halos and for that no activity was found. Regarding *Escherichia coli*, the FC fractions revealed to be less active than the VLC fractions, given that the clear halos had the same size (8 mm) but in FC fractions no blurry halos were found. In *Candida albicans*, *Staphylococcus aureus* and *Bacillus subtilis* the activity increased from VLC to FC fractions. Although the biggest increase in the activity was find in *Bacillus subtilis* (halos varied from 6.5 mm to 11 mm), the data from *Staphylococcus aureus* was equally or more interesting, given that the VLC fractions did not produce a halo and FC fractions presented halos up to 8 mm.

Globally fractions GH04 and GH05 were the most active in the antimicrobial assays while fractions GH09 and GH10 were the most active in the cytotoxic assay. Given that, and after LC-HRESIMS analysis, two separate paths were followed to pursue the isolation of the active compounds responsible for these two biological activities.

For the cytotoxic activity, fractions GH09, GH10 and GH11 were pooled together, and the fractionation was pursued through an HPLC (HPLC 01). Fractions GH09 and GH10 were the ones that presented more activity against the cell lines used and fraction GH11 has a representative amount and shared some of the peaks in the LC-HRESIMS spectrum of these fractions. These HPLC resulted in 8 fractions (GH09-11_01 to GH09-11_08) but no further assays to evaluate the cytotoxicity were conducted given the low amount in all the fractions.

In the antimicrobial path, fractions GH04 and GH05 were the ones with higher activity and fractions GH03 and GH06 were pooled together given that the profile of the LC-HRESIMS was similar and they have a substantial amount.

The first step in this pathway was an HPLC (HPLC 02) of fractions GH03 to GH06 that originated nine subfractions (GH03-06_1 to GH03-06_9). These nine fractions were evaluated for their antimicrobial activity and the first four fractions had effect against the microbial strains tested. After NMR analysis, two similar fractions were pooled together and subjected to another HPLC (HPLC 03). This second HPLC in the antimicrobial pathway originated six fractions (GH03-06_01-02_1 to GH03-06_01-02_6), that were posteriorly evaluated on their antimicrobial activity against *Escherichia coli* and *Bacillus subtilis*. Due to the low amount of the fractions, they were only tested against these two strains which were the ones with higher activity in the previous fractionation.

Four fractions of the HLPC 03 had activity against *Bacillus subtilis*, with fraction GH03-06_1-2_6 being the most active with a halo of 7 mm.

Fraction GH03-06_01-02_2 was chosen to pursue the isolation of a pure compound given that by NMR analysis it seemed to be the less complex fraction with only two or three major compounds. Another HPLC was conducted, HPLC 04, and four fractions were harvested (GH03-06_01-02_2a to GH03-06_01-02_2d) and subjected to proton NMR analysis. Fraction GH03-06_01-02_2d appear to be almost pure, with minor impurities and revealed to be an already know compound, Nostodione A. This compound is a fragment of scytonemin, a cyanobacterial NP with UV screening capability, and plays a role in the scytonemin biosynthetic pathway (Ekebergh et al., 2012).

Scytonemin is one of the most common compounds with photoprotective properties discovered in cyanobacteria and to be used in new cosmetic formulations. It is also one of the major group of compounds present in cyanobacteria exposed to high levels of stress and UV which is one of the reasons that these compounds are frequently discovered in environmental biomasses as in this case (Kumar et al., 2018).

6. Conclusion and future work

The two methods used in this work proved efficient in revealing compounds with bioactivity: the supplementation assays proved to be a new and efficient method to find new natural products that revealed to have cytotoxic and antimicrobial activities; by the evaluation of an environmental biomass, several bioactive fractions were obtained in both assays, and we were able to achieve an almost pure compound.

The new methodology developed in our laboratory could in fact lead to the discovery of new compounds produced by cyanobacteria, namely the nocuolactylates. In the future, the six strains that were subjected to the supplementation assay with deuterium-labelled fatty acid will be thoroughly analyzed in order to verify if there are new compounds containing fatty acid residues being produced by them. Also, new cyanobacterial strains should be subjected to this assay, in order to expand the diversity of chemical structures that incorporate fatty acid residues. Provided that new compounds are discovered, it will be necessary to grow the referred strains in large scale to isolate those compounds.

In the bioactivity-guided approach, where the Cape Verde biomass CV1 was tested, the sequence of chromatographic techniques followed by bioactivity assays and spectrometric analysis led us to isolate an almost pure compound and for that we can consider that we reached the purposed goals.

The structural characterization of the almost pure compounds will also be concluded. In addition, other bioactive fractions that were not further pursued in this experimental work, but that presented bioactivity can also be further studied in an attempt to reach more pure compounds with bioactivity.

In general, the goals for this work were all achieved and it was able to verify the differences, advantages and disadvantages of the two methods we pursue. With this work, the knowledge of cyanobacteria and new NPs that these organisms produce were also enrichment which was also very important.

7. References

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8. Work dissemination

Part of this work, namely relative to the bioactivity of the nocuolactylates, was presented in a poster format in the Blue Think Conference 2020, presented in Figure 8.1.

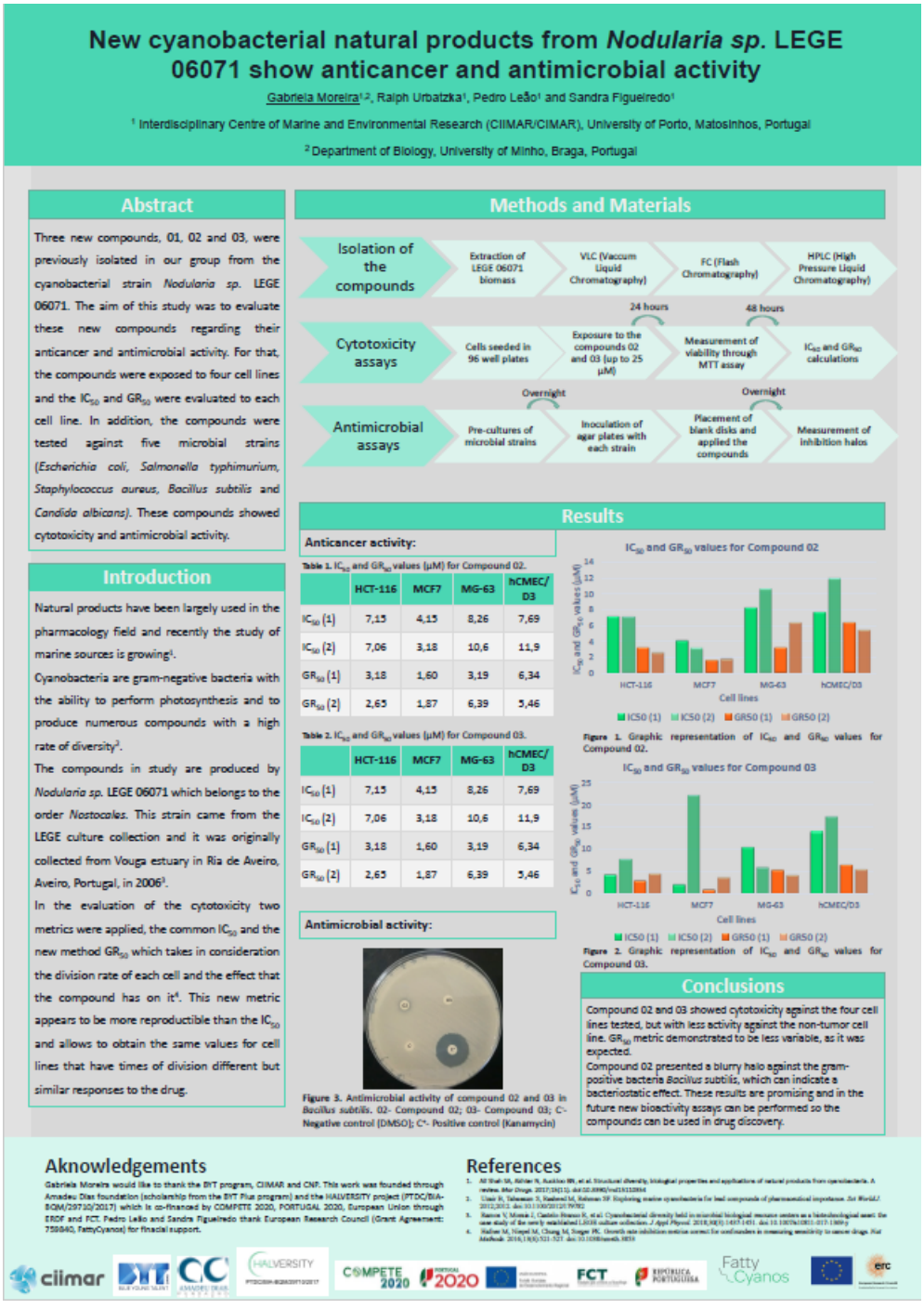

Figure 8.1. Poster presentation in the Blue Think Conference 2020.

These results were also presented in the BYT final session 2019/2020 and a report in article format was submitted to the BYT committee.

The results related to the nocuolactylates were also described in an article, named "Discovery of cyanobacterial natural products containing fatty acid residues" available in https://doi.org/10.1002/anie.202015105.

9. Appendix

Table 9.1. IC_{50} and GR_{50} values for nocuolactylates A and B, paclitaxel and nocuolin A on the cell line HCT-116.

Compound	Assay	IC_{50} (µM)	GR_{50} (µM)
Nocuolactylate A	1	7.15	3.18
	2	7.06	2.65
Nocuolactylate B	1	4.35	2.93
	2	7.74	4.45
Paclitaxel	1	0.0101	0.00615
	2	0.0153	0.0103
Nocuolin A	1	0.997	0.686
	2	1.44	0.403

Table 9.2. IC_{50} and GR_{50} values for nocuolactylates A, B, paclitaxel and nocuolin A on the cell line MG-63.

Compound	Assay	IC_{50} (µM)	GR_{50} (µM)
Nocuolactylate A	1	8.26	3.19
	2	10.6	6.39
Nocuolactylate B	1	10.5	5.25
	2	6.05	4.12
Paclitaxel	1	0.0705	0.0257
	2	0.0334	0.00881
Nocuolin A	1	0.740	0.488
	2	0.779	0.688

Table 9.3. IC_{50} and GR_{50} values for nocuolactylates A and B, paclitaxel and nocuolin A on the cell line MCF7.

Compound	Assay	IC_{50} (µM)	GR_{50} (µM)
Nocuolactylate A	1	4.15	1.60
	2	3.18	1.87
Nocuolactylate B	1	2.15	0.972
	2	22.2	3.63
Paclitaxel	1	0.174	0.0262
	2	0.0172	0.0480
Nocuolin A	1	2.15	0.340
	2	0.620	0.286

Table 9.4. IC_{50} and GR_{50} values for compounds 02, 03, paclitaxel and nocuolin A on the cell line hCMEC/D3.

Compound	Assay	IC_{50} (µM)	GR_{50} (µM)
Nocuolactylate A	1	7.69	6.34
	2	11.9	5.46
Nocuolactylate B	1	13.9	6.39
	2	17.4	5.37
Paclitaxel	1	0.0166	0.00278
	2	0.0559	0.00715
Nocuolin A	1	2.10	0.777
	2	1.33	0.905