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Endophytic Actinobacteria from *Laminaria ochroleuca*: a new source of bioactive compounds

Mariana Girão Silva Martins

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Mariana Girão Silva Martins

Dissertação de Mestrado apresentada à
Faculdade de Ciências da Universidade do Porto em
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Mestrado em Biologia Celular e Molecular

Departamento de Biologia da Faculdade de Ciências da Universidade do Porto (FCUP)

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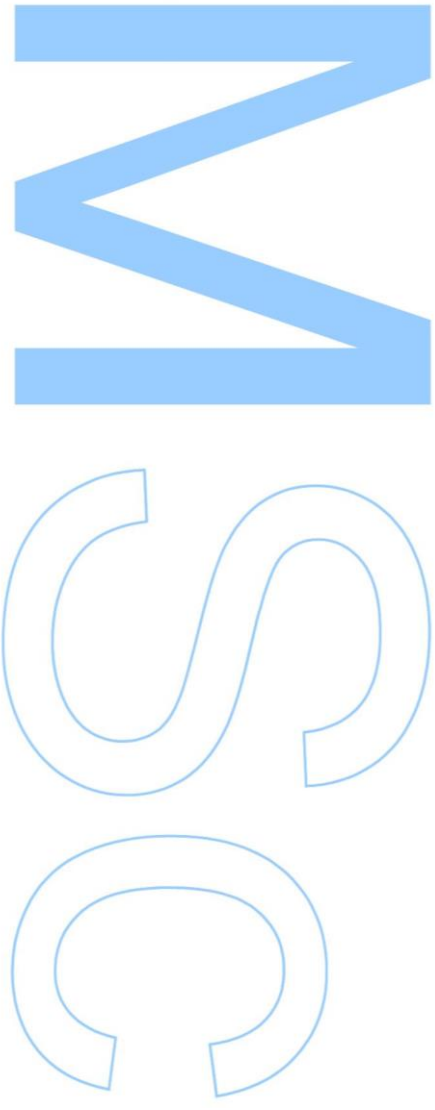

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Abstract

Nature is the major reservoir of biologically active molecules. The urgent need of finding novel molecules with pharmaceutical interest is prompting the research of underexplored environments, such as marine ecosystems. In this regard, marine actinobacteria represent a remarkable source of biologically active compounds. Marine actinobacteria often live in association with other organisms, representing unique ecological niches for the discovery of new bioactive substances. Despite the proven potential of actinobacteria as source of secondary metabolites with pharmacologically-relevant activities, only few studies have focused on actinobacteria associated with macroalgae. This study aimed to investigate the cultivable community of endophytic actinobacteria associated with the macroalgae *Laminaria ochroleuca* and assess its potential to produce compounds with antimicrobial and anticancer activities. Fragments of tissues from different parts of *L. ochroleuca* (holdfast, stipe and blade), collected in a rocky shore in northern Portugal, were surface sterilized and plated in three culture media selective for actinobacteria. A total of 90 actinobacterial strains were isolated, with the majority being affiliated with the genus *Streptomyces*. Isolates associated with the genera *Isoptericola*, *Rhodococcus*, *Nonomuraeae*, *Nocardiopsis*, *Microbispora* and *Microbacterium* were also obtained. Extracts from all actinobacterial isolates were tested for their antimicrobial activity using the agar-based disk diffusion method, followed by determination of MIC values for extracts showing relevant activities. Forty-five isolates inhibited, to some degree, the growth of *Candida albicans* and/or *Staphylococcus aureus*, with MIC values ranging from < 0.487 to 1000 µg/mL. The actinobacterial isolates were also tested for their anticancer potential in two human carcinogenic cell lines (breast carcinoma T-47D and neuroblastoma SH-SY5Y) and compared to their cytotoxicity on a non-carcinogenic endothelial cell line (hCMEC/D3). Thirty isolates exhibited anticancer activity in at least one of the tested cell lines, decreasing their viability to less than 50%. Four isolates were identified with strong cytotoxic activity on SH-SY5Y cells, but with low activity on hCMEC/D3 cells. According to LC-MS/MS dereplication data, the activity of most extracts may be associated with the presence of secondary metabolites antimycins. The active strain *Streptomyces* sp. KENR25 was selected for a bioassay-guided fractionation, following part of a traditional natural product discovery pipeline, with several fractions showing mainly antimicrobial activity. This study reveals that *L. ochroleuca* is a rich source of actinobacteria with promising antimicrobial and anticancer activities and further supports that macroalgae may be a valuable resource of actinobacteria and, consequently, of new molecules with biotechnological importance.

Keywords

marine actinobacteria, endophytic actinobacteria, bioactivity, antimicrobial, anticancer, macroalgae, kelp, *Laminaria ochroleuca*

Resumo

A natureza constitui o maior reservatório de moléculas biologicamente ativas. A urgente necessidade de encontrar novas moléculas com interesse farmacêutico tem incentivado a investigação de ambientes pouco explorados, como os ecossistemas marinhos. Neste sentido, as actinobactérias marinhas são uma fonte notável de compostos biologicamente ativos. As actinobactérias marinhas vivem muitas vezes em associação com variados organismos, representando nichos ecológicos únicos para a descoberta de novas substâncias bioativas. Apesar do enorme potencial das actinobactérias como fonte de metabolitos secundários com atividades farmacológicas relevantes, poucos estudos se têm focado no potencial de actinobactérias associadas a macroalgas. Este estudo teve como objetivo investigar a comunidade cultivável de actinobactérias endófitas associadas à macroalga *Laminaria ochroleuca* e avaliar o seu potencial para produzir compostos com atividades antimicrobiana e anticancerígena. Fragmentos de tecidos de diferentes partes da alga *L. ochroleuca* (rizoide, estipe e lâmina), recolhida na costa rochosa norte de Portugal, foram esterilizados na sua superfície e plaqueados em três meios de cultura seletivos para actinobactérias. Um total de 90 estirpes de actinobactérias foram isoladas, estando a a maioria afiliada com o género *Streptomyces*. Isolados associados com os géneros *Isoptericola*, *Rhodococcus*, *Nonomuraeae*, *Nocardiopsis*, *Microbispora* e *Microbacterium* também foram obtidos. Todos os extratos actinobacterianos foram testados quanto à sua atividade antimicrobiana usando o método de difusão em agar, seguindo-se a determinação do MIC para extratos com atividade relevante. Quarenta e cinco isolados inibiram o crescimento de *Candida albicans* e/ou *Staphylococcus aureus*, com valores de MIC entre < 0.487 e 1000 µg/mL. O potencial anticancerigeno dos isolados foi também testado em duas linhas celulares carcinogénicas humanas (carcinoma da mama T-47D e neuroblastoma SH-SY5Y) e a sua citotoxicidade comparada numa linha celular não-carcinogénica (hCMEC/D3). Trinta isolados exibiram atividade anticancerígena em pelo menos uma das linhas celulares testadas, diminuindo a viabilidade das células para menos de 50%. Quatro isolados foram identificados com uma forte atividade citotóxica nas células SH-SY5Y, mas com pouca atividade nas células hCMEC/D3. De acordo com os dados de desreplicação, a atividade da maioria dos extratos está associada com a presença dos metabolitos secundários antimicinas. A estirpe ativa KENR25 foi selecionada para um fracionamento guiado por ensaios de bioatividade, seguindo parte do procedimento tradicional para a descoberta de produtos naturais, onde várias frações mostraram principalmente atividade antimicrobiana. Este estudo revela que a alga *L. ochroleuca* é uma fonte rica

de actinobactérias com promissoras atividades antimicrobianas e anticancerígenas, suportando a ideia de que as macroalgas podem ser um recurso valioso de actinobactérias e, consequentemente, de novas moléculas com importância biotecnológica.

Palavras-chave

actinobactérias marinhas, actinobactérias endófitas, bioatividade, atividade antimicrobiana, atividade anticancerígena, macroalga, *kelp*, *Laminaria ochroleuca*

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List of Abreviations

16S rRNA	16S ribosomal RNA
BLAST	Basic Local Alignment Search Tool
BP	Base pair
CTAB	Cetyl trimethylammonium bromide
DMEM	Dubelco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EtOAc	Ethyl acetate
FC	Flash Chromatography
GNPS	Global Natural Product Social Molecular Networking
HPLC	High performance liquid chromatography
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MIC	Minimal Inhibitory Concentration
MH	Mueller-Hinton
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
NCBI	National Center for Biotechnology Information
NPS	Nutrient-Poor Sediment
OD	Optical Density
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
PMA	Phosphomolybdic Acid
RH	Raffinose-Histidine
SCN	Starch-Casein-Nitrate
SD	Sabouraud Dextrose
TLC	Thin-Layer Chromatography
VLC	Vacuum Liquid Chromatography

I. Introduction

Human health is constantly and increasingly under the threat of several disorders that demand urgent solutions, such as cancer pathologies or the increasing incidence of antibiotic multi-resistant microbial species. The search for novel bioactive compounds is an effective approach to tackle this problematic, with the natural environment still being the major source to find such molecules [1, 2]. Taking cancer as an example, from 1940 to the end of 2014, about half of the approved drugs for combating this disease were either natural products (NPs) or compounds directly derived from them [3]. Among the different natural sources from which bioactive molecules may derive, microorganisms play a prominent role: from bacteria to fungi, microorganisms have proved their ability to produce a wide range of NPs exhibiting numerous biological activities [4]. Within the domain Bacteria, the phylum Actinobacteria plays a major role in the production of bioactive compounds, especially the species belonging to the order Actinomycetales, commonly known as actinomycetes. So far, more than 10.000 different bioactive compounds produced by actinomycetes have been identified, representing about 50% of all bioactive metabolites obtained from microorganisms, with most of them being derived from the genus *Streptomyces* and a smaller fraction from the so-called rare actinomycetes [5, 6]. From the pharmaceutical, biotechnological, industrial and economic point of view, actinomycetes are amongst the most valuable microorganisms, being capable of producing antibiotics, antimicrobials, anti-inflammatory, anticancer and antitumor agents, immunosuppressive compounds, etc. [3, 7]. Despite the wide range of known bioactive compounds and their significant applications, the discovery of new natural molecules is coming to a stagnation point. However, this reality is not in agreement with the data obtained from genomic analysis which suggest that only about 10% of microbial secondary metabolites are known [8]. The decrease in the discovery of new bioactive compounds and the increase in the re-isolation of known ones, may be explained by the fact that in the last decades, most of the bioactivity screening programmes have focused on highly explored environments, mainly terrestrial ones [7, 9]. Considering the need of finding novel secondary metabolites with bioactive action, it is highly promising to investigate actinobacteria from unexplored or underexplored habitats, such as the marine environment. The conditions found in marine environments are very different from those occurring in terrestrial ones, which translates in different microbial evolutionary mechanisms and, consequently, in different metabolisms that may lead to the production of novel differentiated chemical molecules [10].

1. Phylum Actinobacteria

Actinobacteria are filamentous Gram-positive bacteria, usually with a high guanine/ cytosine content in their DNA that can vary between 50% and 70% according to the species. The phylum Actinobacteria represents one of the largest taxonomic units within the domain Bacteria, both in number and diversity, including 6 classes, 25 orders, 52 families, and 232 genera [7, 11]. Microorganisms belonging to this phylum exhibit a wide variety of morphologies, from coccoid to fragmenting hyphal forms or highly differentiated branched mycelium, with several species being able to reproduce by sporulation. Actinobacteria species also exhibit diverse metabolic, physiological and ecological properties that result in the production of several secondary metabolites, many with biological activity [11]. These microorganisms are widely disseminated in both terrestrial and aquatic habitats, including marine ecosystems. They are usually more common in the soil where they play an important role in the recycling of organic matter but can also be found as plant symbionts (e.g., *Frankia* sp.), animal or plant pathogens (e.g., *Corynebacterium* sp.*, Mycobacterium* sp.*, Propionibacterium* sp. and *Nocardia* sp.) or even gastrointestinal commensals (e.g., *Bifidobacterium* sp.) [11, 12]. Since the 1950s, Actinobacteria from different sources, fundamentally from terrestrial ecosystems, have been studied for their high potential to produce bioactive compounds, resulting in the discovery of a wide range of clinically-relevant compounds. Anticancer, antitumor or antibacterial compounds are representative examples of the wide range of biological active molecules produced by these microorganisms [13, 14]. Actinobacteria also have a role as plant growth-promoting agents [15, 16] and play an important part in bioremediation processes, since they are able to metabolize complex organic compounds and to remove xenobiotics, such as pesticides and heavy metals, from the environment [17, 18]. One other characteristic of these microorganisms is their ability to produce and accumulate polyhydroxyalkanoates (PHAs), biodegradable and renewable polymers that may be used to produce disposable and biodegradable plastics or as scaffold material for tissue engineering or controlled drug delivering [19]. Given the immense biotechnological, industrial, ecological and biopharmaceutical applications of Actinobacteria, there is high interest in studying these microorganisms as potential sources of new compounds. Within the phylum Actinobacteria, the order Actinomycetales comprehends most of the taxonomic units responsible for the synthesis of bioactive secondary metabolites, and the terms actinobacteria or actinomycetes will be hereafter used as synonymous of microorganisms affiliated with this order. Among these taxonomic units, the genus *Streptomyces* is particularly relevant as these microorganisms are considered the primary producers of antibiotics, being highly exploited by the pharmaceutical industry,

with almost all the natural antibiotics currently in clinical use being derived from secondary metabolites of these bacteria [6, 20]. Recognizing the unmatched potential of actinomycetes to produce compounds with relevant bioactivities, these organisms continue to be primary targets to discover novel useful natural products [21].

2. Marine Actinobacteria

Historically, actinobacteria were thought to inhabit exclusively terrestrial environments and it was believed that their occurrence in the oceans was due to the transport of terrestrial spores to those regions. Today, it is known that this is false and that actinobacteria are widely distributed in the marine environment, with several indigenous marine actinobacteria being described. Among several others, representative genera of marine actinobacteria include *Actinomadura*, *Aeromicrobium*, *Dietzia*, *Gordonia*, *Marinophilus*, *Micromonospora*, *Nonomuraea*, *Rhodococcus*, *Saccharomonospora*, *Salinispora*, *Streptomyces*, *Solwaraspora*, *Williamsia*, and *Verrucosispora* [7]. Although their prevalence in sediments, they also exist in association with fishes, sponges, seaweeds or corals (Fig. 1) [7, 22]. Nevertheless, the ecological role, diversity, distribution and bioactive potential of marine actinobacteria are still largely unknown and require further study [13, 23]. The environmental conditions found in marine ecosystems are very different from terrestrial ones, driving unparalleled evolutionary mechanisms. For marine actinobacteria, this is reflected in novel capacities to produce secondary metabolites [7]. Given the fact that the discovery of novel bioactive compounds is reaching a stagnation point, marine actinobacteria pose as a promising source of novel natural products [13].

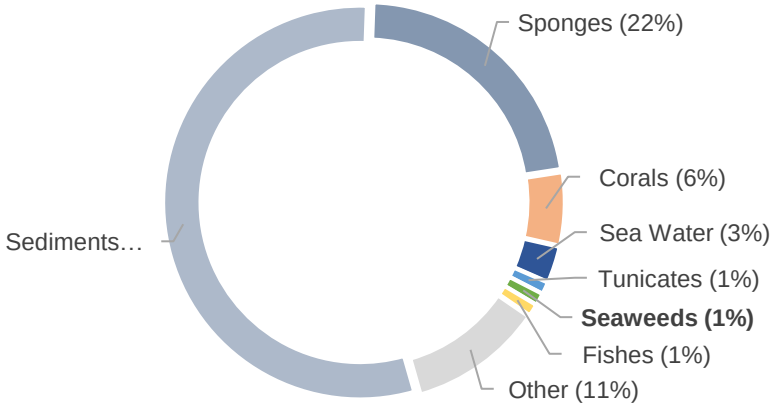


Figure 1. Distribution of actinobacteria in the marine environment, according to a total of 10 400 16S rRNA gene sequences retrieved from marine actinobacteria. Adapted from [22].

3. Marine Actinobacteria as a Source of Bioactive Compounds

The discovery of new drugs to help treating serious human diseases is a constant demanding and, in this respect, marine actinobacteria constitute a precious source for the search of these so needed compounds [1]. It is known that the biosynthesis of these compounds is usually triggered by a wide variety of environmental and physiological signals [24] and, since the last decade, marine actinobacteria have been attracting the attention of the scientists for their ability to produce novel compounds with antibacterial, antifungal, antiviral, antitumor, anticancer, cytotoxic, anti-inflammatory, anti-parasitic, anti-malarial and immunosuppressive activities [13, 14, 25, 26]. Table 1 presents a summary of bioactive compounds reported from marine actinobacteria, until 2017.

Table 1. Bioactive metabolites produced by marine actinobacteria, until 2017 (Adapted from [13, 14, 25])

Compound	Source	Biological Activity	Ref
Abyssomycins	*Verrucosispora* sp.	Antibacterial	[27]
Actinofuranones	*Streptomyces* sp.	Cytotoxic	[28]
Ammosamides	*Streptomyces* sp.	Cytotoxic	[29]
Antimycin A	*Streptomyces* sp.	Antifungal; Anticancer; Anti-inflammatory;	[30]
Arenamides	*Salinipora arenicola*	Cytotoxic	[31]
Arenicolides	*Salinispora arenicola*	Antitumor	[32]
Aureolic acid	*Streptomyces* sp.	Antitumor; Antibacterial	[33]
Aureoverticillactam	*Streptomyces aureoverticillatus*	Anticancer	[34]
Avermectins	*Streptomyces avermitilis*	Anti-parasitic	[35]
Benzastatin C	*Streptomyces nitrosporeus*	Antiviral	[36]
Bisanthraquinone	*Streptomyces* sp.	Antibacterial	[37]
Bonactin	*Streptomyces* sp.	Antibacterial; Antifungal	[38]
Butenolides	*Streptomyces* sp.	Antiviral; Cytotoxic	[39]
Caboxamycin	*Streptomyces* sp.	Anticancer	[40]
Caprolactones	*Streptomyces* sp.	Anticancer	[41]
Chalcomycin A	*Streptomyces* sp.	Antibacterial	[42]
Chandrananimycins	*Actinomadura* sp.	Antibacterial; Antifungal; Anticancer	[43]
Chartreusin	*Streptomyces chartreusis*	Antitumor	[44]
Chinikomycins	*Streptomyces* sp.	Anticancer	[45]
Chloro-dihydroquinones	Novel actinomycete	Antibacterial; Anticancer	[46]
Chromomycins	*Streptomyces griseus*	Antitumor	[33]
Curvularin-7-O-dglucopyranoside	*Pseudonocardia* sp.	Antibacterial; Anticancer	[47]
Cyclomarin A	*Streptomyces* sp.	Anti-inflammatory; Anti-Malarial; Anti-Tuberculosis	[48]
Daryamides	*Streptomyces* sp.	Antitumor; Antifungal	[49]
Diazepinomicin	*Micromonospora* sp.	Antibacterial; Anticancer; Anti-inflammatory	[50]
Essramycin	*Streptomyces* sp.	Antibacterial	[51]
Frigocyclinone	*Streptomyces griseus*	Antibacterial	[52]
Glaciapyrroles	*Streptomyces* sp.	Antibacterial	[53]
Gutingimycin	*Streptomyces* sp.	Antibacterial	[54]
Helquinoline	*Janibacter limosus*	Antibacterial	[55]
Himalomycins A, B	*Streptomyces* sp.	Antibacterial	[56]
IB-00208	*Actinomadura* sp.	Anticancer	[57]
Komodoquinone A	*Streptomyces* sp.	Neuritogenic activity	[58]
Lajollamycin	*Streptomyces nodosus*	Antibacterial	[59]
Lagumycin B	*Micromonospora* sp.	Antibacterial; Cytotoxic	[60]
Leptomycin B	*Streptomyces* sp.	Anti-Parasitic	[61]

Lipoxazolidinones A, B	*Marinispora* sp.	Antibacterial	[62]
Lucentamycins	*Nocardiopsis lucentensis*	Cytotoxic	[63]
Lynamicins	*Marinispora* sp.	Antibacterial	[64]
Manumycins	*Streptomyces* sp.	Antibacterial; Antitumor	[45]
Mansouramycin C	*Streptomyces* sp.	Cytotoxic	[65]
Marinomycins	*Marinispora* sp.	Antibacterial; Cytotoxic;	[66]
Marinopyrroles	*Streptomyces* sp.	Antibacterial; Anticancer	[67]
Mechercharmycins	*Thermoactinomyces* sp.	Anticancer	[68]
Monacyclinones	*Streptomyces* sp.	Antibacterial; Cytotoxic	[69]
N-acetyl-N-demethylmayamycin	*Streptomyces* sp.	Antibacterial; Antitumor	[70]
Neomarinone	Strain CNH-099 C	Cytotoxic	[71]
N-(2-hydroxyphenyl)-2-phenazinamine (NHP)	*Nocardia dassonvillei*	Antifungal; Anticancer	[72]
Pacificanones	*Salinispora pacifica*	Cytotoxic	[73]
Phenazines 1,2	*Streptomyces* sp.	Anti-inflammatory; Anticancer	[74]
Piericidins	*Streptomyces* sp.	Anticancer	[75]
Piperazimycins	*Streptomyces* sp.	Cytotoxic	[76]
Proximicins	*Verrucosispora* sp.	Cytostatic	[77]
Pyridinium	*Amycolatopsis alba*	Antibacterial; Cytotoxic	[78]
Resistoflavine	*Streptomyces chibaensis*	Cytotoxic	[79]
Saadamycin	*Streptomyces* sp.	Antifungal	[80]
Salinamides	*Streptomyces* sp.	Antibacterial; Anti-inflammatory	[81]
Saliniketals	*Salinispora arenicola*	Anticancer	[32]
Salinisporamycin	*Salinispora arenicora*	Antibacterial; Cytotoxic	[82]
Salinipyrones	*Salinispora pacifica*	Cytotoxic	[73]
Salinosporamide A*	*Salinispora tropica*	Anticancer	[83]
Selina-4(14),7(11)-diene-8,9-dio	*Streptomyces* sp.	Anticancer	[84]
Sisomicin	*Streptomyces* sp.	Antibacterial	[85]
Staurosporine	*Streptomyces* sp.	Anti-parasitic	[86]
Strepsesquitriol	*Streptomyces* sp.	Anticancer	[87]
Streptoanthraquinone A	*Streptomyces* sp.	Antibacterial; Anticancer	[70]
Streptochlorin	*Streptomyces* sp.	Anticancer	[88]
Streptokordin	*Streptomyces* sp.	Anticancer	[89]
Streptomyceamide C	*Streptomyces* sp.	Cytotoxic	[90]
Thalassospiramides A, B	*Thalassospira sp.*	Immune-suppressive	[91]
Thiocoraline	*Micromonospora* sp.	Antitumor; Antimicrobial	[92]
Tirandamycin C	*Streptomyces* sp.	Antibacterial	[93]
Trioxacarcins	*Streptomyces* sp.	Antibacterial; Anticancer; Anti-Malarial	[94]
Valinomycin	*Streptomyces* sp.	Anti-Parasitic	[86]
Violapyrones	*Streptomyces* sp.	Antibacterial; Cytotoxic	[95]

*compound in phase I of clinical trials for the treatment of human lymphoma and multiple myeloma

3.1. Antimicrobial Activity

The increasing of antibiotic-resistant bacteria is a worldwide problem and a worrying phenomenon. The microbial resistance to antibiotics, is mainly attributed to their overuse, misuse and to the lack of development of new effective drugs by the pharmaceutical industry [96]. This growing incidence of multi-drug resistant pathogenic microorganisms leads to the urgent need of searching for new antimicrobial compounds [97]. Marine actinobacteria have proved to produce numerous secondary metabolites with antimicrobial activity (Table 1). Some relevant examples illustrated in the literature include compounds with activity against Gram-positive pathogenic bacterial strains, such as abyssomycin C, a polycyclic polyethylene produced by an actinobacteria belonging to the genus *Verrucosispora* exhibiting antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Mycobacterium tuberculosis* [98, 99] or tirandamycin C, a dienoyl tetramic acid produced by a marine *Streptomyces* with activity against vancomycin-resistant *Enterococcus faecalis* (VRE) [93]. Other examples include compounds with a broader spectrum of action, being effective against both Gram-positive and Gram-negative strains, such as essramycin, a pyrimidine produced by a marine strain of *Streptomyces* effective against *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus* and *Micrococcus luteus* [51] or bonactin, an ester also produced by a marine strain of *Streptomyces* with activity against *Bacillus megaterium*, *Micrococcus luteus*, *Kleibsiella pneumoniea*, *Staphylococcus aureus*, *Alicagenes faecalis* and *Escherichia coli* [38]. There are also some compounds described with antifungal activity, as saadamycin, a polyketide produced by an endophyte marine *Streptomyces* [80] or chandrananimycin, a phenoxazin-3-one produced by a marine strain of *Actinomadura* with activity against *Candida albicans* [43]. Considering the numerous antimicrobial compounds produced by marine actinobacteria, these bacteria constitute a hotspot for the screening of novel compounds capable of responding to the urgent problem of antibiotic-resistant strains.

3.2. Anticancer Activity

Cancer is one of the most significant public health problems in the world, being one of the main causes of human death [100]. The pursuit of compounds exhibiting novel anticancer activities and/or that may be more effective while causing less negative effects on healthy cells is one of the major focuses of current scientific research [101]. In this field, marine actinobacteria are also rising to prominence and numerous compounds

synthesized by these organisms have been described to hold activity against cancer cells (Table 1). One of the best examples of success is salinosporamide A. This compound is a rare bicyclic γ-lactam-β-lactone isolated from the marine actinobacteria *Salinispora tropica*. Salinosporamide A revealed an inhibitory effect in several malignant cell types and has entered in clinical trials for the treatment of lymphoma, solid tumors, and multiple myeloma [14]. Another example of a relevant anticancer compound produced by a marine actinobacteria is streptoanthraquinone A, a polycyclic quinone obtained from a marine *Streptomyces* which induces apoptosis in glioma cells and suppresses the proliferation of four different glioma cell lines [70]. Considering the high potential of marine actinobacteria to produce anticancer compounds, bioprospection of these microorganisms in unexplored marine habitats offers very promising results.

4. Actinobacteria in Kelps

Kelps are large brown marine algae that belong to the class Phaeophyceae and to the order *Laminariales*. These algae usually form complex structures, known as kelp forests, which dominate shallow rocky coasts of cold-water marine habitats distributed worldwide, including the north coast of Portugal. Countless organisms live in association with these kelp forests such as marine mammals, fishes, crabs, sea urchins, molluscs and other algae, making this one of the most diverse and productive ecosystems of the world [102]. Kelps are a rich source of bioactive compounds that include polysaccharides, peptides, omega-3 fatty acids, carotenoids, phenolics and vitamins [103]. Many of these molecules have a relevant role in the most varied sectors, such as in the pharmaceutical, agricultural, nutritional and cosmetic industries [104-106].

Macroalgae, including kelps, are known to host diverse species of actinobacteria, both epiphytic and endophytic, but very few studies have been carried out to assess their biotechnological potential, with the majority of the bioactivity screenings targeting epiphytic microorganisms, mainly fungi [107]. Nevertheless, some studies demonstrated that actinobacteria isolated from macroalgae are capable of producing diverse bioactive compounds, including antibiotics and antitumor and anti-inflammatory compounds [108, 109]. Focusing in the endophytic microorganisms, these are characterized by living in the inner tissues of a plant/algae without causing negative damages to the host [110]. During this symbiotic association, endophytes produce secondary metabolites that improve the fitness of the host plant/algae and its resistance against environmental stressors, obtaining in return nutrients and shelter from their host [111]. The interaction between endophytic microorganisms and their host may result in the production of secondary

metabolites with relevant bioactivities [112]. Considering the many bioactive properties of kelp extracts, known and well established for years, one possibility that remains open is the fact that the production of these metabolites can be driven by the community of actinobacteria present in the macroalgae.

Thus, the bioprospection of endophytic actinobacteria in the kelp *L. ochroleuca* is an innovative study that may contribute to the discovery of novel compounds with pharmacological, industrial or ecological interest.

5. Aim and outline of this thesis

Novel drugs are urgently needed to tackle serious illnesses that affect millions of people worldwide, such as cancer pathologies or infections caused by antibiotic-resistant microorganisms. Secondary metabolites produced by marine actinobacteria constitute a promising solution for these alarming problems, due to their clinical relevant properties.

In this context, the present master's thesis aimed to explore the diversity and potential of actinobacterial endophytes of the macroalgae *L. ochroleuca* to produce secondary metabolites with antimicrobial and anticancer activities. The study had as starting point a collection of 112 microbial strains isolated by the student during a work conducted in the last year of her BSc degree.

This thesis is organised in five sections. It starts with an Introduction where the main characteristics of the phylum Actinobacteria, with special emphasis on marine actinobacteria, and their recognized value as producers of antimicrobial and anticancer compounds are addressed. This section is followed by the Materials and Methods where the whole methodology associated to the work performed is detailed, namely the methodology employed for the taxonomic identification of the isolated microorganisms, the bioactivity assays and chemical elucidation of bioactive compounds. The subsequent two sections are relative to the Results and to the Discussion, where the results obtained under the scope of this thesis are presented and discussed. Finally, the last section consists on the main conclusions of the performed work.

II. Materials and Methods

As mentioned in the previous section, this work had as starting point a collection of 112 microbial strains isolated from *L. ochroleuca* in a study conducted by the student during the last year of her BSc degree. Though no actinobacterial strains were isolated in the scope of present thesis, the methodology employed in the previous BSc study to achieve this goal will be here referred in order to allow a better understanding of the whole work performed.

1. Sampling and Bacterial Isolation

Samples of *L. ochroleuca* were collected in the intertidal zone of Mindelo rocky shore, located in northern Portugal (41.309298°; -8.742228°), transported to the laboratory under refrigeration and processed on the same day. The collected specimens were washed with sterile sea water and segmented into three distinct parts: holdfast, stipe and blade (Fig. 2). Each part was cut into small pieces (ca. 2 cm in length) that were subjected to a chemical and enzymatic disinfection treatment for the elimination of the epiphytic microbial community. In this process, samples were incubated in a CTAB buffer solution (diluted 1:100) containing proteinase K (20 mg/mL) for 30 min at 60 °C, and washed for three consecutive times with sterile sea water for 1 min [113]. The effectiveness of the disinfection treatment was evaluated by plating the final wash water and the disinfected tissues of each part of the algae on Plate Count Agar (PCA). To increase the isolation of endophytic actinobacteria, grinded tissues from the different parts of *L. ochroleuca* were inoculated in duplicate in three selective culture media: Starch-Casein-Nitrate agar (SCN: 10 g of soluble starch, 0.3 g of casein, 2 g of K_2HPO_4, 2 g of KNO_3, 2 g of NaCl, 0.05 g of $MgSO_4.7H_2O$, 0.02 g of $CaCO_3$, 0.01 g of $FeSO_4.7H_20$ and 17 g of agar, per litre of distilled water), Raffinose-Histidine agar (RH: 10 g of raffinose, 1 g of L-Histidine, 1 g of K_2HPO_4, 0.5 g of $MgSO_4.7H_2O$, 0.01 g of $FeSO_4.7H_20$ and 17 g of agar, per litre of distilled water) and Nutrient-Poor Sediment Extract agar (NPS: 100 mL of marine sediment extract obtained by washing 900 mL of sediments with 500 mL of seawater and 17 g of agar, per litre of seawater). All culture media were supplemented with cycloheximide (50 mg/L) and nalidixic acid (50 mg/L) to inhibit the growth of fungi and Gram-negative bacteria. The plates were incubated at 28 °C for a period of up to 6

weeks. Morphologically distinct colonies were isolated and cryopreserved at -80 ºC in 30% (v/v) of glycerol.

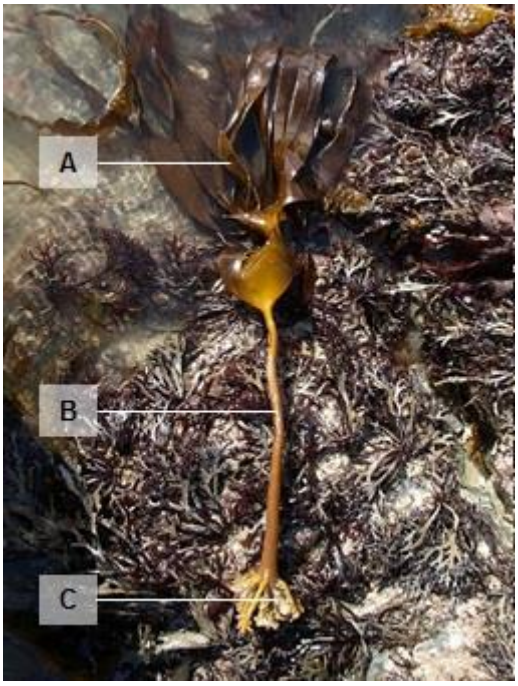


Figure 2. Specimen of *L. ochroleuca* with the indication of (A) blade, (B) stipe and (C) holdfast.

2. Taxonomic Identification of the Isolates

All isolates were taxonomically identified through 16S rRNA gene sequencing. For each isolate, genomic DNA was extracted using the E.Z.N.A. Bacterial DNA Kit (Omega Biotek, Norcross, GA) according to the manufacturer's recommendation. The 16S rRNA gene was amplified by PCR using the universal primers 27F (5'-GAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') [114, 115]. The PCR mixture (final volume of 10 µL) consisted in: 5 µL of Qiagen Multiplex PCR Master Mix (Qiagen, Valencia, CA), 1 µL of each primer (2 µM), 2 µL of DNA template and 1 µL of nuclease-free water. The reaction was started with an initial denaturation at 95 °C for 15 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 48 °C for 90 s and extension at 72 °C for 90 s, and a final extension at 72 °C for 10 min. PCR products were separated on a 1.4% agarose gel containing SYBR Safe (ThermoFisher Scientific, USA). Purification and sequencing of the samples was performed by GenCore, I3S (Instituto de Investigação e Inovação em Saúde, Portugal) as follows: 10 µL reactions were prepared by combining 0.8 µL of Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Life Technologies, United Kingdom) with 0.8 µL of 10 µM primers (the same primers used in the PCR), 1 mL of BigDye

Terminator v1.1 & v3.15X Sequencing Buffer and water DNase, RNase-free (Gibco, USA). Thermal cycler conditions were: 96 °C for 2 min, 35 cycles at 96 °C for 30 s, 50 °C for 15 s and 60 °C for 4 min and one final hold at 60 °C for 10 min. Sequencing reaction products were purified using Sephadex™G-50 Fine DNA Grade columns (GE Healthcare, United Kingdom) according to the manufacturer's recommendations. Purified samples were added to 12 µL Hi-Di™ formamide (Life Technologies, USA). Sequencing was performed in a Genetic Analyzer 3130xl sequencer (Applied Biosystems), according to the manufacturer's recommendations. The obtained 16S rDNA sequences were analysed using the Geneious software, version 11.1.4. The taxonomic affiliation of the isolates was established using the 16S ribosomal RNA (Bacteria and Archaea) database from NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and confirmed using the Identify tool from EzTaxon (http://www.ezbiocloud.net/) and the Sequence Match tool from the Ribosomal Database Project (https://rdp.cme.msu.edu/index.jsp). To complement the taxonomic evaluation of the isolates, a phylogenetic tree was elaborated. According to BLAST results, the 3 closest neighbour sequences for each isolate were selected, choosing only organisms belonging to different species. Once the sequences were obtained for all isolates (128 sequences), a Geneious alignment was performed resulting in an alignment with 1281 bp. The phylogenic tree was made using the Maximum Likelihood method with 1000 bootstraps based on the Tamura-Nei model. Strains KENR90, KENR91 and KENR92 were not included in the tree since their nucleotide sequences were too short (590 bp, 617 bp and 833 bp, respectively). The tree was constructed using the Molecular Evolutionary Genetics Analysis program Version 7.0 (MEGA7) [116].

3. Bioactivity Assays

3.1. Preparation of Crude Extracts

Each actinobacterial isolate was grown in a 100 mL Erlenmeyer flask containing 30 mL of liquid culture medium with a composition identical to the solid medium from which the isolate was obtained (without the addition of cycloheximide and nalidixic acid) (Fig. 3A). The flasks were incubated at 28 ºC, 100 rpm, in the dark. After four days of incubation, 0.5 g of Amberlite XAD16N resin (Sigma-Aldrich, St. Louis, Mo.) were added to the culture medium and the flasks were incubated for another three days (Fig. 3B). The obtained biomass and resin were then harvested by centrifugation (4.600 rpm for 10 min), freeze-dried and preserved at -20 ºC. Each culture was extracted using 30 mL of a

solution of acetone/methanol 1:1 (v/v) and the organic layer was dried in a rotary evaporator. The resulting crude extract (Fig. 3C) was dissolved in dimethyl sulfoxide (≥ 99.9%, DMSO, Sigma-Aldrich, USA) to obtain stock solutions at 10 mg/mL, 3 mg/mL and 1 mg/mL for the bioactivity assays.

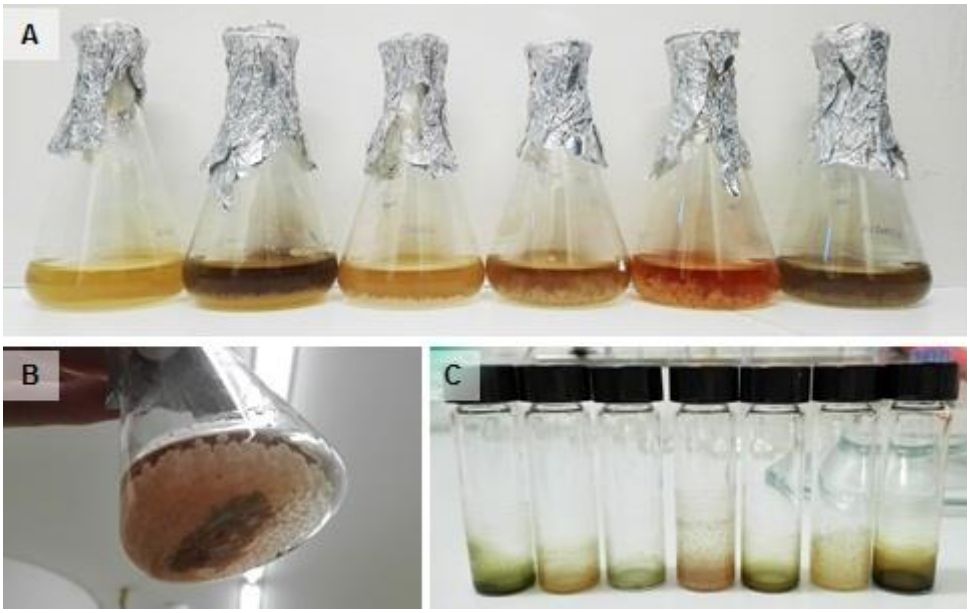


Figure 3. Liquid cultures of some actinobacterial isolates (A) bacterial growth in the Erlenmeyer flasks, (B) Amberlite XAD16N resin added to the cultures and (C) crude extracts obtained from some liquid cultures.

3.2. Screening of Antimicrobial Activity

The antimicrobial activity of the crude extracts obtained from the actinobacterial isolates was tested against five reference microorganisms - *Escherichia coli* (ATCC 25922), *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 29213), *Salmonella typhimurium* (ATCC 25241) and *Candida albicans* (ATCC 10231) - using the agar-based disk diffusion method (Fig. 4). The bacterial strains were grown in Mueller-Hinton agar (MH) and *C. albicans* in Sabouraud Dextrose agar (SD). For the bioassay, reference microorganisms were suspended in the corresponding liquid medium and their turbidity was adjusted to 0.5 McFarland standard (OD_{625} = 0.08−0.13). The suspensions were then used to inoculate agar plates (MH or SD, according to the reference microorganism) by evenly streaking the plates with a swab dipped in the suspension. Blank paper disks (6mm in diameter; Oxoid) were placed on the surface of the inoculated medium and 15 µL of each actinobacterial crude extract (10 mg/mL) were loaded into the disks. Positive control consisted in 15 µL of enrofloxacin (10 mg/mL) for the bacterial

strains, and 15 µL of nystatin (10 mg/mL) for *C. Albicans,* and the negative control consisted in 15 µL of DMSO. Antimicrobial activity was determined by measuring the diameter of the inhibition halo formed around each disk after 24 h of incubation at 37 °C. For each extract, assays were conducted in duplicate against each reference microorganism. For extracts exhibiting antimicrobial activity, the minimal inhibitory concentration (MIC) (Fig. 5) was also determined. Inoculum suspensions of the reference strains were prepared as mentioned above. For each extract, stock solutions at 1 mg/mL were prepared in the appropriate culture medium. Two-fold dilutions in the same medium were sequentially obtained from these stocks, resulting in extracts solutions with concentrations ranging from 1 mg/mL to 0.487 µg/mL. The assay was conducted in 96 well plates by adding 50 µL of the microbial inoculum (diluted 1:100) and 50 µL of each extract dilution to each well, in triplicate for each extract dilution. The MIC was determined by spectrophotometric analysis (625 nm), after 18 h of incubation at 37 °C. The growth control consisted in 50 µL of microbial inoculum and 50 µL of medium broth and the sterility control in 100 µL of medium broth. For each extract, MIC was determined from two independent experiments.

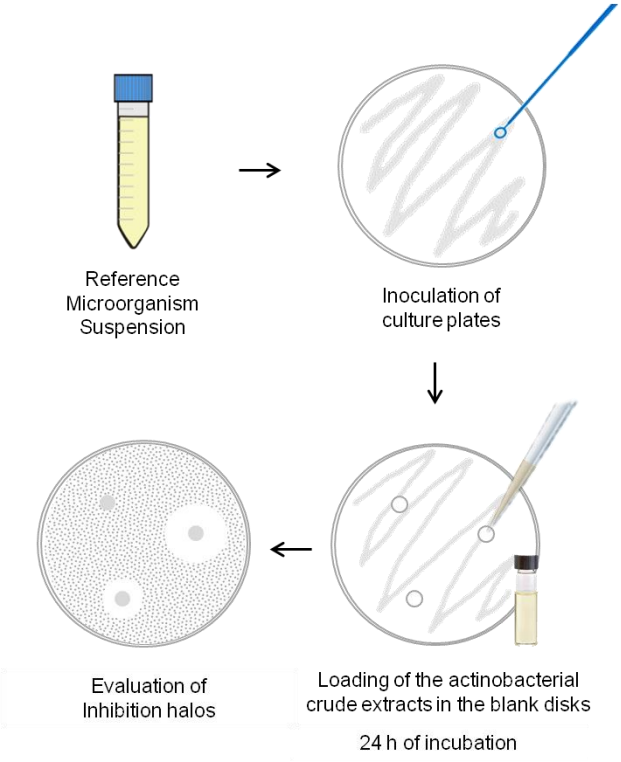


Figure 4. Illustrative diagram of the agar-based disk diffusion assay.

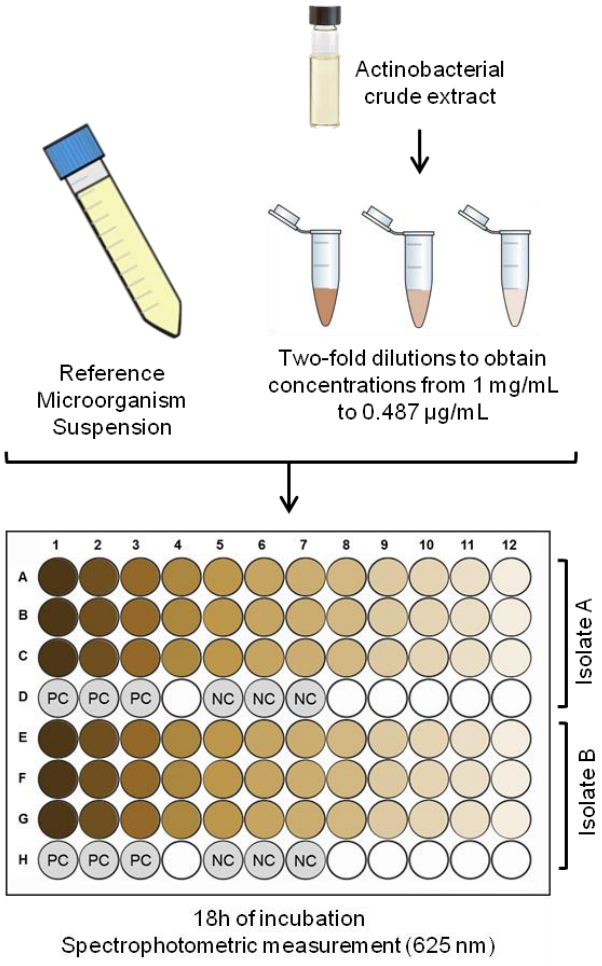


Figure 5. Illustrative diagram of the minimal inhibitory concentration (MIC) assay. PC and NC indicate positive and negative controls, respectively.

3.3. Screening of Anticancer Activity

The anticancer activity of each actinobacterial crude extract was tested in two human cancer cell lines - breast ductal carcinoma (T-47D) and neuroblastoma (SH-SY5Y) (Sigma-Aldrich, St. Louis, Missouri, USA) - using the MTT assay (Fig. 6). T-47D cells were grown in Dulbeco's Modified Eagle Medium (DMEM) (Gibco, Thermo Fischer Scientific, Waltham, Massachusetts, USA) and SH-SY5Y in a 1:1 mixture of MEM/F12 medium, both supplemented with 10% (v/v) fetal bovine serum (Biochrom, Berlin, Germany), 1% (v/v) antibiotics (100 mg/L streptomycin), 100 IU/mL penicillin (Biochrom, Berlin, Germany) and 0.1% (v/v) amphotericin (GE Healthcare, Little Chafont, United Kingdom), at 37 °C in an incubator with 5% carbon dioxide. The cells were seeded in 96-well plates at 6.6×10^4 cells/mL, let to adhere overnight and exposed to the extracts (15 µg/mL) for 24 and 48 h. Positive control consisted in 20% DMSO and solvent control in

0.5% DMSO. After 24 and 48 h of exposure, 20 µL of MTT (final concentration: 0.2 mg/mL) were added per well and the plates were incubated for an additional period of 4 h at 37 °C. After this step, the culture medium was removed from each well and 100 µL of DMSO were added to the wells. The results were obtained by reading the OD of the plates at 570 nm in a plate reader (Biotek, Synergy HT). The assays were performed in triplicate and two independent experiments were performed for each extract. Cellular viability was expressed as a percentage relative to the solvent control. Extracts exhibiting anticancer activity were additionally tested on a non-tumour cell line, human brain capillary endothelial cells (hCMEC/D3) (kindly donated by Dr. P. O. Courad (INSERM, France) following the same procedure described above.

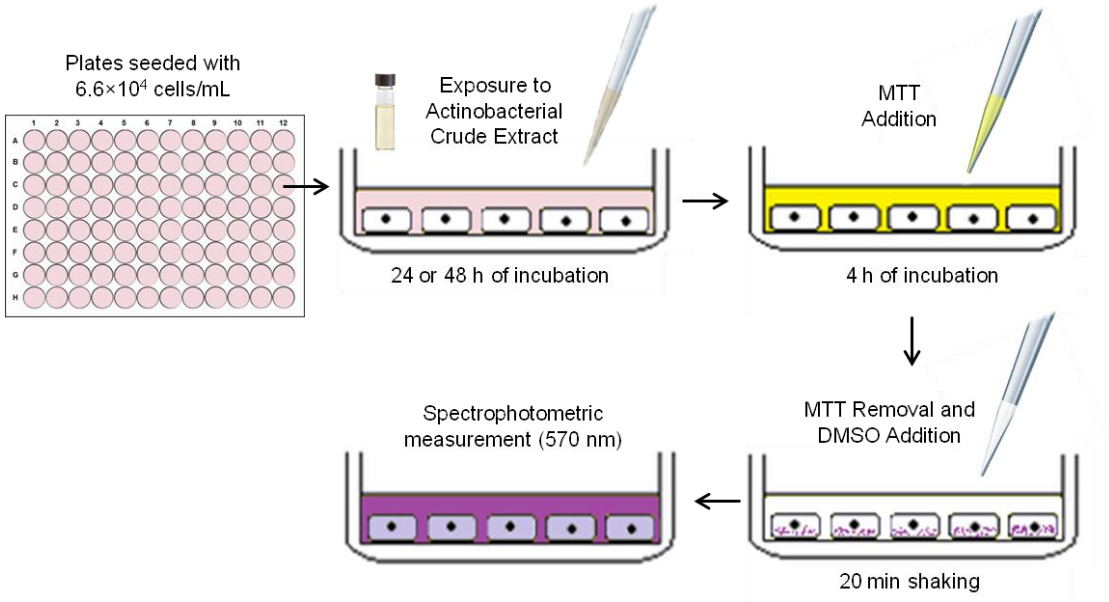


Figure 6. Illustrative diagram of the MTT assay.

4. Dereplication of Active Crude Extracts

A set of 35 actinobacterial crude extracts was selected for dereplication analysis. The selection of these extracts was made according to their performance in the bioactivity screenings: inhibition halos > 1 cm and capability to decrease at least one cancer cell line viability to less than 30% (Annex 1). The selection also took into account the phylogenetic relationship of the actinobacterial isolates producing the bioactive extracts, which was evaluated through the construction of a phylogenetic tree (Annex 2). From this analysis, seven strains (KENR16B, KENR19, KENR21, KENR34, KENR69, KENR79 and KENR93) were discarded once they proved to be highly similar to others,

both in terms of bioactive profile and taxonomic classification. The crude extract of each selected isolate was analysed by Liquid chromatography-tandem mass spectrometry (LC-MS/MS). The samples were prepared in 1.5 mL vials at 1 mg/mL. The eluents used were water/methanol/formic acid 95:5:0.1 (v/v) and acetone/methanol/formic acid 95:5:0.1 (v/v). The separation was carried out on an ACE UltraCore 2.5 Super C18 (75 × 2.1 mm) column. The obtained data were processed using the dereplication workflow from Global Natural Product Social Molecular Networking (GNPS) (https://gnps.ucsd.edu/) [117] using the default parameters, except for the ion mass tolerance precursor which was set at 0.01 Da and fragment ion mass tolerance which was set at 0.04 Da. All compounds that fulfilled the following conditions were considered as likely identities: natural compounds with relevant biological activity (i.e., antimicrobial, anticancer, cytotoxic) and cosine value > 0.85. The dereplication data was matched with the strain's bioactivity profiles, allowing ultimately to select which strains would be more promising to proceed to a bioactivity-guided isolation of novel bioactive compounds.

5. Bioactivity-Guided Study of Bioactive Molecules - *Streptomyces* sp. KENR25

The strategy employed within the present topic corresponds to part of a traditional natural product discovery pipeline relying on the combination of fractionation techniques and submission of the resulting fractions to bioassays (Fig. 7). The fractionation techniques are based on chromatographic procedures which allow the separation, based on selected physical parameters, of different components present in a sample. Strain KENR25, identified as *Streptomyces* sp., was selected due to its performance in the bioactivity screenings: both antimicrobial and cytotoxic activity against the two cancer cell lines tested. This selection was made before the dereplication procedure, therefore the composition of the crude extract and presence of known bioactive compounds was not yet known.

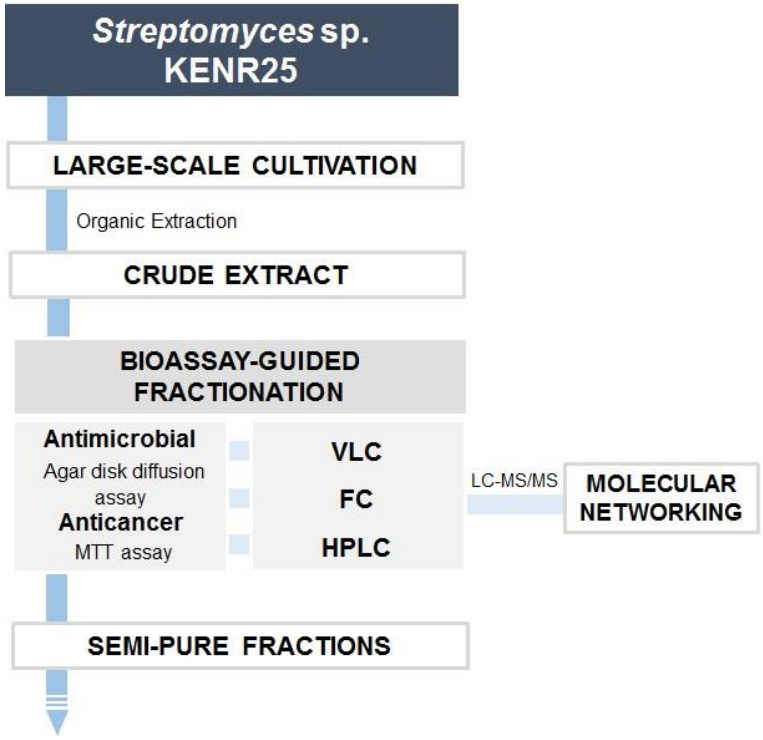


Figure 7. Diagram of the bioassay-guided workflow to study bioactive molecules.

5.1. Large-Scale Cultivation

The cultivation was performed following the previously described procedure for growing actinobacterial isolates in liquid culture by scaling-up the culture volume. The strain was grown at 28 ºC, 100 rpm and in the dark, in a total of 15 L, using 1 L Erlenmeyer flasks, each containing 500 mL of SCN liquid medium. After four days of incubation, 8.3 g of resin were added to each flask and the flasks incubated for three additional days. The obtained biomass and resin were then harvested by centrifugation (4.600 rpm for 10 min), freeze-dried and preserved at -20 ºC until organic extraction and further fractionation.

5.2. Organic Extraction

The extraction apparatus was prepared by assembling a Büchner funnel containing two Whatman grade 1 filter paper (Whatman, Sigma-Aldrich, USA) and a cloth, a vacuum adapter and a round bottom (RB) flask. The freeze-dried biomass and the resin obtained from large-scale cultivation were firstly placed in a stainless-steel goblet and macerated with a spatula. Then, the biomass and resin were entirely immersed in a solution of 500 mL of acetone/methanol 1:1 (v/v). This mixture was left under constant stirring for 2 h, at

30 °C. The ensuing steps consisted in decanting the solvent content into the Büchner funnel, collect the extract in the RB flask and recover into the goblet the biomass that was retained in the cloth. This process was repeated, adapting the solvent mixture and stir time, until the solvent content run out of colour. After the extraction was complete, the RB flask was removed from the assembly and the solvents dried in a rotary evaporator. The content of the RB flask was dissolved using acetone and methanol and filtered through cotton to retain any remaining cells. The filtered extract was finally dried under vacuum to evaluate the obtained mass of the crude extract.

5.3. Bioassay-guided fractionation

5.3.1. Vacuum Liquid Chromatography (VLC)

The crude extract was fractionated by normal phase Vacuum Liquid Chromatography (VLC) (Fig. 8) using a solvent polarity gradient (Table 2) on a glass fritted chromatography column. A thin layer of sand (ca. 1 cm in height) and 300 g of silica gel 60 (0.015-0.040 mm) (Merck, Sigma-Aldrich, USA) were placed in the column and then a mixture of hexane/ethyl acetate 9:1 (v/v) was used to pack the column. The crude extract was dissolved using a mixture of hexane/ethyl acetate 9:1 (v/v) and gently loaded onto the top of the silica column. A second layer of sand was positioned over the extract. The different eluents were sequentially added to the column without letting the silica surface become exposed to air. A total of ten fractions (A-I2, in order of increasing polarity) were collected in RB flasks and dried in a rotary evaporator. The chromatographic fractions were resuspended using dichloromethane and ethyl acetate 1:1 (v/v) (A-E) or methanol (F-I2), filtered through cotton, transferred to pre-weighted 16 mL vials and dried under vacuum. The mass of each fraction was determined, and the vials stored at -80 ºC until further use. The antimicrobial and anticancer activity of the ten VLC fractions was tested following the same protocols previously described for these assays. To gather information on the structure of the potentially active compounds, each active fraction, as well as its adjacent ones, was analysed by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. Each sample was prepared by dissolving the mass in a deuterated solvent methanol or chloroform, according to their polarity, and transferred into 400MHz NMR tubes (Norell Standard SeriesTM 5 mm, Sigma-Aldrich, USA). The samples were then sent to the Laboratory for Structural Elucidation (LAE) of the Materials Centre of the University of Porto (CMUP) where a Resonance Spectrometer Bruker Avance III 400 MHz, 9.4 Tesla was used for the analysis. The data

was analysed using the Mnova software allowing to verify the fractions spectra similarity. At this point it was decided to focus attention on the antimicrobial activity of four fractions - D, E, F and G - that proved to be active against *C. albicans*. Considering the antibiogram results, together with the ^{1}H NMR spectra similarity, these four fractions were dissolved using dichloromethane, pooled together in the same vial and processed as a single fraction (DEFG).

Table 2. Solvent mixtures used for elution of *Streptomyces sp*. KENR25 crude extract on Vacuum Liquid Chromatography. Due to the high polarity of the extract, the last solvent mixture was repeated (fraction I2)

Fraction	Solvent Mixtures	Volume
A	10% EtOAc - 90% Hex	> 500mL
B	30% EtOAc - 70% Hex	500 mL
C	50% EtOAc - 50% Hex	500 mL
D	75% EtOAc - 25% Hex	500 mL
E	100% EtOAc	500 mL
F	10% MeOH - 90% EtOAc	500 mL
G	30% MeOH - 70% EtOAc	500 mL
H	60% MeOH - 40% EtOAc	500 mL
I	100% MeOH	500 mL
I2	100% MeOH	500 mL

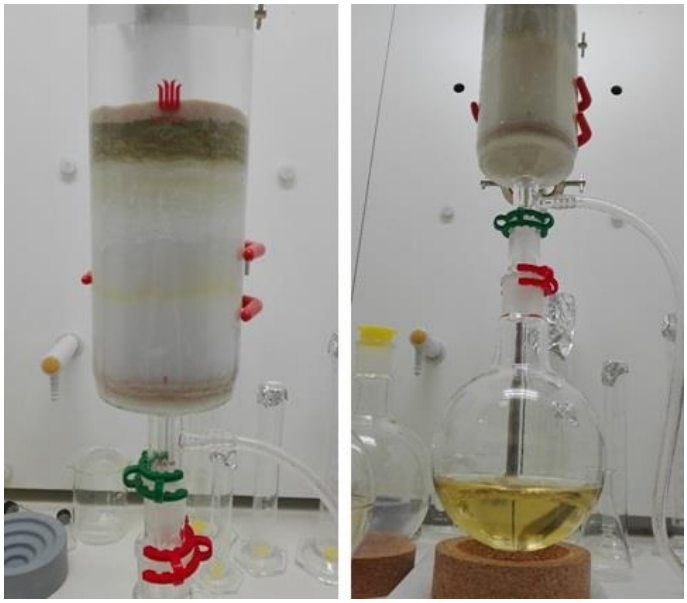


Figure 8. Vacuum Liquid Chromatography fractionation of *Streptomyces* sp. KENR25 crude extract.

5.3.2. Flash Chromatography (FC)

The antimicrobial-active DEFG fraction was fractionated using normal phase Flash Chromatography (FC). A Thin-Layer Chromatography (TLC) (Fig. 9A) was initially performed as a polarity indicator for the compounds present in the DEFG fraction, allowing determining the appropriate first solvent mixture to initiate the FC (Fig. 9B). For the TLC procedure, the fraction was applied onto a silica gel plate using a glass capillary and a mixture of ethyl acetate/hexane 1:1 (v/v) was used as mobile phase. To allow the detection of the compounds, the plate was stained using phosphomolybdic acid (PMA) and heated to activate the stain for visualization. The active DEFG fraction was further fractionated by normal phase FC. According to the TLC results it was decided that the first elution solvent mixture for FC would be ethyl acetate/hexane 2:3 (v/v). This mixture was used to solvate the silica gel (0.040-0.063 mm) in the column. The sample was added to the top of the silica layer, followed by the addition of cotton to protect the silica surface from the impact of solvents addition. Elution was performed using a gradient of increasing polarity (Table 3) and the sample was recovered in 10 mL tubes. Tubes with a similar sample coloration and profile on the TLC plates were combined in separated RB flasks, leading to a total of nine sub-fractions (DEFG-A - DEFG-I). These sub-fractions were transferred to 8 mL pre-weighted vials and dried under vacuum. The mass of each sub-fraction was determined, and the vials stored at -20 ºC. To identify the fraction containing the active compounds responsible for the activity against *C. albicans*, the nine FC fractions were tested following the same protocol previously described for the antimicrobial assay, and the active fractions were also analyzed by ^{1}H NMR.

Table 3. Solvent mixtures used for elution of the DEFG fraction on FC

Solvent Mixtures	Volume
60% Hex - 40% EtOAc	500 mL
50% Hex - 50% EtOAc	100 mL
40% Hex - 60% EtOAc	50 mL
30% Hex - 70% EtOAc	50 mL
20% Hex - 80% EtOAc	50 mL
10% Hex - 90% EtOAc	50 mL
100% EtOAc	50 mL
90% EtOAc: - 10% MeOH	50 mL
80% EtOAc - 20% MeOH	50 mL
60% EtOAc - 40% MeOH	50 mL
70% EtOAc - 30% MeOH	50 mL
100% MeOH	100 mL

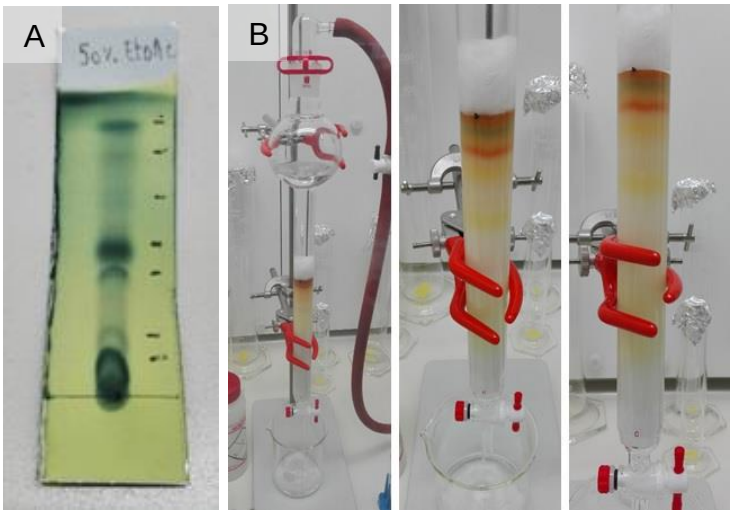


Figure 9. Sub-fractioning of the DEFG fraction. (A) pre-chromatography Thin-Layer Chromatography and (B) Flash Chromatography column.

5.3.3. High-Performance Liquid Chromatography (HPLC)

The data obtained with all the procedures described above for the active fractions, the higher amount of available extract and the chromatogram simplicity of the sample, led to the selection of the fraction DEFG-A for semi-preparative High Performance Liquid Chromatography (HPLC) fractionation in a Waters Alliance HPLC combined with a Photodiode Array (PDA) detector. The sample was first dissolved in approximately 5 mL, using dichloromethane and methanol, and filtered through cotton into a vial. In each run, a sample volume of 250 µL was injected and the separation was carried out on a Luna 10u C18 column (100A, 4 µm, 250 × 10 mm, Phenomenex), with a flow rate of 3 mL/min. The chromatogram was monitored with fixed wavelengths at 225 nm and 254 nm. The elution was performed using HPLC grade acetonitrile and ultra-pure water according to

the following program: acetonitrile/water 3:7 (v/v) for 40 min followed by a linear gradient to 95% acetonitrile, held for 15 min before returning to the initial conditions. The 10 HPLC fractions were transferred to 8 mL pre-weighted vials and dried under vacuum. The mass of each fraction was determined, and the vials stored at -20 ºC.

5.4. Molecular Networking

A molecular networking was performed as an additional approach to study the metabolomic profile of DEFG sub-fractions that showed positive result in the antimicrobial assay. The first step was to analyse the selected fractions by LC-MS/MS, following the same protocol previously described for this technique. The obtained data followed then the “Data analysis” workflow from GNPS using the default parameters, except for the ion mass tolerance precursor which was set at 0.01 Da and fragment ion mass tolerance which was set at 0.04 Da. The GNPS tools were used to get an overview of identified compounds/clusters and then the MS/MS data was downloaded to Cytoscape software, version 3.6.1., where the molecular networking was generated (http://www.cytoscape.org/). A colour layout was applied to the network to allow an easier visualization and interpretation of the results.

III. Results

1. Phylogenetic Identification of Actinobacteria Isolated from *L. ochroleuca*

At the beginning of the present MSc project, a total of 112 bacterial strains were available for the study, being 90 identified as actinobacteria (and the remaining identified as Firmicutes, Proteobacteria and Fungi). These actinobacteria were previously isolated from disinfected tissue fragments obtained from holdfasts, stipes and blades of *L. ochroleuca*. Most of the strains (89%) were isolated from the holdfasts (Fig. 10A). The highest percentage of the isolates was recovered from the medium SCN (63%), followed by RH (27%) and NPS (10%) (Fig. 10B). The isolates exhibited diverse morphological features, with several strains presenting characteristics typical of actinobacteria, such as slow growth, formation of hyphae and production of spores and pigments, the latter sometimes leading to the change of the colour of the culture medium (Fig. 11).

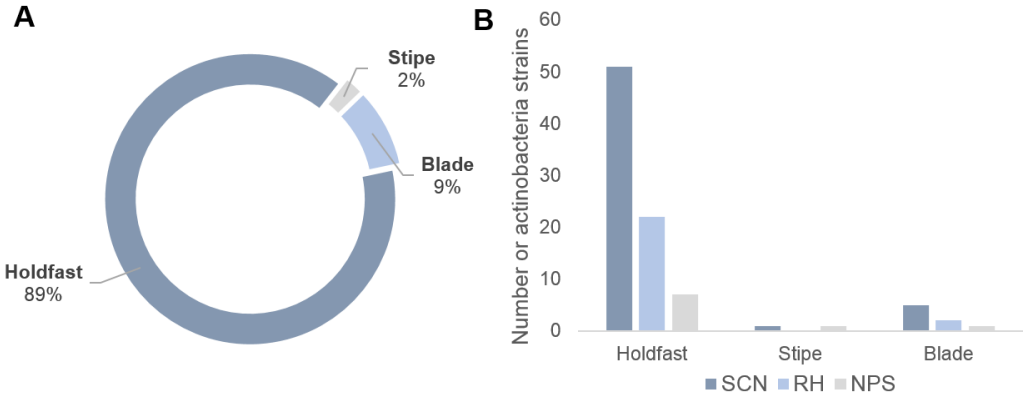


Figure 10. Actinobacterial isolates recovered from *L. ochroleuca.* (A) Percentage of actinobacterial strains isolated from holdfasts, stipes and blades of *L. ochroleuca* and (B) Distribution of the isolates by the selective culture media used in the study (SCN: Starch-Casein-Nitrate agar; RH: Raffinose-Histidine; NPS: Nutrient-Poor Sediment).

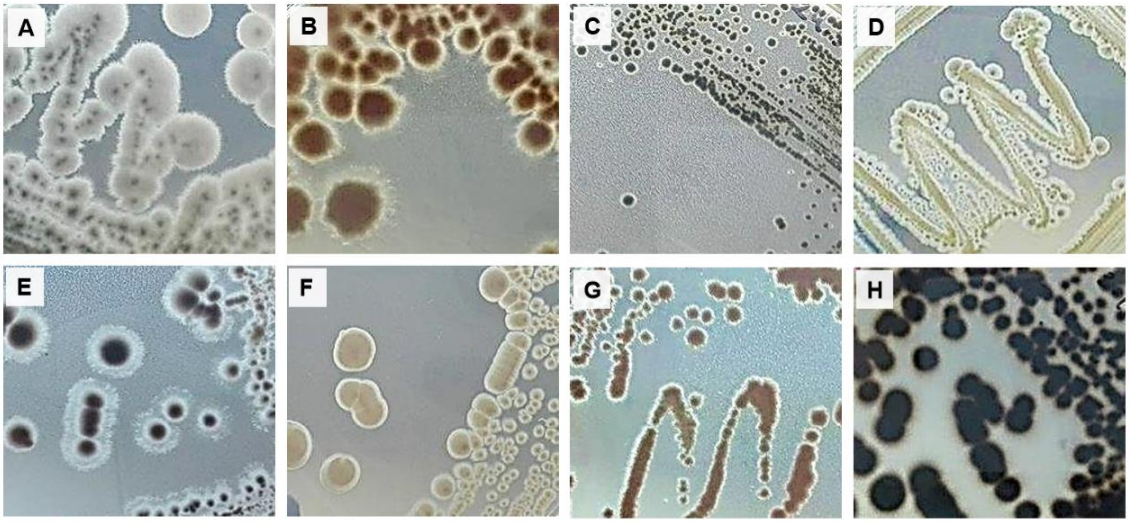


Figure 11. Morphological diversity of some actinobacterial strains isolated from *L. ochroleuca*: (A) strain KENS1, (B) strain KENR91, (C) strain KENR92, (D) strain KENR94, (E) strain KENRB10, (F) strain KENR56, (G) strain KENR81 and (H) strain KENB8.

The disinfection treatment was not entirely effective, as five isolates were recovered from the disinfection controls. Two of these isolates were grown in a PCA plate inoculated with a sterilised holdfast fragment being identified as *Pseudomonas* sp. and *Lysinibacillus* sp., two were obtained in a PCA plate inoculated with the final wash water from the disinfection of a holdfast fragment being identified as *Bacillus* sp. and *Staphylococcus* sp., and one was found in a PCA plate inoculated with the final wash water from the disinfection of a blade fragment being identified as a *Penicillium* sp.

Most actinobacterial isolates identified by 16S rRNA gene sequencing belonged to the *Streptomyces* genus. However, strains belonging to other genera (including some rare) were also retrieved: *Isoptericola, Rhodococcus, Nonomuraeae, Nocardiopsis, Microbispora* and *Microbacterium* (Figs. 12A and 13). All of these genera, except for *Microbispora*, were found in holdfasts of *L. ochroleuca*, while only two actinobacterial genera were recovered from stipes (*Microbispora* and *Microbacterium*) and blades (*Streptomyces* and *Microbacterium*) (Fig. 12B). The selective medium SCN allowed the retrieval of strains belonging to all genera identified in this study, revealing to be the most efficient medium (Fig. 12C). Growth of *Streptomyces* and *Microbacterium* strains was observed in all culture media used. Several of the isolated strains, many of them affiliated with the genus *Streptomyces*, were found to group very closely with high bootstrap values (Fig. 13), indicating a high similarity between them. In addition, the obtained isolates consistently clustered with species already described in the literature, discarding the possibility of some of these isolates constitute a new species (Fig. 13).

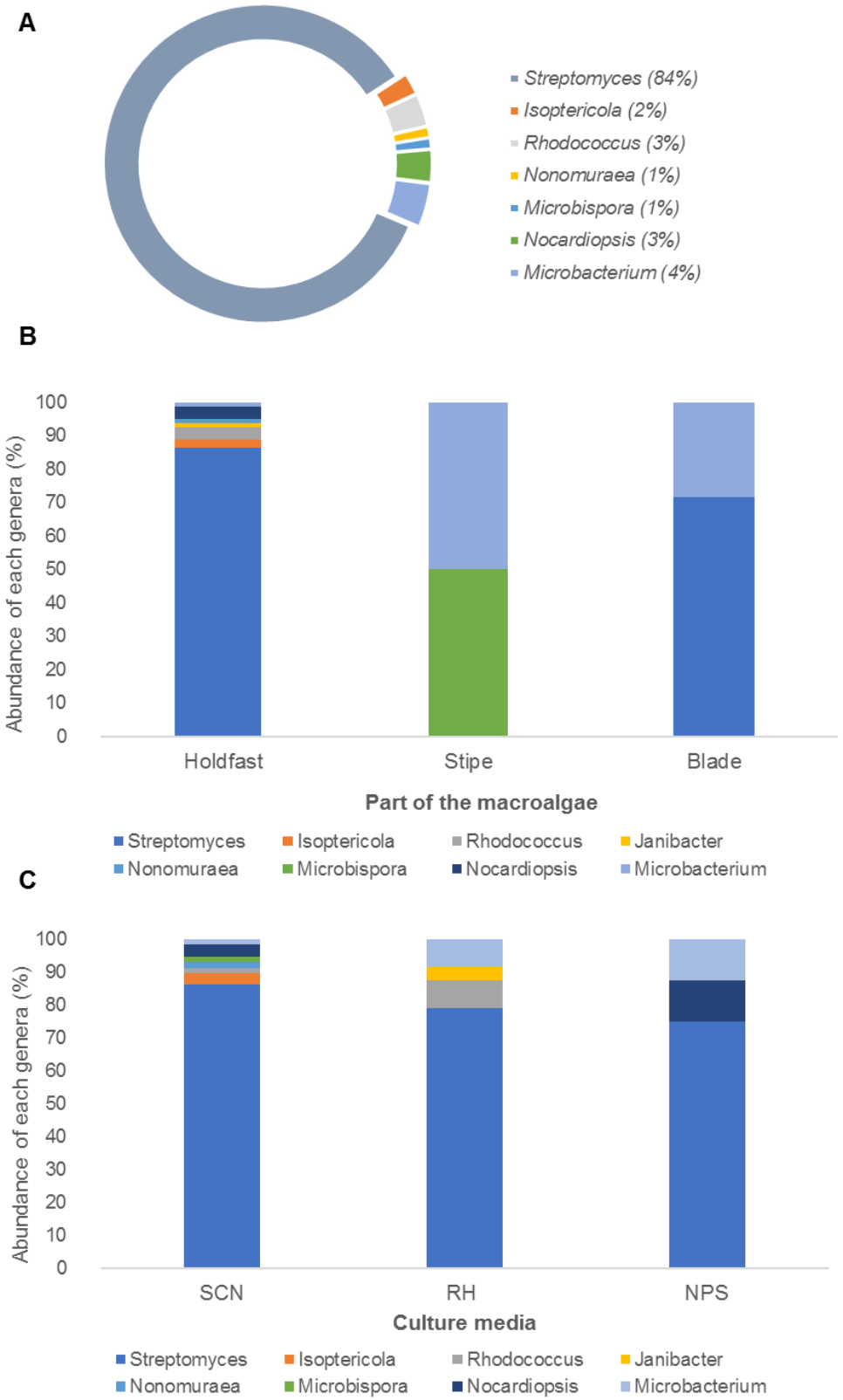


Figure 12. Actinobacterial genera recovered from *L. ochroleuca*. (A) Percentage of actinobacterial genera isolated from *L. ochroleuca*, (B) genera distribution in the holdfast, stipe and blade of the macroalgae and (C) genera distribution according to the selective culture media used for the isolation.

Figure 13. Phylogenetic relationship of the 87 actinobacterial isolates recovered from *L. ochroleuca*, based on 16S rRNA gene homology with their GenBank nearest neighbours. Numbers at nodes represent bootstrap values when higher than 60%. Numbers in parenthesis correspond to GenBank accession numbers. *Bacillus subtilis* was used as outgroup.

2. Bioactive Potential of the Actinobacterial Isolates

Antimicrobial assays identified 45 isolates (half of the total number of the actinobacterial strains isolated) capable of inhibiting the growth of *C. albicans* or *S. aureus*. These isolates were affiliated with the genera *Streptomyces* (39 strains), *Isoptericola* (one strain), *Nonomuraeae* (one strain), *Nocardiopsis* (one strain), *Microbispora* (one strain) and *Microbacterium* (two strains). From these 45 isolates, 37 exhibited activity against *C. albicans*, three showed activity against *S. aureus* and five had activity against both microorganisms (Table 4). The inhibition halos observed had a diameter that varied between 7 and 25 mm, and all of them presented a translucent appearance (Fig. 14). MIC values determined for the actinobacterial extracts ranged between < 0.487 and 1000 µg/mL (Table 4). The lowest MIC value attained against *S. aureus* (3.9 µg/mL) was observed for extracts of the *Streptomyces* strains KENR60 and KENR64, isolated from holdfasts of *L. ochroleuca*. The lowest MIC value attained against *C. albicans* (< 0.487 µg/mL) was observed for crude extract of the *Streptomyces* KENR25, also isolated from holdfasts of *L. ochroleuca*. Although most of the isolates exhibiting antimicrobial activity were recovered from holdfasts (89%), some of them were also obtained from blades (9%) and stipes (2%).

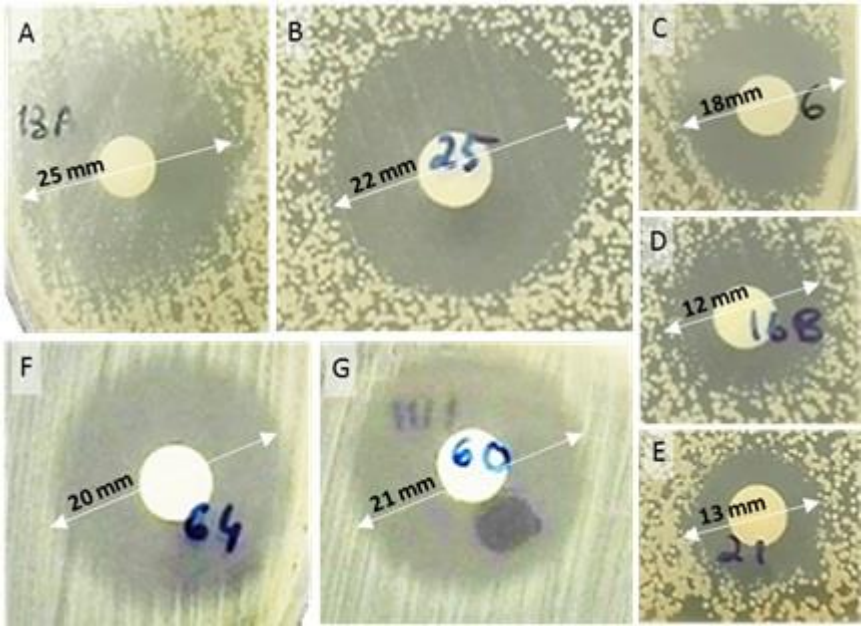


Figure 14. Antimicrobial activity of actinobacterial strains isolated from *L. ochroleuca*. (A-E) Examples of inhibition halos against *C. albicans* and (F-G) against *S. aureus*. (A) Strain KENR13A, (B) strain KENR25, (C) strain KENR6, (D) strain KENR16B, (E) strain KENR21, (F) strain KENR64 and (G) strain KENR60.

Table 4. Actinobacteria isolated from *L. ochroleuca* with antimicrobial activity. The diameter of inhibition halos obtained by the agar disk diffusion method and MIC values are shown

		Diameter of Inhibition Halos					**MIC** (µg/mL)	
ISOLATE	**TAXONOMIC IDENTIFICATION**	*E. coli*	*S. typhimurium*	*S. aureus*	*B. subtilis*	*C. albicans*	*S. aureus*	*C. albicans*
KENR3	*Streptomyces olivochromogenes*							31.25
KENR6	*Streptomyces* sp.						1000	0.975
KENR8	*Streptomyces atratus*							1.95
KENR11A	*Streptomyces* sp.							7.81
KENR13	*Streptomyces* sp.							1.95
KENR13A	*Streptomyces* sp.							0.975
KENR13B	*Streptomyces* sp.						1000	7.81
KENR14	*Streptomyces iamenensis*							7.81
KENR16B	*Streptomyces* sp.							15.62
KENR17A	*Streptomyces* sp.						500	1.95
KENR18	*Streptomyces* sp.						1000	0.975
KENR19	*Streptomyces* sp.							0.975
KENR21	*Streptomyces* sp.							1.95
KENR21A	*Streptomyces* sp.							0.975
KENR24	*Streptomyces* sp.							7.81
KENR25	*Streptomyces* sp.							< 0.487
KENR29	*Nonomuraea sp.*							3.9
KENR31	*Streptomyces* sp.						62.5	1000
KENR33	*Streptomyces lannensis*							1.95
KENR34	*Streptomyces* sp.							15.62
KENR35	*Streptomyces* sp.							0.975
KENR36	*Streptomyces* sp.							125
KENR42	*Streptomyces mirabilis*							125
KENR49	*Streptomyces atratus*							3.9
KENR60	*Streptomyces* sp.						3.9	
KENR64	*Streptomyces* sp.						3.9	
KENR65	*Streptomyces* sp.							125
KENR69	*Streptomyces* sp.							15.62
KENR70	*Nocardiopsis prasina*							15.62
KENR72	*Streptomyces aureus*							1.95
KENR74	*Streptomyces* sp.							1.95
KENR77	*Streptomyces* sp.							< 0.487
KENR79	*Streptomyces* sp.							0.975
KENR80	*Streptomyces* sp.							15.62
KENR81	*Streptomyces* sp.							3.9
KENR84	*Isoptericola sp.*							3.9
KENR86	*Streptomyces* sp.							500
KENR91	*Streptomyces* sp.							7.81
KENR93	*Microbacterium testaceum*							7.81
KENR94	*Streptomyces* sp.						7.81	
KENB3	*Streptomyces* sp.							7.81
KENB8	*Streptomyces* sp.							500
KENB9	*Streptomyces* sp.							3.9
KENB10	*Microbacterium testaceum*						1000	250
KENS2	*Microbispora bryophytorum*							1.95
			No Halo	< 1 cm	1 - 2 cm	> 2 cm		

The screening for anticancer activities revealed 30 isolates that had cytotoxic activity (i.e. reducing viability to less than 50% of the control) against at least one of the tested human cancer cell lines. Fifteen isolates were able to decrease the viability of SH-SY5Y cells to less than 50%, while two isolates produced this effect in T-47D cells and 13 isolates had this effect in both cell lines (Fig. 15A and 15B). Strains KENR18, KENR25, KENR59, KENR60, KENR64, KENR65, KENR91, KENR94 and KENB1 (all *Streptomyces*) had a prominent activity against the cell line SH-5YSY, reducing its viability by more than 90%. Some of these strains (KENR60, KENR64, KENR65, KENR94 and KENB1) also caused a similar effect in the cell line T-47D. Although most of the extracts with high activity had effect in both cell lines, some of them were more effective against only one cell line. This situation was verified for the extracts from the *Streptomyces* strains KENR6, KENR23D, KENR26, KENR31, KENR35, KENR57, KENR71, KENR74, KENR79, KENR80, KENR81, KENR85 and KENR91, and the *Rhodococcus* strains KENR39 and KENR78, that were more effective against SH-5YSY cells, and of the *Streptomyces* strain KENR11A and *Nocardiopsis* KENR70, that were more effective against T-47D cells. Although most of the isolates with anticancer activity were *Streptomyces*, other strains also demonstrated notorious activity. For example, strains of the genera *Rhodococcus* (KENR39 and KENR78), *Microbacterium* (KENB10) and *Nocardiopsis* (KENR70) were also effective in decreasing the cellular viability. Once again, the highest percentage of strains exhibiting cytotoxic activity on cancer cells was retrieved from holdfasts (93%) followed by blades (7%). Effects of the crude extracts were also tested on a non-carcinogenic, endothelial cell line to verify their specificity towards cancer cells. Five extracts demonstrated high cytotoxicity on hCMEC/D3 cells, indicating a more general cytotoxicity. Ten extracts with cytotoxic activity on cancer cells caused a decrease in the viability of the non-tumour cell line less than 30% (KENR16A, KENR23D, KENR26, KENR31, KENR39, KENR57, KENR59, KENR71, KENR85 and KENB10) (Fig. 15C). In particular, extracts from the strains KENR18, KENR25, KENR59 and KENR91 showed a promising profile with high activity on SHSY-5Y cells, but with low activity on normal hCMEC/D3 cells.

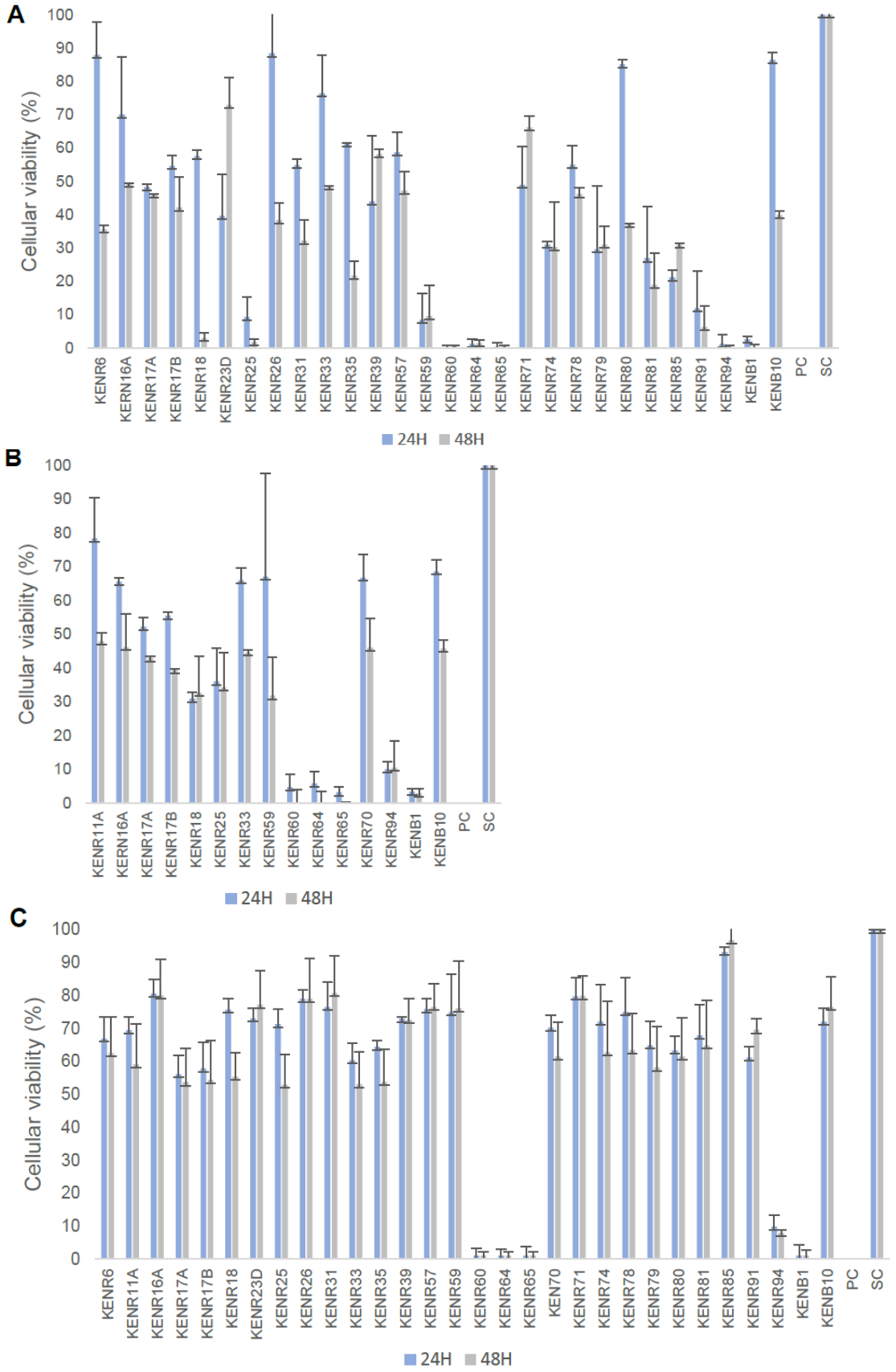


Figure 15. Actinobacterial strains isolated from *L. ochroleuca* with anticancer activity. The graphs show the extracts able to decrease cancer cells viability to less than 50% of the control, after 24 and/or 48 h of exposure, for (A) SH5Y-SH, (B) T-47D, and (C) hCMEC/D3 cell lines. PC and SC indicate positive and solvent controls, respectively. Error bars represent standard deviation of the mean of at least duplicate assays performed in triplicates each.

3. Bioactive Compounds Produced by Actinobacterial Strains

According to the dereplication data, the metabolites present in most of the crude extracts tested are antimycins (Table 5). These compounds were present in 26 out of the 35 tested extracts (74%), being antimycin A_2 the most common, followed by antimycin A_3, antimycin A_1 and finally antimycin A_4. This latter antimycin was found only in the crude extract of the strain *Streptomyces* sp. KENR13B, with this extract being the only one where the presence of the 4 antimycins was detected. Antimycin was produced by strains of all tested genera: Streptomyces, *Isoptericola*, *Microbispora* and *Microbacterium*. For the remaining 9 extracts (26%) no match was found, according to the established criteria (natural compounds with relevant biological activity and cosine value > 0.85). In addition to antimycin, no other compound with known bioactivity was recorded.

Table 5. Dereplication results for the 35 actinobacterial crude extracts selected, indicating the compounds recorded for each one and the correspondent cosine value

Strain	Identification	Compound	Cosine
KENR6	*Streptomyces* sp.	Antimycin A_2	0.92
		Antimycin A_3	0.92
KENR8	*Streptomyces atratus*	Antimycin A_2	0.91
KENR11A	*Streptomyces* sp.	Antimycin A_2	0.93
		Antimycin A_3	0.91
KENR13	*Streptomyces* sp.	Antimycin A_2	0.92
		Antimycin A_3	0.92
KENR13A	*Streptomyces* sp.	Antimycin A_2	0.92
		Antimycin A_3	0.92
KENR13B	*Streptomyces* sp.	Antimycin A_2	0.91
		Antimycin A_3	0.91
		Antimycin A_1	0.85
		Antimycin A_4	0.85
KENR14	*Streptomyces iamenensis*	Antimycin A_2	0.91
KENR17A	*Streptomyces* sp.	Antimycin A_2	0.92
		Antimycin A_3	0.91
KENR18	*Streptomyces* sp.	Antimycin A_2	0.92
		Antimycin A_3	0.92
KENR21A	*Streptomyces* sp.	Antimycin A_2	0.92
		Antimycin A_3	0.92
KENR25	*Streptomyces* sp.	Antimycin A_2	0.92
		Antimycin A_3	0.91
KENR29	*Streptomyces* sp.	Antimycin A_2	0.91
		Antimycin A_3	0.90
KENR31	*Streptomyces* sp.	No match	
KENR33	*Streptomyces lannensis*	Antimycin A_2	0.92
		Antimycin A_3	0.92
KENR35	*Streptomyces* sp.	Antimycin A_3	0.93
		Antimycin A_2	0.92
KENR49	*Streptomyces atratus*	Antimycin A_2	0.91
KENR59	*Streptomyces* sp.	No match	
KENR60	*Streptomyces* sp.	No match	
KENR64	*Streptomyces* sp.	No match	
KENR65	*Streptomyces* sp.	No match	
KENR72	*Streptomyces aureus*	Antimycin A_2	0.92
		Antimycin A_3	0.90
KENR74	*Streptomyces* sp.	Antimycin A_2	0.92
		Antimycin A_3	0.92
KENR77	*Streptomyces* sp.	Antimycin A_3	0.92
		Antimycin A_2	0.90
KENR80	*Streptomyces* sp.	Antimycin A_2	0.92
		Antimycin A_3	0.91
KENR81	*Streptomyces* sp.	Antimycin A_2	0.92
		Antimycin A_3	0.91
		Antimycin A_1	0.85
KENR84	*Isoptericola sp.*	Antimycin A_2	0.92
		Antimycin A_3	0.91
		Antimycin A_1	0.87
KENR85	*Streptomyces diastaticu*	No match	
KENR86	*Streptomyces* sp.	No match	
KENR91	*Streptomyces* sp.	No match	
KENR94	*Streptomyces* sp.	Antimycin A_3	0.88
KENS2	*Microbispora bryophytorum*	Antimycin A_2	0.90
KENB1	*Streptomyces atratus*	No match	
KENB3	*Streptomyces* sp.	Antimycin A_2	0.92
		Antimycin A_3	0.92
KENB9	*Streptomyces* sp.	Antimycin A_2	0.91
KENB10	*Microbacterium testaceum*	Antimycin A_2	0.88

4. Bioassay-guided Study of Bioactive Molecules Produced by *Streptomyces* sp. KENR25

Streptomyces sp. KENR25 was selected for further analysis based on its good performance in the bioactivity screenings: this strain was responsible for an inhibition halo of 22 mm against *C. albicans* and in the anticancer assay proved to be effective against both tested human cancer cell lines (i.e. decreased the cellular viability to less than 50%). To study in more detail the metabolites responsible for the bioactivity, the first step was to grow strain KENR25 in a larger scale to obtain enough biomass for the following procedures. 15 L of the strain were grown, which resulted in 45 g of biomass. The next step consisted in extracting the culture using methanol and acetone, yielding 7.8 g of crude extract.

Once the crude extract was obtained, it was fractionated by VLC. From this procedure 10 fractions (A-I2) (Table 6) were generated and, considering the initial crude extract and the mass of each fraction, the yield of the procedure was approximately 85.5%. In the antimicrobial assay where all the fractions were tested, 5 of them - D, E, F, G and I2 – were able to inhibit the growth of *C. albicans,* with inhibition halos between 15 and 19 mm of diameter. Fractions D, E, F and G presented more diffuse halos while fraction I2 presented a halo with a translucent appearance (Fig. 16).

Table 6. VLC fractions with the indication of the mass amount yield for each one

Fraction	Mass (mg)	Mass (%)
A	28.0	0.42
B	36.4	0.55
C	17.8	0.27
D	65.1	0.98
E	8.4	0.13
F	6.1	0.09
G	22.0	0.33
H	421.3	6.32
I	4639.3	69.58
I2	1423.3	21.35
Total	6666.7	

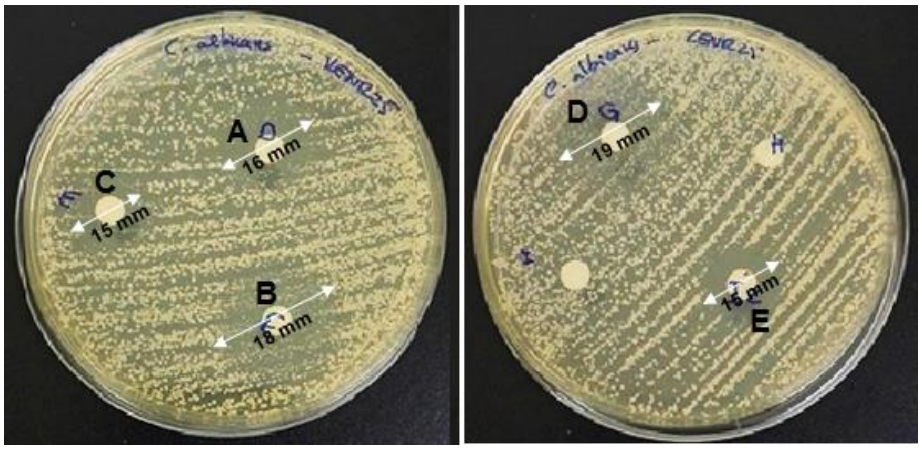


Figure 16. Inhibition halos of the antimicrobial active VLC fractions: (A) fraction D, (B) fraction E, (C) fraction F, (D) fraction G, and (E) fraction I2.

In the assay of anticancer activity, none of the fractions was able to decrease the viability of the cells lines neuroblastoma SH5Y-SH and breast ductal carcinoma T-47D to values below 50% (Figs. 17A and 17B). Nevertheless, the fractions that seemed to have a greater potential were fraction D that led to a decrease in the viability of the cell line SH5Y-SH to 59% after 48 h of exposure, and fraction H that decreased the viability of the cell line T-47D for 57% after 48 h of exposure (Fig. 17B).

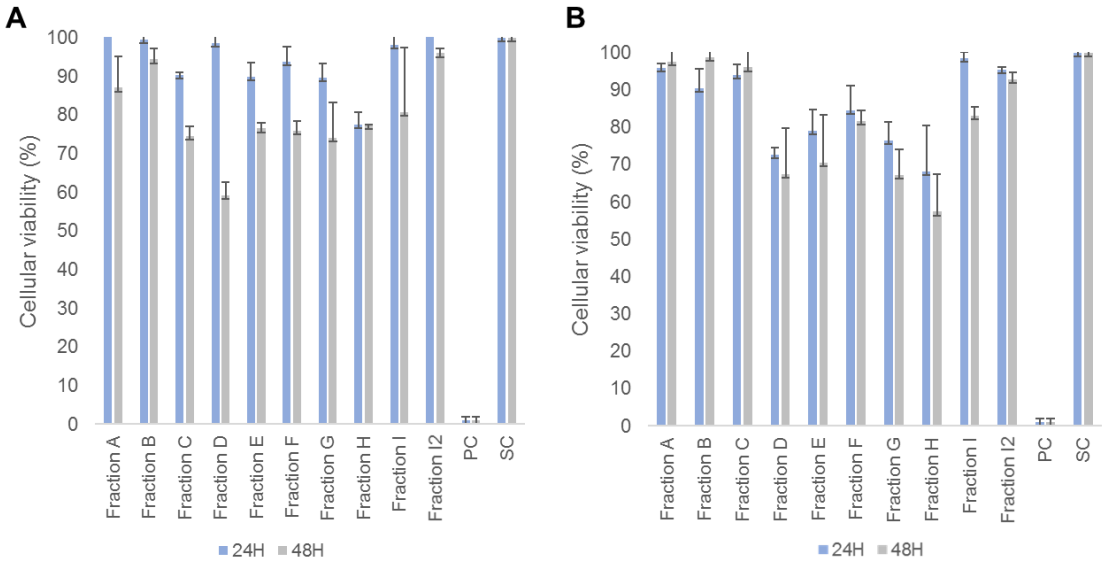


Figure 17. MTT results of VLC fractions. The graphs show the cellular viability after 24 and/or 48 h of exposure for (A) SH5Y-SH, (B) T-47D cell lines. PC and NC indicate positive and negative controls, respectively. Error bars represent standard deviation of the mean of at least duplicate assays.

E

At this point attention became focused on the antimicrobial activity of the fractions, that proved to be more relevant than the anticancer activity. The active fractions (D, E, F, G and I2) and the adjacent ones (C, H and I) were selected to ^{1}H NMR analysis. This analysis showed that the profiles of fractions D, E, F, and G are more similar to each other, as are those of fractions I and I2. The fractions C and H appear to be the ones that differ most from the rest (Fig. 18).

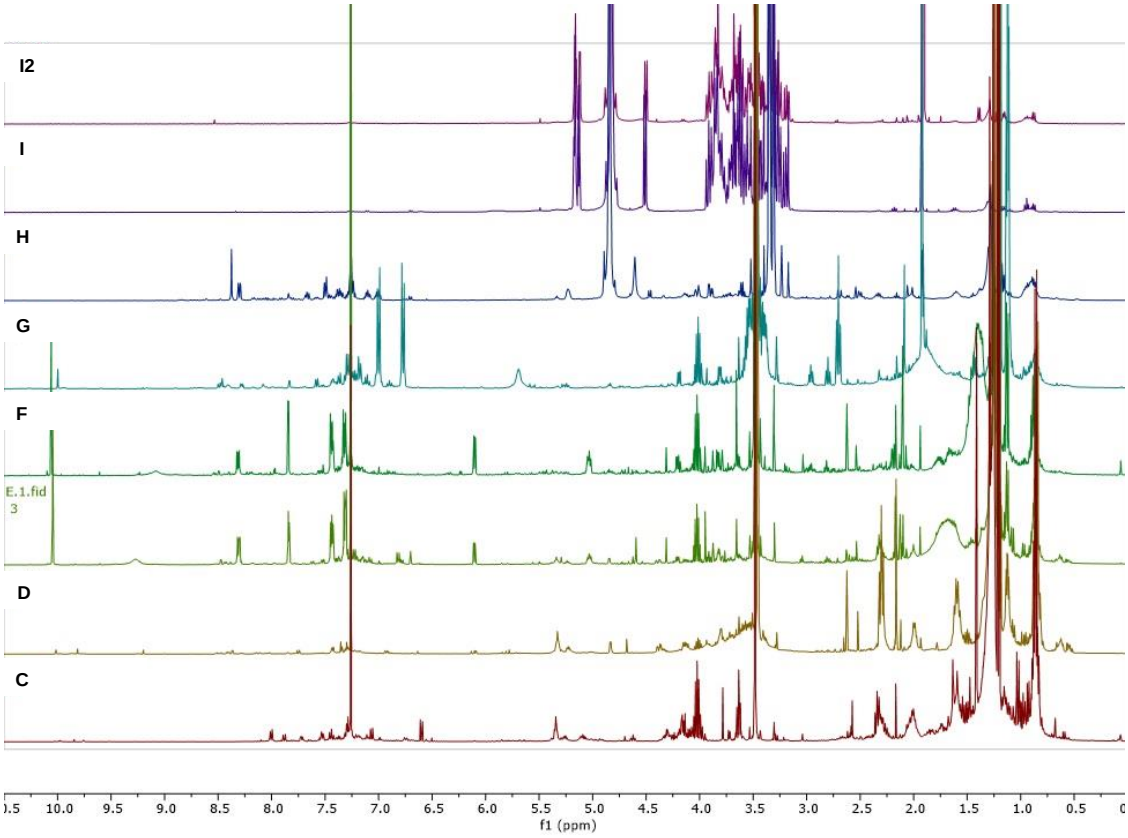


Figure 18. ^{1}H NMR spectra of *Streptomyces* sp. KENR25 VLC fractions C-I2.

According to the results of the antimicrobial assay and the ^{1}H NMR data it was decided to proceed with fractions D, E, F, and G. These 4 fractions were pooled and processed as a single fraction (DEFG), totalizing a mass of 101.64 mg. DEFG fraction was further fractionated by FC, assisted by TLC. From this procedure, 9 fractions (DEFG-A - DEGF-I) were generated (Table 7) and, bearing in mind the DEFG fraction and the mass of each sub-fraction, the yield of the procedure was approximately 96.8%. Once again, all the fractions were tested for their activity against *C. albicans* and 5 fractions - DEFG-A - DEFG-E – showed to be able to inhibit the growth of this microorganism, with inhibition halos between 13 and 25 mm of diameter. All the halos presented a diffuse appearance (Fig. 19).

Table 7. FC fractions with the indication of the mass amount yield for each one

Fraction	Mass (mg)	Mass (%)
DEFG-A	23.8	24.26
DEFG-B	18.2	18.48
DEFG-C	14.8	15.03
DEFG-D	6.3	6.40
DEFG-E	1.9	1.93
DEFG-F	4.3	4.37
DEFG-G	4.1	4.16
DEFG-H	14.5	14.72
DEFG-I	10.5	10.66
Total	98.5	

Figure 19. Inhibition halos caused by of the antimicrobial active FC fractions: (A) fraction DEFG-A, (B) fraction DEFG-B, (C) fraction DEFG-C, (D) fraction DEFG-D and (E) fraction DEFG-E.

The active fractions were again subjected to ^{1}H NMR analysis (Fig. 20). According to the similarity of the obtained spectra it was considered that the fractions DEFG-A and DEFG-B should be processed individually, and the remainder could eventually be grouped.

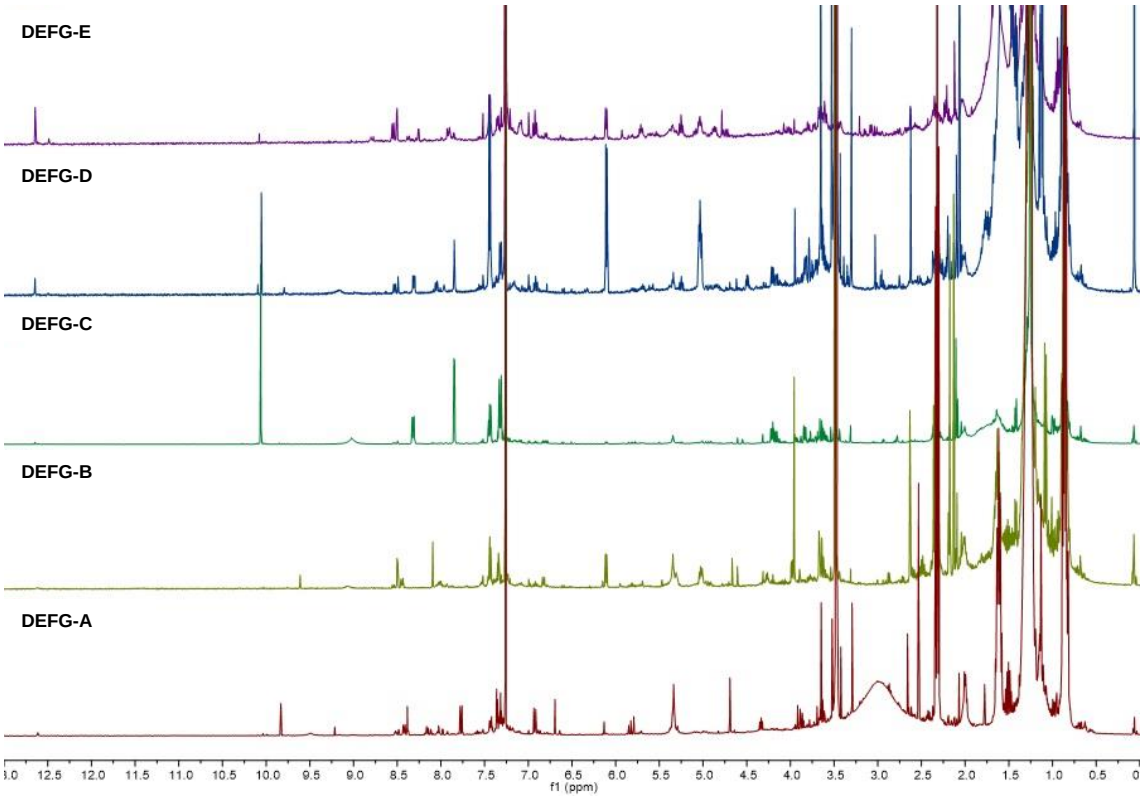
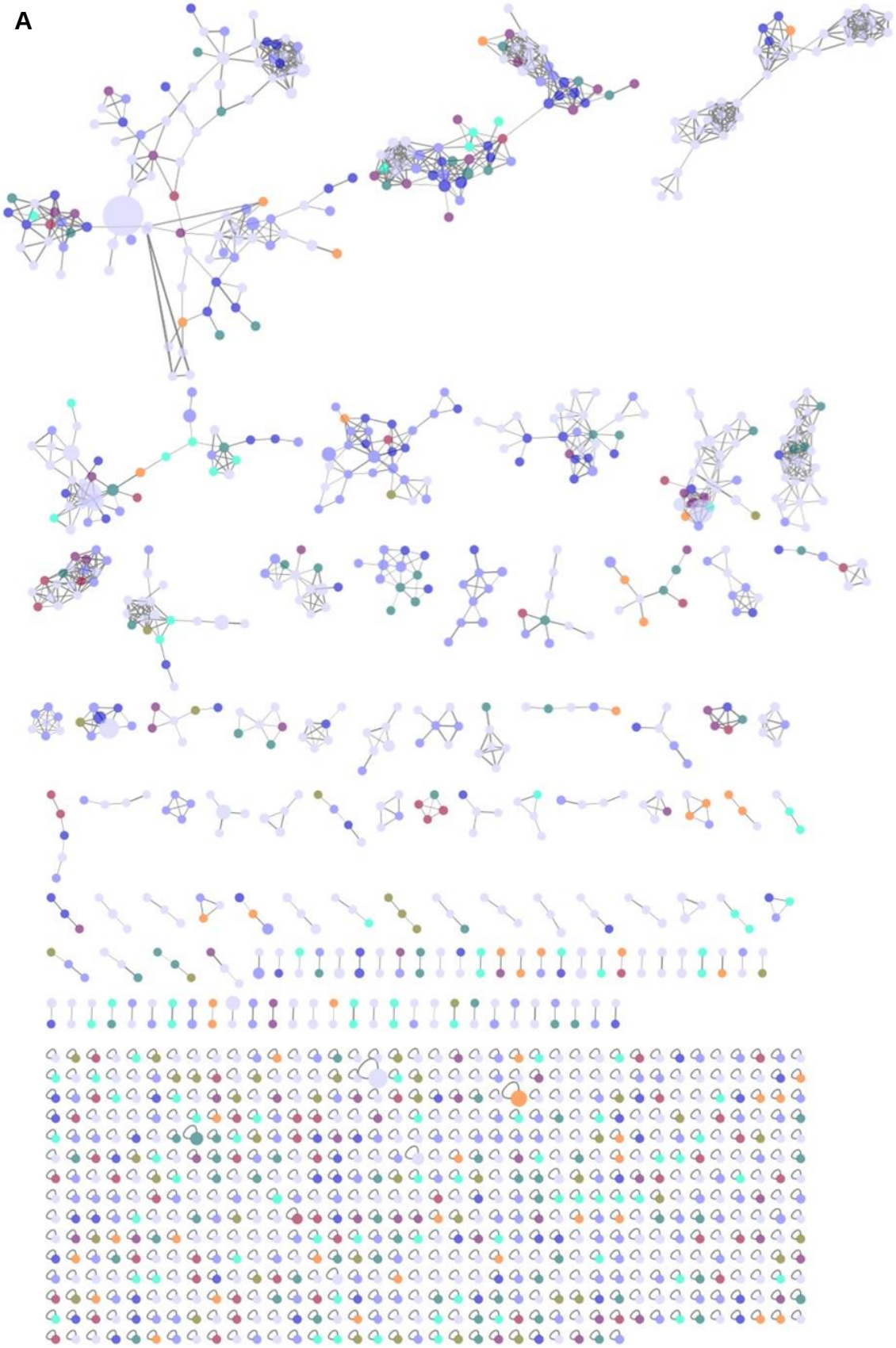


Figure 20. ^{1}H NMR spectra of *Streptomyces* sp. KENR25 FC fractions DEFG-A – DEFG-E.

The five active DEFG fractions were also analysed by LC-MS/MS. The resultant files were uploaded to GNPS directory and two protocols were performed: data analysis and dereplication. According to the results of these procedures and, focusing on the elucidation of the molecules responsible for the bioactivity of the strain, the compounds present in the sample that stood out were the antibiotics Antimycin A_2, A_3, and A_4. Using the MS/MS data, a molecular networking was also developed in Cytoscape (Fig. 21).


A

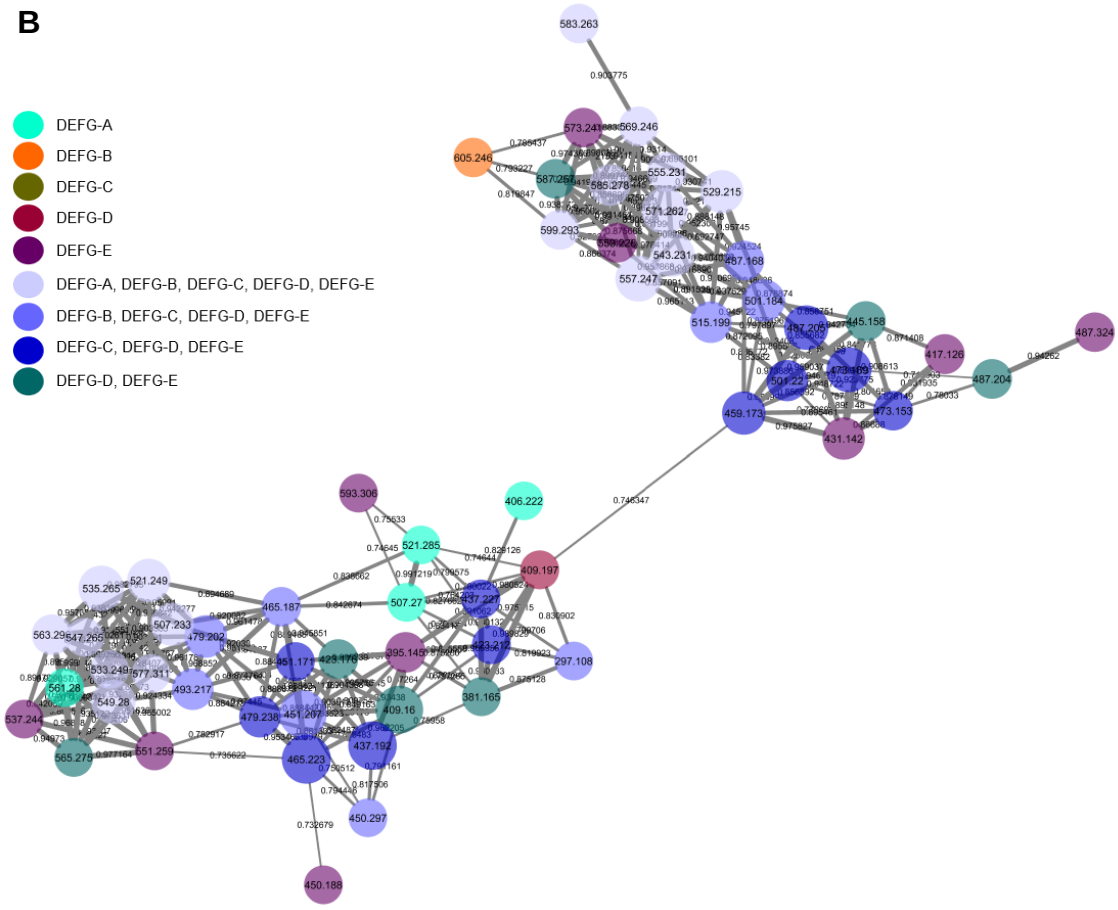


Figure 21. Molecular networking developed with the MS/MS data obtained for the fractions DEFG-A – DEFG-E. (A) Complete molecular networking and (B) cluster corresponding to the antimycins. The numbers within the nodes correspond to the mass of each fragment. The size of the nodes indicates the abundance of the fragment. The similarity between two nodes was computed based on the cosine score defining the connecting edges. The colours match the fragments found in each of the DEFG sub-fractions.

Regarding all data - ^{1}H NMR, LC-MS/MS, antimicrobial activity in the assay, amount of available mass and simplicity of the sample - DEFG-A fraction was selected for HPLC fractionation. From this procedure 10 fractions were obtained (DEFG-A-A – DEFG-A-J) (Table 8), according to the peaks recorded in the spectra (Fig. 22). Concerning the DEFG-A fraction and the mass of each sub-fraction, the yield of the procedure was approximately 72.4 %. Each fraction will be later tested for its action against the growth of *C. albicans,* following the procedures already described for this bioassay.

Table 8. HPLC fractions with the indication of the mass amount yield for each one (BL - baseline)

Fraction	Mass (mg)	Mass (%)
DEFG-A-A	0.1	0.58
DEFG-A-B	0.2	1.16
DEFG-A-C	0.7	4.05
DEFG-A-D	1.5	8.67
DEFG-A-E	2.3	13.29
DEFG-A-F	0.3	1.73
DEFG-A-G	0.2	1.16
DEFG-A-H	0.1	0.58
DEFG-A-I	0.1	0.58
DEFG-A-J	11.2	64.74
BL	0.6	3.47
Total	17.3	

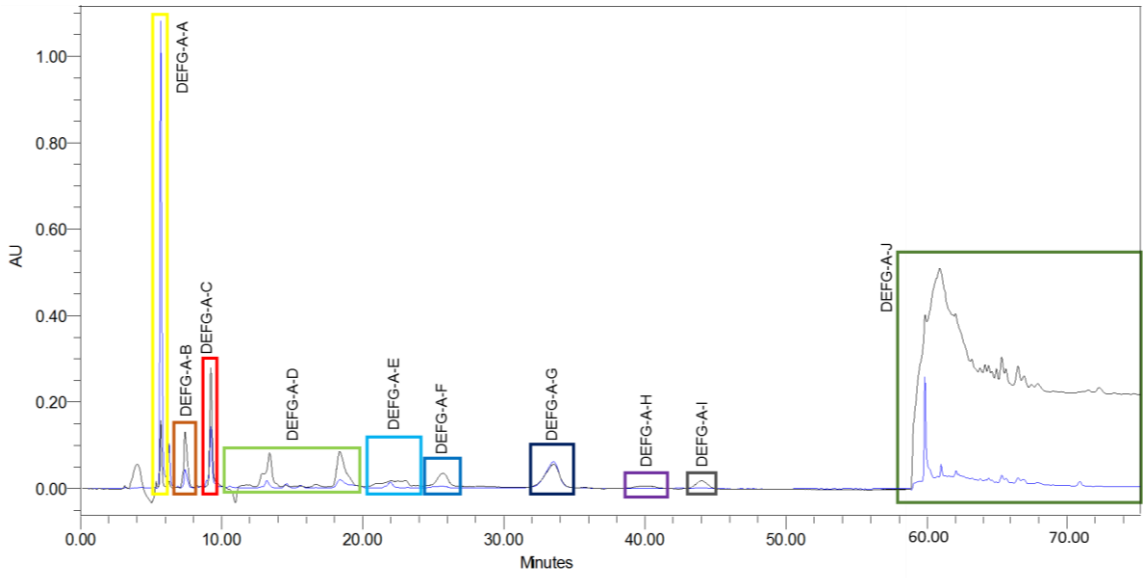


Figure 22. HPLC chromatogram of the peaks corresponding to the fractions DEFG-A-A to DEFG-A-J.

IV. Discussion

Macroalgae offer a wealthy habitat for several epiphytic and endophytic microorganisms [118]. Although actinobacterial endophytes of terrestrial plants have been proven to be a valuable source of bioactive compounds [119, 120] , their presence and diversity in macroalgae and their biotechnological potential is largely unknown. The collection of microorganisms previously isolated from the kelp *L. ochroleuca*, revealed to contain a high number of actinobacterial strains, indicating that this macroalgae is a rich reservoir of actinobacteria. Although it is not possible to guarantee that the recovered isolates are all endophytes (the disinfection process was not entirely effective), only five isolates grew in the disinfection control plates and none was identified as Actinobacteria. Achieving complete microbial sterilization of an organism's surface, such as algae or plants, is difficult and rarely achieved. Several studies focusing on the isolation of endophytic microorganisms have been trying to optimize the disinfection methods, but in general were unable to obtain total efficiency [113, 121, 122]. The efficiency of our sterilization method is in line with the pattern of results generally obtained with these procedures. Though the endophytic nature of the actinobacterial strains isolated from *L. ochroleuca* could not be completely established, the low number of isolates grown in the disinfection controls leads us to accept that many of the recovered strains are in fact endophytes.

The majority of the isolated strains was obtained from holdfasts of *L. ochroleuca*, which may result from the permanent contact of this part of the algae with marine sediments or coastal rocks, where actinobacteria are known to predominate [22, 123]. The largest fraction of actinobacterial isolates was recovered from the culture medium SCN, followed by the RH medium. The success of these media in the recovering of marine actinobacteria has been reported in other studies, reason why they were chosen for the isolation process [124, 125]. The NPS medium allowed a lower recovery of actinobacteria from *L. ochroleuca*, which may be due to the poor nutrients composition of this medium, suggesting that actinobacteria associated with this kelp species may prefer richer carbon sources to grow.

The 16S rRNA gene sequencing analysis revealed that most of the recovered isolates (84%) were affiliated with the genus *Streptomyces*. Microorganisms belonging to this genus have been frequently isolated from several marine ecological niches [126, 127]. It is quite promising to have isolated so many *Streptomyces* strains from *L. ochroleuca* considering that this genus is the most important source of bioactive molecules. Previous studies have also reported the isolation of several *Streptomyces* strains with notorious

biological activity from various algae species, including species belonging to the class Phaeophyceae where *L. ocholeuca* belongs [108, 128, 129]. *Streptomyces* are amongst the actinobacteria most commonly isolated and cultivated under laboratory conditions [127, 128]. Nonetheless, the experimental approach adopted in the present study also allowed the isolation of actinobacterial strains affiliated with six rare actinobacterial genera – *Isoptericola, Rhodococcus, Nonomuraeae, Nocardiopsis, Microbispora* and *Microbacterium*. Rare actinobacterial genera are gaining increasing attention in the field of drug discovery owning to their underexplored metabolic potential [130-132]. Some of the rare actinobacterial genera identified in the present study, namely *Isoptericola, Nocardiopsis* and *Microbacterium*, have also been isolated from other macroalgae species, including some from the class Phaeophyceae [133-136]. The bioactivity assays revealed that half of the total recovered strains exhibited antimicrobial activity. Most of these isolates showed antifungal activity against *C. albicans*, with four *Streptomyces* strains exhibiting the highest activities (inhibition halos > 20 mm and MIC values between < 0.487 and 1.95 µg/mL). *Streptomyces* strains isolated in other studies from diverse marine sources have also been reported to produce secondary metabolites with antifungal properties against *C. albicans*, like isoikarugamycin, a novel polycyclic tetramic acid macrolactam produced by *Streptomyces zhaozhouensis*, with a MIC value of 2–4 µg/mL [137] or saadamycin, a polyketide produced by a marine sponge-associated *Streptomyces* sp., with a MIC value of 1–5 µg/mL [80]. Eight actinobacterial strains isolated in the present study also exhibited antibacterial activity against *S. aureus*, showing inhibition halos of 10-20 mm and MIC values between 3.90 and 7.81 µg/mL. These results are promising, considering that *S. aureus* is a human pathogen responsible for an extensive range of clinical infections, some of them associated with resistance to antibiotics, constituting an important target for the development of novel therapeutic agents [138]. The capacity of marine actinobacteria to produce secondary metabolites active against *S. aureus* has also been previously reported, as is the case of the compounds arenimycin and abyssomicin C, two antibiotics produced respectively by *Salinispora* and *Verrucosispora* strains, active against multidrug-resistant *S. aureus* and with MIC values of < 1 and 13 µg/mL, respectively [98, 139]. Recently, an actinobacterial strain identified as *Kocuria marina* CMG S2, isolated from the brown macroalgae *Pelvetia canaliculata*, was reported to produce a novel antibiotic, named as kocumarin, exhibiting notorious activity against fungi and pathogenic bacteria, including methicillin-resistant *S. aureus* [109].

In this study, the anticancer activity of all actinobacterial isolates was also tested against two human carcinogenic cell lines – neuroblastoma SH-SY5Y and breast ductal carcinoma T-47D. A total of 30 extracts demonstrated capacity to decrease the viability

of the cell lines by more than 50% (at 15 µg/mL) and, in some cases, to values approaching 0%, after 24 h or 48 h of exposure (Fig. 15). Many of those potent extracts demonstrated cytotoxicity also on the non-carcinogenic cell line hCMEC/D3, which indicates general cytotoxicity. However, some of the extracts with strong anticancer activity did have little impact on non-tumour cells, showing an interesting specificity towards neuroblastoma cells (KENR18, KENR25, KENR59, KENR91). These results suggest that some of the isolates might produce secondary metabolites with strong cytotoxicity towards cancer cells, which should be explored in future works. Marine actinobacteria have been already recognised as prolific producers of anticancer compounds, with an increasing number of studies reporting actinobacterial strains isolated from different marine sources, including macroalgae, capable of producing secondary metabolites with anticancer activity [25, 100, 110, 139]. Many of these strains are *Streptomyces* but genera exclusive from marine environments like *Salinispora, Salinibacterium* or *Marinispora* are also wealthy reservoirs of promising anticancer molecules [25, 140].

According to the dereplication procedure, where the metabolomic profile of 35 crude extracts with bioactive properties was investigated, it was possible to notice that antimycins were the mostly abundant compounds. In natural products research, dereplication is an imperative step since it allows to understand if the active compounds present in a sample might be or not already known [141]. Antimicyins are a group of bioactive secondary metabolites exhibiting antifungal activity that were isolated for the first time in 1949 from a soil *Streptomyces* strain [142]. This substance was initially named as antimycin A, but later it was proved that this was a mixture of at least four bioactive components, named as antimycin A_1, A_2, A_3 and A_4 [143]. To date, more than 40 naturally occurring antimycins, and additional ring-expanded antimycins, have been isolated [144]. Since their initial isolation, antimicyins have been target of extensive research for their powerful and diverse biological activities. As a result of their ability to inhibit mitochondrial electron transport chains [145, 146], these macrolide antibiotics have been shown to have antifungal, insecticidal, nematocidal, and piscicidal properties [147-149]. In addition to all these biological features, some classes of antimycins have also proved to have promising anticancer and anti-inflammatory activities [144, 150, 151]. Antimycins-producing actinobacteria have been isolated from numerous environments, including marine ecosystems, with marine *Streptomyces* being especially preponderant in the synthesis of this antibiotic [152-154]. Considering the established antimicrobial and anticancer properties of the antimycins, their presence in 26 out of the 35 extracts selected in this study for dereplication is the most likely explanation for the biological activities observed (Table 5). The occurrence of this antibiotic in so many extracts raised the question of a

possible contamination during the analysis, however, extracts of cyanobacteria were concomitantly examined during the same experimental procedure and, for these extracts, the presence of antimycins was not detected, allowing validating the dereplication results. Although the synthesis of antimycins is usually associated with *Streptomyces*, from the dereplication data it was possible to verify that strains belonging to other genera - *Isoptericola* (KENR84), *Microbacterium* (KENB10), *Microbispora* (KENS2) - also seem to produce them. In addition, there was no correlation between the part of the macroalgae from which the actinobacterial strains were isolated and the production of antimycin, since strains isolated from the holdfast, stipe and blade of *L. ochroleuca* seem to be producers of these metabolites. It may be speculated that macroalgae represents a favourable ecological niche for the synthesis of antimycins, not only by *Streptomyces* but also by other actinobacteria, however these two assumptions are still unclear and may represent future research paths. Apart from identifying the bioactive compounds most probably present in the extracts, dereplication also allows selecting the more promising strains for the eventual discovery of novel bioactive compounds. Therefore, when early used in NPs research, dereplication can minimize time, effort, and costs [155]. The fact that the bioactivities of nine actinobacterial extracts could not be attributed to a known active secondary metabolite in the dereplication process, makes these extracts the most promising ones for future research. All these extracts were derived from *Streptomyces* strains – KENR31, KENR59, KENR60, KENR64, KENR65, KENR85, KENR86, KENR91 and KENRB1 – and displayed strong bioactivities that may possibly be associated with compounds not yet described. By analysing the bioactivity results of these isolates (Table 4, Fig. 15, Annex 2), it is possible to observe that these strains display a very dissimilar profile: KENR31 have antimicrobial activity against both *C. albicans* and *S. aureos (*MIC values of 62.5 and 500 µg/mL, respectively); KENR59 has anticancer activity, decreasing SH5Y-SY cells viability to values below 10%; KENR65 exhibits both antifungal and anticancer activity, being effective against *C. albicans* (MIC of 125 µg/mL) and decreasing SH5Y-SY and T-47D cells viability for values below 3%; KENR85 holds anticancer activity, decreasing SH5Y-SY cells viability to values below 31%; KENR86 has antifungal activity against *C. albicans* (MIC of 1 mg/mL); KENB1 has anticancer activity, decreasing both SH5Y-SY and T-47D cells viability to values below 3%; KENR60 and KENR64 are the most similar strains in terms of bioactivity, as both display antibacterial activity against *S. aureus (*MIC of 3.9 µg/mL) and anticancer activity against SH5Y-SY and T-47D cells, decreasing their viability to values below 5%. In this scenario, the investigation of the bioactive compounds present in the extracts from strains KENR85 and KENR86 may not be a priority, as their activities are not as outstanding as those of the remaining extracts, but

all nine strains mentioned here would be important for future research on bioactive NPs prospecting.

Streptomyces sp. KENR25 was selected for a bioactivity-guided study of the produced bioactive molecules. A strategy based on the traditional natural product discovery pipeline was applied. The strain selection was made before the dereplication procedure, being the presence of antimycin in its extract not yet known. Focusing on the antimicrobial activity of the strain, it was verified that the capacity of inhibiting *C. albicans* growth was preserved throughout the fractionation procedures. In the initial assay, the strain's crude extract presented an inhibition halo with a diameter of 22 mm and a MIC value < 0.487 (Table 4, Fig. 14). From the VLC fractionation, 5 active fractions - D, E, F, G and I2 – were obtained, with inhibition halos between 15-19 mm of diameter (Fig. 16). From the FC fractionation, also 5 active fractions - DEFG-A, DEFG-B, DEFG-C, DEFG-D and DEFG-E - were obtained, with inhibition halos between 13-25 mm of diameter (Fig. 19). In contrast with antimicrobial activity, it seems that anticancer activity was lost with the fractionation process. In the initial anticancer assays, the performance of KENR25 was very strong, especially when the crude extract was tested on the SH-SY5Y cell line, where the viability of the cells decreased to values below 10%. In the T-47D cell line, the cellular viability decreased to values below 36%. However, the best values obtained with the VLC fractions were 59% (fraction D) and 57% (fraction H), for SH-SY5Y and T-47D cells viability, respectively (Fig. 17). To complement the bioactivity results, a molecular networking of the active antimicrobial FC fractions was developed. Molecular networking is an organizational approach to MS/MS data that has been recently introduced in the drug discovery field. Among its various applications, it allows to identify unknown natural products with potential pharmaceutical significance. This approach relies on the molecules chemistry: two related molecules are likely to share similar MS/MS fragmentation patterns, being a molecular network a visual representation of the molecular relatedness (chemical similarity) of any set of compounds [155, 156]. This tool has been increasingly used in the search for novel bioactive compounds, namely produced by marine actinobacteria [157]. By examining the molecular network, it was possible to observe the cluster corresponding to antimycin (Fig. 21B), as would be expected according to the dereplication data. Nevertheless, in this cluster several nodes with slightly different masses from those corresponding to antimycins A_2 and A_3 occur. Some of these might correspond to internal fragments and adducts formed during MS analysis, but others may possibly be new antimycin analogues (a few dozen have already been described, so the existence of more is conceivable). From the molecular networking it was also possible to infer that KENR25 may produce other active metabolites than antimycins due to the presence of other unidentified clusters. A more

detailed study will be necessary in order to evaluate their abundance and distribution within the fractions, but they can be associated with a novel secondary metabolite. The strategy applied for KENR25 strain may be used in the future with promising strains, namely those for which the replication process found no known compounds, leading eventually to the discovery of a novel bioactive substance.

The combination of microbiology, molecular biology, chemistry and *in silico* techniques allowed to understand the bioactive potential of endophytic actinobacteria associated with the macroalgae *L. ochroleuca*. Although challenging and demanding, the possibility of discovering, isolating and characterizing a new pharmaceutically relevant compound from the studied strains remains open.

V. Conclusion

The objective of this MSc thesis was to investigate the community of endophytic actinobacteria associated with the macroalgae *L. ochroleuca* and its respective antimicrobial and anticancer potential. The results presented here suggest that marine macroalgae, such as *L. ochroleuca*, represent a valuable source of actinobacteria, both in terms of abundance and diversity and of natural products with powerful bioactivities. Using an interdisciplinary approach, it was possible not only to evaluate the antimicrobial and anticancer potential of the 90 actinobacterial strains isolated from the studied macroalgae, but also to understand the main compounds responsible for their bioactivities and realise that some of the metabolites produced by them may not yet have been described and constitute new compounds.

In conclusion, this work adds to the yet-meagre knowledge available on actinobacteria associated with marine macroalgae and their bioactive potential. The promising results presented in this thesis pave the way for further studies, namely the analysis of the chemical composition of the bioactive extracts for the identification of the chemical entities responsible for the observed activities.

VI. References

1. Bull, A.T. and J.E. Stach, *Marine actinobacteria: new opportunities for natural product search and discovery.* Trends in microbiology, 2007. **15**(11): p. 491-499.
2. Fisch, K.M. and T.F. Schaeberle, *Toolbox for Antibiotics Discovery from Microorganisms.* Archiv der Pharmazie, 2016. **349**(9): p. 683-691.
3. Newman, D.J. and G.M. Cragg, *Natural products as sources of new drugs from 1981 to 2014.* Journal of natural products, 2016. **79**(3): p. 629-661.
4. Bhatnagar, I. and S.-K. Kim, *Immense essence of excellence: marine microbial bioactive compounds.* Marine drugs, 2010. **8**(10): p. 2673-2701.
5. Azman, A.-S., I. Othman, C.-M. Fang, K.-G. Chan, B.-H. Goh, and L.-H. Lee, *Antibacterial, anticancer and neuroprotective activities of rare Actinobacteria from mangrove forest soils.* Indian journal of microbiology, 2017. **57**(2): p. 177-187.
6. Berdy, J., *Bioactive microbial metabolites.* J Antibiot (Tokyo), 2005. **58**(1): p. 1-26.
7. Dharmaraj, S., *Marine Streptomyces as a novel source of bioactive substances.* World Journal of Microbiology and Biotechnology, 2010. **26**(12): p. 2123-2139.
8. Skinnider, M.A., C.A. Dejong, P.N. Rees, C.W. Johnston, H. Li, A.L. Webster, M.A. Wyatt, and N.A. Magarvey, *Genomes to natural products prediction informatics for secondary metabolomes (PRISM).* Nucleic acids research, 2015. **43**(20): p. 9645-9662.
9. Fenical, W. and P.R. Jensen, *Developing a new resource for drug discovery: marine actinomycete bacteria.* Nature chemical biology, 2006. **2**(12): p. 666.
10. David, B., J.-L. Wolfender, and D.A. Dias, *The pharmaceutical industry and natural products: historical status and new trends.* Phytochemistry Reviews, 2015. **14**(2): p. 299-315.
11. Ventura, M., C. Canchaya, A. Tauch, G. Chandra, G.F. Fitzgerald, K.F. Chater, and D. van Sinderen, *Genomics of Actinobacteria: tracing the evolutionary history of an ancient phylum.* Microbiology and molecular biology reviews, 2007. **71**(3): p. 495-548.
12. Barka, E.A., P. Vatsa, L. Sanchez, N. Gaveau-Vaillant, C. Jacquard, H.-P. Klenk, C. Clément, Y. Ouhdouch, and G.P. van Wezel, *Taxonomy, physiology, and natural products of Actinobacteria.* Microbiology and Molecular Biology Reviews, 2016. **80**(1): p. 1-43.

13. Manivasagan, P., K.-H. Kang, K. Sivakumar, E.C. Li-Chan, H.-M. Oh, and S.-K. Kim, *Marine actinobacteria: an important source of bioactive natural products.* Environmental toxicology and pharmacology, 2014. **38**(1): p. 172-188.
14. ul Hassan, S.S., K. Anjum, S.Q. Abbas, N. Akhter, B.I. Shagufta, S.A.A. Shah, and U. Tasneem, *Emerging biopharmaceuticals from marine actinobacteria.* Environmental toxicology and pharmacology, 2017. **49**: p. 34-47.
15. Sathya, A., R. Vijayabharathi, and S. Gopalakrishnan, *Plant growth-promoting actinobacteria: a new strategy for enhancing sustainable production and protection of grain legumes.* 3 Biotech, 2017. **7**(2): p. 102.
16. Toumatia, O., S. Compant, A. Yekkour, Y. Goudjal, N. Sabaou, F. Mathieu, A. Sessitsch, and A. Zitouni, *Biocontrol and plant growth promoting properties of Streptomyces mutabilis strain IA1 isolated from a Saharan soil on wheat seedlings and visualization of its niches of colonization.* South African Journal of Botany, 2016. **105**: p. 234-239.
17. Alvarez, A., J.M. Saez, J.S.D. Costa, V.L. Colin, M.S. Fuentes, S.A. Cuozzo, C.S. Benimeli, M.A. Polti, and M.J. Amoroso, *Actinobacteria: current research and perspectives for bioremediation of pesticides and heavy metals.* Chemosphere, 2017. **166**: p. 41-62.
18. Aparicio, J.D., E.E. Raimondo, R.A. Gil, C.S. Benimeli, and M.A. Polti, *Actinobacteria consortium as an efficient biotechnological tool for mixed polluted soil reclamation: experimental factorial design for bioremediation process optimization.* Journal of hazardous materials, 2018. **342**: p. 408-417.
19. Matias, F., D. Bonatto, G. Padilla, M.F.d.A. Rodrigues, and J.A.P. Henriques, *Polyhydroxyalkanoates production by actinobacteria isolated from soil.* Canadian journal of microbiology, 2009. **55**(7): p. 790-800.
20. Devine, R., M.I. Hutchings, and N.A. Holmes, *Future directions for the discovery of antibiotics from actinomycete bacteria.* Emerging Topics in Life Sciences, 2017: p. ETLS20160014.
21. Gao, B. and R.S. Gupta, *Phylogenetic framework and molecular signatures for the main clades of the phylum Actinobacteria.* Microbiology and Molecular Biology Reviews, 2012. **76**(1): p. 66-112.
22. Abdelmohsen, U.R., K. Bayer, and U. Hentschel, *Diversity, abundance and natural products of marine sponge-associated actinomycetes.* Natural product reports, 2014. **31**(3): p. 381-399.
23. Jensen, P.R., E. Gontang, C. Mafnas, T.J. Mincer, and W. Fenical, *Culturable marine actinomycete diversity from tropical Pacific Ocean sediments.* Environmental microbiology, 2005. **7**(7): p. 1039-1048.

24. Bibb, M.J., *Regulation of secondary metabolism in streptomycetes.* Current opinion in microbiology, 2005. **8**(2): p. 208-215.
25. Lam, K.S., *Discovery of novel metabolites from marine actinomycetes.* Current opinion in microbiology, 2006. **9**(3): p. 245-251.
26. ul Hassan, S.S. and A.L. Shaikh, *Marine actinobacteria as a drug treasure house.* Biomedicine & Pharmacotherapy, 2017. **87**: p. 46-57.
27. Riedlinger, J., A. Reicke, H. Zähner, B. Krismer, A.T. Bull, L.A. Maldonado, A.C. Ward, M. Goodfellow, B. Bister, and D. Bischoff, *Abyssomicins, inhibitors of the para-aminobenzoic acid pathway produced by the marine Verrucosispora strain AB-18-032.* The Journal of antibiotics, 2004. **57**(4): p. 271-279.
28. Cho, J.Y., H.C. Kwon, P.G. Williams, C.A. Kauffman, P.R. Jensen, and W. Fenical, *Actinofuranones A and B, Polyketides from a Marine-Derived Bacterium Related to the Genus Streptomyces (Actinomycetales)⊥.* Journal of natural products, 2006. **69**(3): p. 425-428.
29. Hughes, C.C., J.B. MacMillan, S.P. Gaudêncio, P.R. Jensen, and W. Fenical, *The ammosamides: structures of cell cycle modulators from a marine-derived Streptomyces species.* Angewandte Chemie International Edition, 2009. **48**(4): p. 725-727.
30. Raveh, A., P.C. Delekta, C.J. Dobry, W. Peng, P.J. Schultz, P.K. Blakely, A.W. Tai, T. Matainaho, D.N. Irani, and D.H. Sherman, *Discovery of potent broad spectrum antivirals derived from marine actinobacteria.* PLoS One, 2013. **8**(12): p. e82318.
31. Asolkar, R.N., K.C. Freel, P.R. Jensen, W. Fenical, T.P. Kondratyuk, E.-J. Park, and J.M. Pezzuto, *Arenamides A− C, Cytotoxic NFκB Inhibitors from the Marine Actinomycete Salinispora arenicola.* Journal of Natural Products, 2010. **73**(4): p. 796-796.
32. Williams, P.G., E.D. Miller, R.N. Asolkar, P.R. Jensen, and W. Fenical, *Arenicolides A− C, 26-Membered Ring Macrolides from the Marine Actinomycete Salinispora arenicola.* The Journal of organic chemistry, 2007. **72**(14): p. 5025-5034.
33. Lu, J., Y. Ma, J. Liang, Y. Xing, T. Xi, and Y. Lu, *Aureolic acids from a marine-derived Streptomyces sp. WBF16.* Microbiological research, 2012. **167**(10): p. 590-595.
34. Mitchell, S.S., B. Nicholson, S. Teisan, K.S. Lam, and B.C. Potts, *Aureoverticillactam, a novel 22-atom macrocyclic lactam from the marine actinomycete Streptomyces aureoverticillatus.* Journal of natural products, 2004. **67**(8): p. 1400-1402.

35. Burg, R.W., B.M. Miller, E.E. Baker, J. Birnbaum, S.A. Currie, R. Hartman, Y.-L. Kong, R.L. Monaghan, G. Olson, and I. Putter, *Avermectins, new family of potent anthelmintic agents: producing organism and fermentation.* Antimicrobial agents and Chemotherapy, 1979. **15**(3): p. 361-367.
36. Lee, J.-G., I.-D. Yoo, and W.-G. Kim, *Differential antiviral activity of benzastatin C and its dechlorinated derivative from Streptomyces nitrosporeus.* Biological and Pharmaceutical Bulletin, 2007. **30**(4): p. 795-797.
37. Socha, A.M., K.L. LaPlante, and D.C. Rowley, *New bisanthraquinone antibiotics and semi-synthetic derivatives with potent activity against clinical Staphylococcus aureus and Enterococcus faecium isolates.* Bioorganic & medicinal chemistry, 2006. **14**(24): p. 8446-8454.
38. Schumacher, R.W., S.C. Talmage, S.A. Miller, K.E. Sarris, B.S. Davidson, and A. Goldberg, *Isolation and structure determination of an antimicrobial ester from a marine sediment-derived bacterium.* Journal of natural products, 2003. **66**(9): p. 1291-1293.
39. Strand, M., M. Carlsson, H. Uvell, K. Islam, K. Edlund, I. Cullman, B. Altermark, Y.-F. Mei, M. Elofsson, and N.-P. Willassen, *Isolation and characterization of anti-adenoviral secondary metabolites from marine actinobacteria.* Marine drugs, 2014. **12**(2): p. 799-821.
40. Hohmann, C., K. Schneider, C. Bruntner, E. Irran, G. Nicholson, A.T. Bull, A.L. Jones, R. Brown, J.E. Stach, and M. Goodfellow, *Caboxamycin, a new antibiotic of the benzoxazole family produced by the deep-sea strain Streptomyces sp. NTK 937.* The Journal of antibiotics, 2009. **62**(2): p. 99-104.
41. Stritzke, K., S. Schulz, H. Laatsch, E. Helmke, and W. Beil, *Novel caprolactones from a marine streptomycete.* Journal of natural products, 2004. **67**(3): p. 395-401.
42. Wu, S.J., S. Fotso, F. Li, S. Qin, and H. Laatsch, *Amorphane Sesquiterpenes from a Marine Streptomyces sp.⊥, 1.* Journal of natural products, 2007. **70**(2): p. 304-306.
43. Maskey, R.P., F.C. Li, S. Qin, H.H. Fiebig, and H. Laatsch, *Chandrananimycins AC: production of novel anticancer antibiotics from a marine Actinomadura sp. isolate M048 by variation of medium composition and growth conditions.* The Journal of antibiotics, 2003. **56**(7): p. 622-629.
44. Xu, Z., K. Jakobi, K. Welzel, and C. Hertweck, *Biosynthesis of the antitumor agent chartreusin involves the oxidative rearrangement of an anthracyclic polyketide.* Chemistry & biology, 2005. **12**(5): p. 579-588.

45. Li, F., R.P. Maskey, S. Qin, I. Sattler, H.H. Fiebig, A. Maier, A. Zeeck, and H. Laatsch, *Chinikomycins A and B: isolation, structure elucidation, and biological activity of novel antibiotics from a marine Streptomyces sp. isolate M045#, 1.* Journal of natural products, 2005. **68**(3): p. 349-353.
46. Soria-Mercado, I.E., A. Prieto-Davo, P.R. Jensen, and W. Fenical, *Antibiotic terpenoid chloro-dihydroquinones from a new marine actinomycete.* Journal of natural products, 2005. **68**(6): p. 904-910.
47. Ye, X., K. Anjum, T. Song, W. Wang, S. Yu, H. Huang, X.-Y. Lian, and Z. Zhang, *A new curvularin glycoside and its cytotoxic and antibacterial analogues from marine actinomycete Pseudonocardia sp. HS7.* Natural product research, 2016. **30**(10): p. 1156-1161.
48. Bürstner, N., S. Roggo, N. Ostermann, J. Blank, C. Delmas, F. Freuler, B. Gerhartz, A. Hinniger, D. Hoepfner, and B. Liechty, *Gift from nature: cyclomarin A kills mycobacteria and malaria parasites by distinct modes of action.* ChemBioChem, 2015. **16**(17): p. 2433-2436.
49. Asolkar, R.N., P.R. Jensen, C.A. Kauffman, and W. Fenical, *Daryamides A− C, weakly cytotoxic polyketides from a marine-derived actinomycete of the genus Streptomyces strain CNQ-085.* Journal of natural products, 2006. **69**(12): p. 1756-1759.
50. Charan, R.D., G. Schlingmann, J. Janso, V. Bernan, X. Feng, and G.T. Carter, *Diazepinomicin, a new antimicrobial alkaloid from a marine Micromonospora sp.* Journal of natural products, 2004. **67**(8): p. 1431-1433.
51. El-Gendy, M.M., M. Shaaban, K.A. Shaaban, A.M. El-Bondkly, and H. Laatsch, *Essramycin: a first triazolopyrimidine antibiotic isolated from nature.* The Journal of antibiotics, 2008. **61**(3): p. 149.
52. Bruntner, C., T. Binder, W. Pathom-aree, M. Goodfellow, A.T. Bull, O. Potterat, C. Puder, S. Hörer, A. Schmid, and W. Bolek, *Frigocyclinone, a novel angucyclinone antibiotic produced by a Streptomyces griseus strain from Antarctica.* The Journal of antibiotics, 2005. **58**(5): p. 346-349.
53. Macherla, V.R., J. Liu, C. Bellows, S. Teisan, B. Nicholson, K.S. Lam, and B.C. Potts, *Glaciapyrroles A, B, and C, pyrrolosesquiterpenes from a Streptomyces sp. isolated from an Alaskan marine sediment.* Journal of natural products, 2005. **68**(5): p. 780-783.
54. Maskey, R.P., M. Sevvana, I. Usón, E. Helmke, and H. Laatsch, *Gutingimycin: a highly complex metabolite from a marine streptomycete.* Angewandte Chemie International Edition, 2004. **43**(10): p. 1281-1283.

55. Asolkar, R.N., D. Schroeder, R. Heckmann, S. Lang, I. Wagner-Doebler, and H. Laatsch, *Helquinoline, a new tetrahydroquinoline antibiotic from Janibacter limosus Hel 1.* The Journal of antibiotics, 2004. **57**(1): p. 17-23.
56. Maskey, R.P., E. Helmke, and H. Laatsch, *Himalomycin A and B: isolation and structure elucidation of new fridamycin type antibiotics from a marine Streptomyces isolate.* The Journal of antibiotics, 2003. **56**(11): p. 942-949.
57. Malet-Cascon, L., F. Romero, F. Espliego-Vazquez, D. Grávalos, and J.L. Fernández-Puentes, *IB-00208, a new cytotoxic polycyclic xanthone produced by a Marine-derived Actinomadura.* The Journal of antibiotics, 2003. **56**(3): p. 219-225.
58. Itoh, T., M. Kinoshita, S. Aoki, and M. Kobayashi, *Komodoquinone A, a novel neuritogenic anthracycline, from marine Streptomyces sp. KS3.* Journal of natural products, 2003. **66**(10): p. 1373-1377.
59. Manam, R.R., S. Teisan, D.J. White, B. Nicholson, J. Grodberg, S.T. Neuteboom, K.S. Lam, D.A. Mosca, G.K. Lloyd, and B.C. Potts, *Lajollamycin, a Nitro-tetraene Spiro-β-lactone-γ-lactam Antibiotic from the Marine Actinomycete Streptomyces n odosus.* Journal of natural products, 2005. **68**(2): p. 240-243.
60. Mullowney, M.W., E. Ó hAinmhire, U. Tanouye, J.E. Burdette, V.C. Pham, and B.T. Murphy, *A Pimarane Diterpene and Cytotoxic Angucyclines from a Marine-Derived Micromonospora sp. in Vietnam's East Sea.* Marine drugs, 2015. **13**(9): p. 5815-5827.
61. Yin, F., P. Sun, B. Tang, H. Gong, Q. Ke, and A. Li, *Anti-parasitic effects of Leptomycin B isolated from Streptomyces sp. CJK17 on marine fish ciliate Cryptocaryon irritans.* Veterinary parasitology, 2016. **217**: p. 89-94.
62. Macherla, V.R., J. Liu, M. Sunga, D.J. White, J. Grodberg, S. Teisan, K.S. Lam, and B.C. Potts, *Lipoxazolidinones A, B, and C: antibacterial 4-oxazolidinones from a marine actinomycete isolated from a Guam marine sediment.* The Journal of Natural Products, 2007. **70**(9): p. 1454-1457.
63. Cho, J.Y., P.G. Williams, H.C. Kwon, P.R. Jensen, and W. Fenical, *Lucentamycins A− D, cytotoxic peptides from the marine-derived actinomycete Nocardiopsis lucentensis.* Journal of natural products, 2007. **70**(8): p. 1321-1328.
64. McArthur, K.A., S.S. Mitchell, G. Tsueng, A. Rheingold, D.J. White, J. Grodberg, K.S. Lam, and B.C. Potts, *Lynamicins a− e, chlorinated bisindole pyrrole antibiotics from a novel marine actinomycete.* Journal of natural products, 2008. **71**(10): p. 1732-1737.
65. Hawas, U.W., M. Shaaban, K.A. Shaaban, M. Speitling, A. Maier, G. Kelter, H.H. Fiebig, M. Meiners, E. Helmke, and H. Laatsch, *Mansouramycins A− D, Cytotoxic*

Isoquinolinequinones from a Marine Streptomycete (1). Journal of natural products, 2009. **72**(12): p. 2120-2124.

66. Kwon, H.C., C.A. Kauffman, P.R. Jensen, and W. Fenical, *Marinomycins A− D, Antitumor-Antibiotics of a New Structure Class from a Marine Actinomycete of the Recently Discovered Genus "Marinispora".* Journal of the American Chemical Society, 2006. **128**(5): p. 1622-1632.
67. Hughes, C.C., A. Prieto-Davo, P.R. Jensen, and W. Fenical, *The marinopyrroles, antibiotics of an unprecedented structure class from a marine Streptomyces sp.* Organic letters, 2008. **10**(4): p. 629-631.
68. Kanoh, K., Y. Matsuo, K. Adachi, H. Imagawa, M. Nishizawa, and Y. Shizuri, *Mechercharmycins A and B, cytotoxic substances from marine-derived Thermoactinomyces sp. YM3-251.* The Journal of antibiotics, 2005. **58**(4): p. 289-292.
69. Vicente, J., A.K. Stewart, R.M. Van Wagoner, E. Elliott, A.J. Bourdelais, and J.L. Wright, *Monacyclinones, new angucyclinone metabolites isolated from Streptomyces sp. M7_15 associated with the Puerto Rican sponge Scopalina ruetzleri.* Marine drugs, 2015. **13**(8): p. 4682-4700.
70. Liang, Y., X. Xie, L. Chen, S. Yan, X. Ye, K. Anjum, H. Huang, X. Lian, and Z. Zhang, *Bioactive polycyclic quinones from marine Streptomyces sp. 182SMLY.* Marine drugs, 2016. **14**(1): p. 10.
71. Hardt, I.H., P.R. Jensen, and W. Fenical, *Neomarinone, and new cytotoxic marinone derivatives, produced by a marine filamentous bacterium (actinomycetales).* Tetrahedron Letters, 2000. **41**(13): p. 2073-2076.
72. Gao, X., Y. Lu, Y. Xing, Y. Ma, J. Lu, W. Bao, Y. Wang, and T. Xi, *A novel anticancer and antifungus phenazine derivative from a marine actinomycete BM-17.* Microbiological research, 2012. **167**(10): p. 616-622.
73. Oh, D.-C., E.A. Gontang, C.A. Kauffman, P.R. Jensen, and W. Fenical, *Salinipyrones and pacificanones, mixed-precursor polyketides from the marine actinomycete Salinispora pacifica.* Journal of natural products, 2008. **71**(4): p. 570-575.
74. Kondratyuk, T.P., E.-J. Park, R. Yu, R.B. Van Breemen, R.N. Asolkar, B.T. Murphy, W. Fenical, and J.M. Pezzuto, *Novel marine phenazines as potential cancer chemopreventive and anti-inflammatory agents.* Marine drugs, 2012. **10**(2): p. 451-464.
75. Hayakawa, Y., S. Shirasaki, S. Shiba, T. Kawasaki, Y. Matsuo, K. Adachi, and Y. Shizuri, *Piericidins C7 and C8, new cytotoxic antibiotics produced by a marine Streptomyces sp.* The Journal of antibiotics, 2007. **60**(3): p. 196-200.

76. Miller, E.D., C.A. Kauffman, P.R. Jensen, and W. Fenical, *Piperazimycins: cytotoxic hexadepsipeptides from a marine-derived bacterium of the genus Streptomyces.* The Journal of organic chemistry, 2007. **72**(2): p. 323-330.
77. Schneider, K., S. Keller, F.E. Wolter, L. Röglin, W. Beil, O. Seitz, G. Nicholson, C. Bruntner, J. Riedlinger, and H.P. Fiedler, *Proximicins A, B, and C—antitumor furan analogues of netropsin from the marine actinomycete Verrucosispora induce upregulation of p53 and the cyclin kinase inhibitor p21.* Angewandte Chemie International Edition, 2008. **47**(17): p. 3258-3261.
78. Dasari, V.R.R.K., M.K.K. Muthyala, M.Y. Nikku, and S.R.R. Donthireddy, *Novel Pyridinium compound from marine actinomycete, Amycolatopsis alba var. nov. DVR D4 showing antimicrobial and cytotoxic activities in vitro.* Microbiological research, 2012. **167**(6): p. 346-351.
79. Gorajana, A., M. Venkatesan, S. Vinjamuri, B.V. Kurada, S. Peela, P. Jangam, E. Poluri, and A. Zeeck, *Resistoflavine, cytotoxic compound from a marine actinomycete, Streptomyces chibaensis AUBN 1/7.* Microbiological research, 2007. **162**(4): p. 322-327.
80. El-Gendy, M.M. and A.M. EL-Bondkly, *Production and genetic improvement of a novel antimycotic agent, saadamycin, against dermatophytes and other clinical fungi from endophytic Streptomyces sp. Hedaya48.* Journal of industrial microbiology & biotechnology, 2010. **37**(8): p. 831-841.
81. Moore, B.S., J.A. Trischman, D. Seng, D. Kho, P.R. Jensen, and W. Fenical, *Salinamides, antiinflammatory depsipeptides from a marine streptomycete.* The Journal of Organic Chemistry, 1999. **64**(4): p. 1145-1150.
82. Matsuda, S., K. Adachi, Y. Matsuo, M. Nukina, and Y. Shizuri, *Salinisporamycin, a novel metabolite from Salinispora arenicora.* The Journal of antibiotics, 2009. **62**(9): p. 519-526.
83. Feling, R.H., G.O. Buchanan, T.J. Mincer, C.A. Kauffman, P.R. Jensen, and W. Fenical, *Salinosporamide A: a highly cytotoxic proteasome inhibitor from a novel microbial source, a marine bacterium of the new genus Salinospora.* Angewandte Chemie International Edition, 2003. **42**(3): p. 355-357.
84. Wu, S.J., S. Fotso, F. Li, S. Qin, G. Kelter, H.H. Fiebig, and H. Laatsch, *N-Carboxamido-staurosporine and Selina-4 (14), 7 (11)-diene-8, 9-diol, New Metabolites from a Marine Streptomyces sp.* The Journal of antibiotics, 2006. **59**(6): p. 331-337.
85. Lu, Y., X. Dong, S. Liu, and X. Bie, *Characterization and identification of a novel marine Streptomyces sp. produced antibacterial substance.* Marine biotechnology, 2009. **11**(6): p. 717.

86. Pimentel-Elardo, S.M., S. Kozytska, T.S. Bugni, C.M. Ireland, H. Moll, and U. Hentschel, *Anti-parasitic compounds from Streptomyces sp. strains isolated from Mediterranean sponges.* Marine drugs, 2010. **8**(2): p. 373-380.
87. Yang, X.-W., K. Peng, Z. Liu, G.-Y. Zhang, J. Li, N. Wang, A. Steinmetz, and Y. Liu, *Strepsesquitriol, a rearranged zizaane-type sesquiterpenoid from the deep-sea-derived actinomycete Streptomyces sp. SCSIO 10355.* Journal of natural products, 2013. **76**(12): p. 2360-2363.
88. Shin, H.J., H.S. Jeong, H.-S. Lee, S.-K. Park, H.M. Kim, and H.J. Kwon, *Isolation and structure determination of streptochlorin, an antiproliferative agent from a marine-derived Streptomyces sp. 04DH110.* Journal of microbiology and biotechnology, 2007. **17**(8): p. 1403-1406.
89. Jeong, S.-Y., H.J. Shin, T.S. Kim, H.-S. Lee, S.-k. Park, and H.M. Kim, *Streptokordin, a new cytotoxic compound of the methylpyridine class from a marine-derived Streptomyces sp. KORDI-3238.* The Journal of antibiotics, 2006. **59**(4): p. 234-240.
90. Fu, S., F. Wang, H. Li, Y. Bao, Y. Yang, H. Shen, B. Lin, and G. Zhou, *Secondary metabolites from marine-derived Streptomyces antibioticus strain H74-21.* Natural product research, 2016. **30**(21): p. 2460-2467.
91. Oh, D.-C., W.K. Strangman, C.A. Kauffman, P.R. Jensen, and W. Fenical, *Thalassospiramides A and B, immunosuppressive peptides from the marine bacterium Thalassospira sp.* Organic letters, 2007. **9**(8): p. 1525-1528.
92. Romero, F., F. Espliego, J.P. BAZ, T.G. DE QUESADA, D. GRÁVALOS, F. DE LA CALLE, and J.L. FERNÁNDEZ-PUENTES, *Thiocoraline, a new depsipeptide with antitumor activity produced by a marine micromonospora.* The Journal of antibiotics, 1997. **50**(9): p. 734-737.
93. Carlson, J.C., S. Li, D.A. Burr, and D.H. Sherman, *Isolation and characterization of tirandamycins from a marine-derived Streptomyces sp.* Journal of natural products, 2009. **72**(11): p. 2076-2079.
94. Maskey, R.P., E. Helmke, O. Kayser, H.H. Fiebig, A. Maier, A. Busche, and H. Laatsch, *Anti-cancer and antibacterial trioxacarcins with high anti-malaria activity from a marine streptomycete and their absolute stereochemistry.* The Journal of antibiotics, 2004. **57**(12): p. 771-779.
95. Shin, H.J., H.-S. Lee, J.S. Lee, J. Shin, M.A. Lee, H.-S. Lee, Y.-J. Lee, J. Yun, and J.S. Kang, *Violapyrones H and I, new cytotoxic compounds isolated from Streptomyces sp. associated with the marine starfish Acanthaster planci.* Marine drugs, 2014. **12**(6): p. 3283-3291.

96. Ventola, C.L., *The antibiotic resistance crisis: part 1: causes and threats.* Pharmacy and Therapeutics, 2015. **40**(4): p. 277.
97. Chelvan, Y., T. Chelvan, A.C. Pushpam, R. Karthik, and K. Ramalingam, *Extraction and Purification of Antimicrobial Compounds from Marine Actinobacteria.* Research Journal of Pharmacy and Technology, 2016. **9**(4): p. 381.
98. Bister, B., D. Bischoff, M. Ströbele, J. Riedlinger, A. Reicke, F. Wolter, A.T. Bull, H. Zähner, H.P. Fiedler, and R.D. Süssmuth, *Abyssomicin C—A Polycyclic Antibiotic from a Marine Verrucosispora Strain as an Inhibitor of the p-Aminobenzoic Acid/Tetrahydrofolate Biosynthesis Pathway.* Angewandte Chemie International Edition, 2004. **43**(19): p. 2574-2576.
99. Freundlich, J.S., M. Lalgondar, J.-R. Wei, S. Swanson, E.J. Sorensen, E.J. Rubin, and J.C. Sacchettini, *The abyssomicin C family as in vitro inhibitors of Mycobacterium tuberculosis.* Tuberculosis, 2010. **90**(5): p. 298-300.
100. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer statistics, 2016.* CA: a cancer journal for clinicians, 2016. **66**(1): p. 7-30.
101. El-Naggar, M., S. El-Assar, and A. Shata, *Production of Antitumor Agents from Novel Marine Actinomycetes Strains Isolated from Alexandria, Egypt.* J Antimicro, 2017. **3**(145): p. 2472-1212.1000145.
102. Steneck, R.S., M.H. Graham, B.J. Bourque, D. Corbett, J.M. Erlandson, J.A. Estes, and M.J. Tegner, *Kelp forest ecosystems: biodiversity, stability, resilience and future.* Environmental conservation, 2002. **29**(4): p. 436-459.
103. Kadam, S.U., B.K. Tiwari, and C.P. O'donnell, *Extraction, structure and biofunctional activities of laminarin from brown algae.* International Journal of Food Science & Technology, 2015. **50**(1): p. 24-31.
104. Khan, W., U.P. Rayirath, S. Subramanian, M.N. Jithesh, P. Rayorath, D.M. Hodges, A.T. Critchley, J.S. Craigie, J. Norrie, and B. Prithiviraj, *Seaweed extracts as biostimulants of plant growth and development.* Journal of Plant Growth Regulation, 2009. **28**(4): p. 386-399.
105. Wang, H.-M.D., C.-C. Chen, P. Huynh, and J.-S. Chang, *Exploring the potential of using algae in cosmetics.* Bioresource technology, 2015. **184**: p. 355-362.
106. Wells, M.L., P. Potin, J.S. Craigie, J.A. Raven, S.S. Merchant, K.E. Helliwell, A.G. Smith, M.E. Camire, and S.H. Brawley, *Algae as nutritional and functional food sources: revisiting our understanding.* Journal of applied phycology, 2017. **29**(2): p. 949-982.

107. Egan, S., T. Thomas, and S. Kjelleberg, *Unlocking the diversity and biotechnological potential of marine surface associated microbial communities.* Current opinion in microbiology, 2008. **11**(3): p. 219-225.
108. Braña, A.F., H.-P. Fiedler, H. Nava, V. González, A. Sarmiento-Vizcaíno, A. Molina, J.L. Acuña, L.A. García, and G. Blanco, *Two Streptomyces species producing antibiotic, antitumor, and anti-inflammatory compounds are widespread among intertidal macroalgae and deep-sea coral reef invertebrates from the central Cantabrian Sea.* Microbial ecology, 2015. **69**(3): p. 512-524.
109. Uzair, B., F. Menaa, B.A. Khan, F.V. Mohammad, V.U. Ahmad, R. Djeribi, and B. Menaa, *Isolation, purification, structural elucidation and antimicrobial activities of kocumarin, a novel antibiotic isolated from actinobacterium Kocuria marina CMG S2 associated with the brown seaweed Pelvetia canaliculata.* Microbiological research, 2018. **206**: p. 186-197.
110. Qin, S., K. Xing, J.-H. Jiang, L.-H. Xu, and W.-J. Li, *Biodiversity, bioactive natural products and biotechnological potential of plant-associated endophytic actinobacteria.* Applied Microbiology and Biotechnology, 2011. **89**(3): p. 457-473.
111. Dinesh, R., V. Srinivasan, M. Anandaraj, and H. Srambikkal, *Endophytic actinobacteria: diversity, secondary metabolism and mechanisms to unsilence biosynthetic gene clusters.* Critical reviews in microbiology, 2017. **43**(5): p. 546-566.
112. Strobel, G. and B. Daisy, *Bioprospecting for microbial endophytes and their natural products.* Microbiology and molecular biology reviews, 2003. **67**(4): p. 491-502.
113. Hollants, J., F. Leliaert, O. De Clerck, and A. Willems, *How endo-is endo-? Surface sterilization of delicate samples: a Bryopsis (Bryopsidales, Chlorophyta) case study.* Symbiosis, 2010. **51**(1): p. 131-138.
114. Stackebrandt, E. and M. Goodfellow, *Nucleic acid techniques in bacterial systematics*. 1991: Wiley.
115. Weisburg, W.G., S.M. Barns, D.A. Pelletier, and D.J. Lane, *16S ribosomal DNA amplification for phylogenetic study.* Journal of bacteriology, 1991. **173**(2): p. 697-703.
116. Kumar, S., G. Stecher, and K. Tamura, *MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets.* Molecular biology and evolution, 2016. **33**(7): p. 1870-1874.
117. Wang, M., J.J. Carver, V.V. Phelan, L.M. Sanchez, N. Garg, Y. Peng, D.D. Nguyen, J. Watrous, C.A. Kapono, and T. Luzzatto-Knaan, *Sharing and*

community curation of mass spectrometry data with Global Natural Products Social Molecular Networking. Nature biotechnology, 2016. **34**(8): p. 828.

118. Egan, S., T. Harder, C. Burke, P. Steinberg, S. Kjelleberg, and T. Thomas, *The seaweed holobiont: understanding seaweed–bacteria interactions.* FEMS Microbiology Reviews, 2013. **37**(3): p. 462-476.
119. Strobel, G.A., *Endophytes as sources of bioactive products.* Microbes and infection, 2003. **5**(6): p. 535-544.
120. Zhao, K., P. Penttinen, T. Guan, J. Xiao, Q. Chen, J. Xu, K. Lindström, L. Zhang, X. Zhang, and G.A. Strobel, *The diversity and anti-microbial activity of endophytic actinomycetes isolated from medicinal plants in Panxi plateau, China.* Current microbiology, 2011. **62**(1): p. 182-190.
121. Ramalashmi, K., K. Prasanna Vengatesh, K. Magesh, R. Sanjana, S. Siril Joe, and K. Ravibalan, *A potential surface sterilization technique and culture media for the isolation of endophytic bacteria from Acalypha indica and its antibacterial activity.* Journal of Medicinal Plants, 2018. **6**(1): p. 181-184.
122. Waheeda, K. and K. Shyam, *Formulation of Novel Surface Sterilization Method and Culture Media for the Isolation of Endophytic Actinomycetes from Medicinal Plants and its Antibacterial Activity.* J Plant Pathol Microbiol, 2017. **8**(399): p. 2.
123. Maldonado, L.A., J.E. Stach, W. Pathom-aree, A.C. Ward, A.T. Bull, and M. Goodfellow, *Diversity of cultivable actinobacteria in geographically widespread marine sediments.* Antonie Van Leeuwenhoek, 2005. **87**(1): p. 11-18.
124. Duncan, K., B. Haltli, K. Gill, H. Correa, F. Berrué, and R. Kerr, *Exploring the diversity and metabolic potential of actinomycetes from temperate marine sediments from Newfoundland, Canada.* Journal of industrial microbiology & biotechnology, 2015. **42**(1): p. 57-72.
125. Webster, N.S., K.J. Wilson, L.L. Blackall, and R.T. Hill, *Phylogenetic diversity of bacteria associated with the marine sponge Rhopaloeides odorabile.* Applied and environmental microbiology, 2001. **67**(1): p. 434-444.
126. Rashad, F.M., H.M. Fathy, A.S. El-Zayat, and A.M. Elghonaimy, *Isolation and characterization of multifunctional Streptomyces species with antimicrobial, nematicidal and phytohormone activities from marine environments in Egypt.* Microbiological research, 2015. **175**: p. 34-47.
127. Xiong, Z.-Q., Q.-X. Liu, Z.-L. Pan, N. Zhao, Z.-X. Feng, and Y. Wang, *Diversity and bioprospecting of culturable actinomycetes from marine sediment of the Yellow Sea, China.* Archives of microbiology, 2015. **197**(2): p. 299-309.
128. Ismail, A., L. Ktari, M. Ahmed, H. Bolhuis, B. Bouhaouala-Zahar, L. Stal, A. Boudabbous, and M. El Bour, *Heterotrophic bacteria associated with the green*

alga Ulva rigida: identification and antimicrobial potential. Journal of Applied Phycology, 2018: p. 1-17.

129. Wiese, J., V. Thiel, K. Nagel, T. Staufenberger, and J.F. Imhoff, *Diversity of antibiotic-active bacteria associated with the brown alga Laminaria saccharina from the Baltic Sea.* Marine Biotechnology, 2009. **11**(2): p. 287-300.
130. Dhakal, D., A.R. Pokhrel, B. Shrestha, and J.K. Sohng, *Marine rare Actinobacteria: isolation, characterization, and strategies for harnessing bioactive compounds.* Frontiers in microbiology, 2017. **8**: p. 1106.
131. Subramani, R. and W. Aalbersberg, *Marine actinomycetes: an ongoing source of novel bioactive metabolites.* Microbiological research, 2012. **167**(10): p. 571-580.
132. Subramani, R. and W. Aalbersberg, *Culturable rare Actinomycetes: diversity, isolation and marine natural product discovery.* Applied microbiology and biotechnology, 2013. **97**(21): p. 9291-9321.
133. Hollants, J., F. Leliaert, O. De Clerck, and A. Willems, *What we can learn from sushi: a review on seaweed–bacterial associations.* FEMS microbiology ecology, 2013. **83**(1): p. 1-16.
134. Martin, M., D. Portetelle, G. Michel, and M. Vandenbol, *Microorganisms living on macroalgae: diversity, interactions, and biotechnological applications.* Applied microbiology and biotechnology, 2014. **98**(7): p. 2917-2935.
135. Santhi, V.S., A.K. Bhagat, S. Saranya, G. Govindarajan, and S.R.D. Jebakumar, *Seaweed (Eucheuma cottonii) associated microorganisms, a versatile enzyme source for the lignocellulosic biomass processing.* International Biodeterioration & Biodegradation, 2014. **96**: p. 144-151.
136. Wang, M., L. Chen, Z. Zhang, X. Wang, S. Qin, and P. Yan, *Screening of alginate lyase-excreting microorganisms from the surface of brown algae.* AMB Express, 2017. **7**(1): p. 74.
137. Lacret, R., D. Oves-Costales, C. Gómez, C. Díaz, M. de la Cruz, I. Pérez-Victoria, F. Vicente, O. Genilloud, and F. Reyes, *New ikarugamycin derivatives with antifungal and antibacterial properties from Streptomyces zhaozhouensis.* Marine drugs, 2014. **13**(1): p. 128-140.
138. Tong, S.Y., J.S. Davis, E. Eichenberger, T.L. Holland, and V.G. Fowler, *Staphylococcus aureus infections: epidemiology, pathophysiology, clinical manifestations, and management.* Clinical microbiology reviews, 2015. **28**(3): p. 603-661.
139. Asolkar, R.N., T.N. Kirkland, P.R. Jensen, and W. Fenical, *Arenimycin, an antibiotic effective against rifampin-and methicillin-resistant Staphylococcus*

aureus from the marine actinomycete Salinispora arenicola. The Journal of antibiotics, 2010. **63**(1): p. 37.

140. Zotchev, S.B., *Marine actinomycetes as an emerging resource for the drug development pipelines.* Journal of biotechnology, 2012. **158**(4): p. 168-175.
141. Nielsen, K.F., M. Månsson, C. Rank, J.C. Frisvad, and T.O. Larsen, *Dereplication of microbial natural products by LC-DAD-TOFMS.* Journal of natural products, 2011. **74**(11): p. 2338-2348.
142. Dunshee, B.R., C. Leben, G. Keitt, and F. Strong, *The isolation and properties of antimycin A.* Journal of the American Chemical Society, 1949. **71**(7): p. 2436-2437.
143. Yang, Y.-Q. and Y. Wu, *Synthesis of antimycins. A review.* Organic preparations and procedures international, 2007. **39**(2): p. 135-152.
144. Liu, J., X. Zhu, S.J. Kim, and W. Zhang, *Antimycin-type depsipeptides: discovery, biosynthesis, chemical synthesis, and bioactivities.* Natural product reports, 2016. **33**(10): p. 1146-1165.
145. Rasimus-Sahari, S., R. Mikkola, M.A. Andersson, M. Jestoi, and M. Salkinoja-Salonen, *Streptomyces strains producing mitochondriotoxic antimycin A found in cereal grains.* International journal of food microbiology, 2016. **218**: p. 78-85.
146. Slater, E., *The mechanism of action of the respiratory inhibitor, antimycin.* Biochimica et Biophysica Acta (BBA)-Reviews on Bioenergetics, 1973. **301**(2): p. 129-154.
147. Hosotani, N., K. Kumagai, H. Nakagawa, T. Shimatani, and I. Saji, *Antimycins A 10～A 16, seven new antimycin antibiotics produced by Streptomyces spp. SPA-10191 and SPA-8893.* The Journal of antibiotics, 2005. **58**(7): p. 460.
148. Lennon, R.E. and C. Vézina, *Antimycin A, a Piscicidal Antibiotic1*, in *Advances in applied microbiology*. 1973, Elsevier. p. 55-96.
149. Shiomi, K., K. Hatae, H. Hatano, A. Matsumoto, Y. Takahashi, C.-L. Jiang, H. Tomoda, S. Kobayashi, H. Tanaka, and S. Ōmura, *A new antibiotic, antimycin A 9, produced by Streptomyces sp. K01-0031.* The journal of antibiotics, 2005. **58**(1): p. 74.
150. Arsianti, A., H. Tanimoto, T. Morimoto, and K. Kakiuchi, *Design and molecular docking study of antimycin A3 analogues as inhibitors of anti-apoptotic Bcl-2 of breast cancer.* Open Journal of Medicinal Chemistry, 2014. **4**(3): p. 79.
151. Strangman, W.K., H.C. Kwon, D. Broide, P.R. Jensen, and W. Fenical, *Potent inhibitors of pro-inflammatory cytokine production produced by a marine-derived bacterium.* Journal of medicinal chemistry, 2009. **52**(8): p. 2317-2327.

152. Han, Z., Y. Xu, O. McConnell, L. Liu, Y. Li, S. Qi, X. Huang, and P. Qian, *Two antimycin A analogues from marine-derived actinomycete Streptomyces lusitanus.* Marine drugs, 2012. **10**(3): p. 668-676.
153. IMAMURA, N., M. NISHIJIMA, K. ADACHI, and H. SANO, *Novel antimycin antibiotics, urauchimycins A and B, produced by marine actinomycete.* The Journal of antibiotics, 1993. **46**(2): p. 241-246.
154. Xu, L.-Y., X.-S. Quan, C. Wang, H.-F. Sheng, G.-X. Zhou, B.-R. Lin, R.-W. Jiang, and X.-S. Yao, *Antimycins A 19 and A 20, two new antimycins produced by marine actinomycete Streptomyces antibioticus H74-18.* The Journal of antibiotics, 2011. **64**(10): p. 661.
155. Yang, J.Y., L.M. Sanchez, C.M. Rath, X. Liu, P.D. Boudreau, N. Bruns, E. Glukhov, A. Wodtke, R. De Felicio, and A. Fenner, *Molecular networking as a dereplication strategy.* Journal of natural products, 2013. **76**(9): p. 1686-1699.
156. Quinn, R.A., L.-F. Nothias, O. Vining, M. Meehan, E. Esquenazi, and P.C. Dorrestein, *Molecular networking as a drug discovery, drug metabolism, and precision medicine strategy.* Trends in pharmacological sciences, 2017. **38**(2): p. 143-154.
157. Duncan, K.R., M. Crüsemann, A. Lechner, A. Sarkar, J. Li, N. Ziemert, M. Wang, N. Bandeira, B.S. Moore, and P.C. Dorrestein, *Molecular networking and pattern-based genome mining improves discovery of biosynthetic gene clusters and their products from Salinispora species.* Chemistry & biology, 2015. **22**(4): p. 460-471.

VII. ANNEX

Annex 1. Identification and bioactive profile of all actinobacterial strains isolated from *L.ochroleuca.*

STRAIN	16S rRNA	ANTIMICROBIAL ASSAY				ANTICANCER ASSAY						DEREPLICATION
		S. aureus		*C. albicans*		SH-SY5Y		T-47D		hCMEC/D3		
		Inhibition Halo (cm)	MIC (µg/mL)	Inhibition Halo (cm)	MIC (µg/mL)	Cellular Viability (% 24 h)	Cellular Viability (% 48 h)	Cellular Viability (% 24 h)	Cellular Viability (% 48 h)	Cellular Viability (% 24 h)	Cellular Viability (% 48 h)	
KENR1	*Streptomyces aureus*	-	-	-	-	92	87	94	77	-	-	-
KENR3	*Streptomyces olivochromogenes*	-	-	1	31.25	95	93	86	88	-	-	-
KENR4	*Streptomyces olivochromogenes*	-	-	-	-	95	69	81	70	-	-	-
KENR5	*Streptomyces sp.*	-	-	-	-	100	100	92	85	-	-	-
KENR6	*Streptomyces sp.*	1	1000	1.8	0.975	87	36	77	60	67	51	Antimycin A_2, A_3
KENR7	*Streptomyces sp.*	-	-	-	-	90	63	80	58	-	-	-
KENR8	*Streptomyces atratus*	-	-	1.7	1.95	100	94	79	86	-	-	Antimycin A_2
KENR10	*Streptomyces diastaticus*	-	-	-	-	68	56	83	69	-	-	-
KENR11	*Streptomyces diastaticus*	-	-	-	-	99	97	100	99	-	-	-
KENR11A	*Streptomyces sp.*	-	-	2	7.81	68	57	78	48	65	47	-
KENR13	*Streptomyces sp.*	-	-	1.7	1.95	77	58	70	57	-	-	Antimycin A_2, A_3
KENR13A	*Streptomyces sp.*	-	-	2.5	0.975	86	72	70	57	-	-	Antimycin A_2, A_3
KENR13B	*Streptomyces sp.*	1	1000	1.2	7.81	89	70	75	67	-	-	Antimycin A_1, A_2, A_3, A_4
KENR13C	*Streptomyces iastaticus*	-	-	-	-	100	88	98	100	-	-	-
KENR14	*Streptomyces iamenensis*	-	-	1.1	7.81	100	53	76	64	-	-	Antimycin A_2
KENR16A	*Streptomyces thermoiolaceus*	-	-	-	-	70	49	65	46	76	69	-
KENR16B	*Streptomyces sp.*	-	-	1.2	15.62	95	97	87	71	-	-	-

KENR17A	*Streptomyces sp.*	-	-	2	1.95	48	46	53	43	50	44	Antimycin $A_{2,}$ A_3
KENR17B	*Streptomyces sp.*	-	-	-	-	55	42	55	39	-	-	-
KENR18	*Streptomyces sp.*	0.8	1000	1.8	0.975	58	3	31	33	73	48	Antimycin $A_{2,}$ A_3
KENR19	*Streptomyces sp.*	-	-	2	0.975	96	79	73	66	-	-	-
KENR21	*Streptomyces sp.*	-	-	1.3	1.95	99	77	87	68	-	-	-
KENR21A	*Streptomyces sp.*	-	-	2	0.975	99	73	80	52	-	-	Antimycin $A_{2,}$ A_3
KENR23A	*Streptomyces sp.*	-	-	-	-	97	55	73	72	-	-	-
KENR23B	*Streptomyces achromogenes*	-	-	-	-	90	81	74	81	-	-	-
KENR23C	*Streptomyces sp.*	-	-	-	-	100	71	86	76	-	-	-
KENR23D	*Streptomyces sp.*	-	-	-	-	39	72	88	81	70	67	-
KENR24	*Streptomyces sp.*	-	-	0.8	7.81	96	75	90	74	67	44	-
KENR25	*Streptomyces sp.*			2.2	< 0.487	9	2	36	34	76	27	Antimycin $A_{2,}$ A_3
KENR26	*Streptomyces sp.*	-	-	-	-	88	38	81	66	-	-	-
KENR27	*Streptomyces sp.*	-	-	-	-	100	98	87	89	-	-	-
KENR28	*Streptomyces atratus*	-	-	-	-	99	91	100	93	-	-	-
KENR29	*Nonomuraea sp.*	-	-	1.4	3.9	50	56	71	77	-	-	Antimycin $A_{2,}$ A_3
KENR30	*Nocardiopsis prasina*	-	-	-	-	59	58	64	57	-	-	-
KENR31	*Streptomyces sp.*	1	500	1.3	62.5	55	32	76	56	69	70	No Match
KENR32	*Streptomyces achromogenes*	-	-	-	-	91	90	98	89	-	-	-
KENR33	*Streptomyces lannensis*	-	-	2.3	1.95	76	48	66	44	55	43	Antimycin $A_{2,}$ A_3
KENR34	*Streptomyces sp.*	-	-	2	15.62	100	64	81	54	-	-	-
KENR35	*Streptomyces sp.*	-	-	2.5	0.975	61	22	58	50	62	43	Antimycin $A_{2,}$ A_3
KENR36	*Streptomyces sp.*	-	-	1	125	89	76	72	65	-	-	-
KENR38	*Streptomyces flaveolus*	-	-	-	-	57	57	75	86	-	-	-
KENR39	*Rhodococcus erythropolis*	-	-	-	-	44	58	68	74	72	66	-
KENR40	*Streptomyces sp.*	-	-	-	-	98	88	90	82	-	-	-
KENR41	*Streptomyces tenae*	-	-	-	-	100	95	89	90	-	-	-
KENR42	*Streptomyces mirabilis*	-	-	1	125	89	68	100	96	-	-	-
KENR45A	*Streptomyces sp.*	-	-	-	-	100	71	92	89	-	-	-
KENR47	*Streptomyces sp.*	-	-	-	-	100	71	75	77	-	-	-

KENR49	*Streptomyces atratus*	-	-	1.8	3.9	70	53	72	71	-	-	Antimycin A_2
KENR50	*Streptomyces sanglieri*	-	-	-	-	73	54	68	60	-	-	-
KENR51	*Streptomyces sanglieri*	-	-	-	-	79	62	77	80	-	-	-
KENR52	*Streptomyces mirabilis*	-	-	-	-	94	78	87	85	-	-	-
KENR55	*Streptomyces sp.*	-	-	-	-	100	78	94	95	-	-	-
KENR57	*Streptomyces sp.*	-	-	-	-	59	47	71	71	72	70	-
KENR59	*Streptomyces sp.*	-	-	-	-	8	10	67	32	43	42	No Match
KENR60	*Streptomyces sp.*	-	-	2.1	3.9	0	1	4	0	0	0	No Match
KENR64	*Streptomyces sp.*	-	-	2	3.9	1	1	5	0	0	0	No Match
KENR65	*Streptomyces sp.*	1.2	125	-	-	0	0	3	0	0	0	No Match
KENR69	*Streptomyces sp.*	1.2	15.62	-	-	73	56	72	61	-	-	-
KENR70	*Nocardiopsis prasina*	1	15.62	-	-	94	83	66	45	66	51	-
KENR71	*Streptomyces brevispora*	-	-	-	-	49	66	75	81	74	74	-
KENR72	*Streptomyces aureus*	-	-	1.7	1.95	100	68	78	71	74	74	Antimycin A_2, A_3
KENR74	*Streptomyces sp.*	-	-	1.6	1.95	31	30	71	67	61	47	Antimycin A_2, A_3
KENR75	*Streptomyces sp.*	-	-	-	-	85	82	100	100	-	-	-
KENR76	*Rhodococcus erythropolis*	-	-	-	-	62	59	69	75	-	-	-
KENR77	*Streptomyces sp.*	-	-	2	< 0.487	100	72	83	66	-	-	Antimycin A_2, A_3
KENR78	*Rhodococcus sp.*	-	-	-	-	55	46	72	73	64	52	-
KENR79	*Streptomyces sp.*	-	-	1.8	0.975	27	31	70	65	58	46	-
KENR80	*Streptomyces sp.*	-	-	1.2	15.82	85	32	62	53	59	49	Antimycin A_2, A_3
KENR81	*Streptomyces sp.*	-	-	1.5	3.9	27	19	65	63	58	51	Antimycin A_1, A_2, A_3
KENR82	*Isoptericola chiayiensis*	-	-	-	-	100	62	82	77	-	-	-
KENR84	*Isoptericola sp.*	-	-	1.8	3.9	73	78	74	76	-	-	Antimycin A_1, A_3
KENR85	*Streptomyces diastaticus*	-	-	-	-	21	31	100	100	95	90	No Match
KENR86	*Streptomyces sp.*	-	-	1.6	1000	100	100	92	89	-	-	No Match
KENR87	*Streptomyces xiamenensis*	-	-	-	-	83	83	84	82	-	-	-
KENR89	*Streptomyces atratus*	-	-	-	-	97	97	77	70	-	-	-
KENR90	*Nocardiopsis prasina*	-	-	-	-	90	94	77	100	-	-	-
KENR91	*Streptomyces sp.*	-	-	-	-	12	6	89	81	64	66	No Match

KENR92	*Streptomyces sp.*	-	-	-	-	95	92	100	100	-	-	-
KENR93	*Microbacterium testaceum*	-	-	1.5	7.81	100	63	88	88	-	-	-
KENR94	*Streptomyces sp.*	-	-	1.7	7.81	1	0	10	10	6	8	Antimycin A_3
KENS1	*Microbacterium testaceum*	-	-	-	-	55	57	90	62	-	-	-
KENS2	*Microbispora bryophytorum*	-	-	1.2	1.95	100	86	100	90	-	-	Antimycin A_2
KENB1	*Streptomyces atratus*	-	-	-	-	2	0	3	3	0	0	No Match
KENB3	*Streptomyces sp.*	-	-	1.3	7.81	100	80	100	84	-	-	Antimycin A_2, A_3
KENB5	*Streptomyces sp.*	-	-	-	-	100	97	95	90	-	-	-
KENB6	*Streptomyces sp.*	-	-	-	-	86	91	69	68	-	-	-
KENB7	*Microbacterium testaceum*	-	-	-	-	100	87	100	88	-	-	-
KENB8	*Streptomyces sp.*	-	-	1	500	65	59	82	80	-	-	-
KENB9	*Streptomyces sp.*	-	-	1.5	3.9	100	56	88	87	-	-	Antimycin A_2
KENB10	*Microbacterium testaceum*	1	1000	1	250	86	40	68	46	68	67	Antimycin A_2

Annex 2. Phylogenetic relationship of the 41 actinobacterial isolates considered for dereplication, based on 16S rRNA gene homology. DNA sequences of the isolates were aligned using the Geneious software, version 11.1.4, resulting in an alignment with 1240 bp. The phylogenic tree was made using the Maximum Likelihood method with 1000 bootstraps based on the Tamura-Nei model. The tree was constructed using the Molecular Evolutionary Genetics Analysis Program, Version 7.0 (MEGA7). Numbers at nodes represent the percentage levels of bootstrap support.