Universidade de Aveiro Departamento de Biologia
Ano 2019

CELSO MIGUEL DA MAIA ALVES

S. CORONOPIFOLIUS BROMOTERPENOS: ATIVIDADE ANTITUMORAL E CARATERIZAÇÃO DE VIAS DE SINALIZAÇÃO INTRACELULARES EM MODELOS *IN VITRO*

S. CORONOPIFOLIUS BROMOTERPENES: ANTITUMOR ACTIVITY AND INTRACELLULAR SIGNAL PATHWAYS CHARACTERIZATION ON *IN VITRO* CANCER MODELS

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“Cheio de Deus, não temo o que virá,
Pois venha o que vier, nunca será
Maior do que a minha alma”

Fernando Pessoa

o júri

presidente

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palavras-chave

Algas, apoptose, células estaminais tumorais, espécies reativas de oxigénio, produtos naturais marinhos, terapias do cancro, vias de sinalização intracelular.

resumo

Atualmente, o cancro representa um dos maiores desafios para a saúde humana e, devido a diversos fatores, é expectável que a sua incidência aumente nas próximas décadas tornando-se de extrema importância desenvolver novas abordagens terapêuticas, incluindo o desenvolvimento de novos fármacos. Neste âmbito, o ambiente marinho tem revelado albergar uma elevada diversidade de estruturas químicas incomuns e distintas com potencial para serem usadas como "scaffolds" no design e desenvolvimento de novos fármacos com grande eficácia e especificidade para o tratamento de doenças humanas, como o cancro. Desta forma, o principal objetivo da presente tese consistiu em estudar o perfil químico da alga vermelha *Sphaerococcus coronopifolius* recolhida na Reserva Natural da Berlenga (Peniche, Portugal) assim como avaliar as atividades antitumorais dos seus principais metabolitos.

O estudo do perfil químico da alga *S. coronopifolius,* através de *screening* bioguiado, permitiu identificar sete compostos, incluindo cinco terpenos previamente descritos e denominados como alloaromadendrene (**1**), bromosphaerol (**3**), sphaerococcenol A (**4**), 12*S*-hydroxy-bromosphaerol (**5**) e 12*R*-hydroxy-bromosphaerol (**7**), assim como duas novas moléculas de origem natural, nomeadamente um diterpeno dactilomelano bromado, designado sphaerodactylomelol (**2**), e um novo sesquiterpeno 7-epi-eudesmano bromado, designado 6-acetyl-sphaeroeudesmanol (**6**). Os compostos (**2**-**5** e **7**) exibiram atividade antiproliferativa num modelo *in vitro* de carcinoma hepatocelular humano (HepG2) num intervalo de IC_{50} entre 42.87 e 279.93 µM, sendo a atividade mais potente induzida pelo sphaerococcenol A (**4**) (IC_{50}: 42.87 µM). Por sua vez, o novo diterpeno, sphaerodactylomelol (**2**), foi o único composto que induziu citotoxicidade (IC_{50}: 719.85 µM) nas células HepG2. Consequentemente, devido às atividades exibidas pelos compostos **2**-**5** e **7** nas células HepG2, o seu potencial antitumoral foi avaliado em diferentes modelos celulares *in vitro* de cancro humano derivados de diferentes tecidos (SH-SY5Y; MCF-7; A549; NCI-H226; PC-3; HCT-15; CACO-2; SK-MEL-28; RenG2) de modo a definir a sua seletividade e potência (0.1 - 100 µM; 24 h). Fibroblastos derivados de tecidos murinos (3T3) foram usados como modelo não tumoral. Os compostos não demonstraram seletividade nem para células tumorais nem entre os modelos derivados de diferentes tecidos. No que diz respeito à capacidade citotóxica, os compostos exibiram um intervalo de IC_{50} entre 4.47 e 89.41 µM. O sphaerococcenol A (**4**) induziu o maior efeito citotóxico exibindo um intervalo de IC_{50} entre 4.47 e 16.59 µM enquanto o sphaerodactylomelol (**2**) demonstrou ser o composto menos citotóxico exibindo um intervalo de IC_{50} entre 33.04 e 89.41 µM. De modo a compreender as vias de sinalização intracelular envolvidas nas atividades citotóxicas observadas, biomarcadores associados à produção de espécies reativas de oxigénio, nomeadamente a produção de peróxido de hidrogénio (H_2O_2) em tempo real, e apoptose (translocação da

fosfatidilserina, potencial mitocondrial membranar, atividade da Caspase-9, condensação de ADN e/ ou fragmentação) foram estudados num modelo *in vitro* de carcinoma humano da mama (MCF-7). As atividades genotóxicas foram avaliadas em fibroblastos derivados de tecidos murinos (L929). O tratamento realizado nas células MCF-7 com os compostos isolados induziu alterações no potencial mitocondrial membranar, aumentou a atividade da Caspase-9 e promoveu a condensação e/ ou a fragmentação de ADN. Com a exceção do bromosphaerol (**3**), todos os compostos promoveram um aumento da produção dos níveis de H_2O_2. No que diz respeito aos ensaios de genotoxicidade, apenas o bromosphaerol (**3**) mediou danos no ADN das células L929. Tendo em vista avaliar os supracitados compostos num sistema que mais se aproximasse a um tecido foram implementadas co-culturas de fibroblastos bronquiais humanos não malignos e células do epitélio bronquial humano malignizadas tendo o 12*R*-hydroxy-bromosphaerol (**7**) induzido citotoxicidade e capacidade de impedir a formação de células estaminais malignas.

Resumindo, os bromoterpenos isolados da alga vermelha *Sphaerococcus coronopifolius* exibiram atividades citotóxicas relevantes, as quais parecem estar associadas aos processos de apoptose, produção de H_2O_2 e danos de ADN. Por sua vez, o composto 12*R*-hydroxy-bromosphaerol (**7**) demonstrou ser o composto mais promissor nos ensaios realizados em sistema de co-cultura impedindo a formação de células tumorais com fenótipo estaminal. Apesar dos resultados obtidos, este trabalho consistiu numa abordagem inicial sendo de extrema importância caraterizar profundamente as vias de sinalização intracelular associadas às atividades antitumorais mediadas por estes compostos de modo a compreender o seu verdadeiro potencial como agentes farmacológicos na terapia do cancro.

keywords

Algae, apoptosis, cancer stem cells, cancer therapeutics, intracellular signaling pathways, marine natural products, reactive oxygen species.

abstract

Nowadays, cancer is one of the major threats to human health and, due to distinct factors, it is expected that its incidence will increase in the next decades leading to an urgent need to develop new approaches to fight it, including the development of new anticancer drugs. Marine environment has revealed to harbor a vast diversity of unusual and distinct chemical structures with high potential to be used as scaffolds for the design and development of new drugs with great effectiveness and specificity to human illnesses, such as cancer. Therefore, the main goal of the present thesis was to study the chemical profile of the red alga *Sphaerococcus coronopifolius* collected in the Berlenga Nature Reserve (Peniche, Portugal) as well as to evaluate the antitumor activities of its major metabolites.

The study of *S. coronopifolius* chemical profile allowed, through antitumor bioguied screening, to identify seven compounds, including five already known terpenes, alloaromadendrene (**1**), bromosphaerol (**3**), sphaerococcenol A (**4**),12*S*-hydroxy-bromosphaerol (**5**), and 12*R*-hydroxy-bromosphaerol (**7**), as well as two new natural molecules, a new brominated dactylomelane diterpene, named sphaerodactylomelol (**2**) and a new brominated 7-epi-eudesmane sesquiterpene, named 6-acetyl-sphaeroeudesmanol (**6**). Compounds (**2**-**5**, **7**) exhibited antiproliferative activity on an *in vitro* model of human hepatocellular carcinoma (HepG2) in an IC_{50} range between 42.87 to 279.93 µM being the highest activity induced by sphaerococcenol A (**4**) (IC_{50}: 42.87 µM). The new diterpene, sphaerodactylomelol (**2**), was the only compound that induced cytotoxicity (IC_{50}: 719.85 µM) on HepG2 cells. Hence, due to the cytotoxic activities exhibited by the compounds **2**-**5** and **7** on HepG2 cells, their antitumor potential was evaluated on several *in vitro* human cancer cells derived from distinct tissues (SH-SY5Y; MCF-7; A549; NCI-H226; PC-3; HCT-15; CACO-2; SK-MEL-28; RenG2) to define their selectivity and potency (0.1 - 100 µM; 24 hours). Murine fibroblasts (3T3) were used as non-tumor cells. *S. coronopifolius* compounds did not displayed selective activity for specific tumor tissue as well as for cancer cells. Concerning the potency of the effects, *S. coronopifolius* metabolites exhibited an IC_{50} range between 4.47 to 89.41 µM. Sphaerococcenol A (**4**) showed the highest cytotoxicity exhibiting a range of IC_{50} between 4.47 to 16.59 µM and sphaerodactylomelol (**2**) displayed the lowest cytotoxicity showing a range of IC_{50} between 33.04 and 89.41 µM. In addition, to understand the intracellular signaling pathways linked to their cytotoxic activities, hallmarks associated to reactive oxygen species production, namely through hydrogen peroxide (H_2O_2) real-time production, and apoptosis (membrane translocation of phosphatidylserine, mitochondrial membrane potential, Caspase-9 activity, and DNA condensation and/ or fragmentation) were studied on an *in vitro* breast carcinoma model (MCF-7 cells). Genotoxic activities were evaluated on fibroblasts derived from mouse tissues (L929 cells). The treatment of MCF-7 cells with compounds induced changes in the mitochondrial

membrane potential, increased Caspase-9 activity, and promoted DNA condensation and/or fragmentation. In addition, with the exception of bromosphaerol (**3**), all compounds promoted the increase of H_2O_2 levels production. Regarding genotoxic effects, only bromosphaerol (**3**) mediated DNA damage in L929 cells. Additionally, to evaluate the compounds' effects in a system similar to a tissue, co-cultures of non-malignant human bronchial fibroblasts and malignant human bronchial epithelial cells were implemented. 12*R*-hydroxy-bromosphaerol (**7**) revealed to be the compound with highest potential exhibiting cytotoxicity and ability to prevent the formation of malignant stem cells.

Summarizing, *Sphaerococcus coronopifolius* bromoditerpenes exhibited cytotoxic activities, which seems to be linked to apoptosis, H_2O_2 generation and induction of DNA damage. Despite the interesting results achieved, this work is an initial approach being of utmost importance to deeply characterize the intracellular signaling pathways associated with antitumor activities mediated by these compounds in order to define their effective pharmacological potential in cancer therapeutics.

Thesis publications

The present work is based on the following manuscripts:

Major publications contribution:

Alves, C**.**, Silva, J., Pinteus, S., Gaspar, H., Alpoim, M.C., Botana, L.M., Pedrosa, R. (2018) From marine origin to therapeutics: The antitumor potential of marine algae-derived compounds. *Frontiers in Pharmacology*. 9, 777 (doi: 10.3389/fphar.2018.00777).

Rodrigues*, D., Alves, C.*****., Horta, A., Pinteus, S., Silva, J., Culioli, G., Thomas, O.P., Pedrosa, R. (2015) Antitumor and Antimicrobial Potential of Bromoditerpenes Isolated from the Red Alga, *Sphaerococcus coronopifolius*. *Marine Drugs*. 13, (2), 713-726 (doi:10.3390/md13020713). *These authors contributed equally to this work.

Minor publications contribution:

Alves, C., Pinteus, S., Rodrigues, A., Horta, A., Pedrosa, R. (2018) Algae from Portuguese Coast Presented High Cytotoxicity and Antiproliferative Effects on an *In vitro* Model of Human Colorectal Cancer. *Pharmacognosy Research*. 10(1): 24-30 (doi: 10.4103/pr.pr_151_16).

Alves, C., Pinteus, S., Horta, A and Pedrosa, R. (2016) High cytotoxicity and anti-proliferative activity of algae extracts on an *in vitro* model of human hepatocellular carcinoma. *SpringerPlus*, 1-13 (doi.org/10.1186/s40064-016-2938-2).

Alves, C., Pinteus, S., Simões, T., Horta, A., Silva, J., Tecelão, C., Pedrosa, R. (2016) *Bifurcaria bifurcata*: a key macro-alga as a source of bioactive compounds and functional ingredients. *International Journal of Food Science & Technology*. 2016:1-9 (doi.org/10.1111/ijfs.13135).

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List of figures and tables

Figures

Tables

Abbreviations and acronyms

5-FU - 5-Fluorouracil

ADC - Antibody–drug conjugate

AIDS - Acquired immunodeficiency syndrome

Akt - Protein kinase B

Ara-A - Vidarabine

Ara-C - Arabinosyl cytosine

Ara-G - Guanosine nucleoside

Ara-GTP - Ara-G triphosphate

ATCC - American Type Culture Collection

ax - Axial

BDDE - Bis(2,3-dibromo-4,5- dihydroxybenzyl) ether

BDDPM - Bis-(2,3-dibromo-4,5-dihydroxy-phenyl)-methane

bFGF - Fibroblast growth factor-basic

brs - Broad singlet

BV - Brentuximab vedotin

C - Carbon

C10 - (E)-9- oxooctadec-10-enoic acid

$CDCl_3$ - Chloroform-*d*

CDK - Cyclin-dependent kinase

Cf-GP - *Capsosiphon fulvescens* glycoprotein

Cf-PS - *Capsosiphon fulvescens* polysaccharide

CH_2 - Methylene

CH_2Cl_2 - Dichloromethane

CH_3 - Methyl

CH_3CN - Acetonitrile

CNS - Central nervous system

CSC - Cancer stem cell

CSC´s - Cancer stem cells

d - Doublet

DAPI - 4′,6-diamidino-2-phenylindole

dd - Doublet of doublets

DDSD - 5(*R*), 19-diacetoxy-15,18(*R* and *S*), dihydro spata-13, 16(*E*)-diene

DMSO - Dimethyl sulfoxide

DMSP - Dimethylsulfoniopropionate

DNA - Deoxyribonucleic acid

DSMZ - The German Collection of Microorganisms and Cell Cultures from the Leibniz Institute (from the German *Deutsche Sammulung von Mikroorganismen und Zelkulturen Gmb H*)

EDTA - Ethylenediaminetetraacetic acid

EGF - Epidermal growth factor

EGFR - Epidermal growth factor receptor

El-SP - *Enteromorpha intestinalis* - sulfated polysaccharide

EM - Eribulin mesylate

EMA - European Medicines Agency

EMS - Ethyl methanesulfonate

EMT - Epithelial-to-mesenchymal transition

ERK - Extracellular-signal-regulated kinase

ESA - *Eucheuma serra* agglutinin

ET-143 - Ecteinascidine-143 (also know as trabectedin)

EtOAc - Ethyl acetate

F-Ara-A - Fludarabine

FBS - Fetal bovine serum

FCCP - Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone

FDA - Food and Drug Administration

FGFR - Fibroblast growth factor receptor

FM-Ara-A - Fludarabine 5-monophosphate

GLP - *Grateloupia longifolia* polysaccharide

GTP - Guanosine-5'-triphosphate

H_2O - Water

H_2O_2 – Hydrogen peroxide

HCl - Hydrochloric acid

HIF-1 - Hypoxia-inducible factor 1

HPLC - High performance liquid chromatography

IC_{50} - Concentration that causes 50% of cell viability reduction

IGF-IR - Insulin-like growth factor-I receptor

IL-6 - Interleukin 6

IL-8 - Interleukin 8

J - Coupling constant

JC-1 - 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide

LDH - Lactate dehydrogenase

LPS - Lipopolysaccharide

m - Multiplet

MAPK - Mitogen-activated protein kinase

MeOH - Methanol

MMAE - Monomethyl auristatin E

MMP - Mitochondrial membrane potential

MMPs - Matrix metalloproteinases

MNP - Marine natural products

MS - Mass spectrometry

MSP - Marine-derived sulfated polysaccharide

MTT - 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAC - *N*-acetyl-cysteine

NFκB - Factor nuclear kappa B

***n*-Hex** - *n*-Hexane

NMR - Nuclear magnetic resonance

NO - Nitric oxide

NPs - Natural products

OTC - Over-the-counter drug

PARP - Poly (ADP-ribose) polymerase

PBS - Phosphate-buffered saline

PDGFR - Platelet-derived growth factor receptor

PI - Propidium iodide

PI3K - Phosphatidylinositol 3-kinase

poli-HEMA - poli-(2-hydroxyethyl methacrylate)

ppm - Parts per million

PS - Phosphatidylserine

R&D - Research and Development

ROS - Reactive oxygen species

ROVs - Remotely operated vehicles

***s* -** Singlet

SDS - Sodium dodecyl sulfate

SEM - Standard error of the mean

SQDG - Sulfoquinovosyldiacylglycerol

td - Triplet of doublets

TDB - 2,3,6-tribromo-4,5-dihydroxybenzyl methyl ether

TNF-α - Tumor necrosis factor-alpha

TTB - Tuberatolide B

UK - United Kingdom

USA - United States of America

UV - Ultraviolet

VEGF - Vascular endothelial growth factor

VEGFR - Vascular endothelial growth factor receptor

VLC - Vacuum liquid chromatography

δ - Chemical shift

$\boldsymbol{\delta_C}$ - ^{13}C chemical shift

$\boldsymbol{\delta_H}$ - ^{1}H chemical shift

Chapter 1

General introduction

This chapter is based on the following manuscript with some modifications:

Alves, C., Silva, J., Pinteus, S., Gaspar, H., Alpoim, M.C., Botana, L.M., Pedrosa, R. (2018). From marine origin to therapeutics: The antitumor potential of marine algae-derived compounds. *Frontiers in Pharmacology* 9, 777 (doi: 10.3389/fphar.2018.00777).

1.1. Introduction

Natural products (NPs) have been used as therapeutic agents for the treatment of a wide spectrum of illnesses for thousands of years, playing an important role in meeting the basic needs of human populations. In 1985, World Health Organization estimated that approximately 65% of world population ensured their primary health care using predominantly plant-derived traditional medicines, with lower prevalence in developed countries (Cragg and Newman, 2013). Due to their unusual chemical features, NPs have functioned as scaffolds for the development of new products with huge therapeutic and industrial potential. Moreover, these compounds present a greater efficiency and specificity towards the targets since they were originated in co-evolution with biological systems. These compounds result from the interactions between organisms and their environment, which promote the production of diverse complex chemical compounds by the organisms to increase their survival and competitiveness (Mishra and Tiwari, 2011).

Comparing with terrestrial organisms, marine organisms do not have a distinguished history of use in traditional medicine. However, in the last 50 years, advances in new technologies and engineering such as scuba diving techniques, manned submersibles and remotely operated vehicles (ROVs) opened up the marine environment to scientific exploration (Cragg and Newman, 2013). The coexistence of several species in these habitats of limited extent increases their competitiveness and complexity. For example, sessile organisms such as algae, corals, sponges and other invertebrates are in constant competition and many of them have evolved chemical weapons to defend themselves against predation or overgrowth of competing species or, conversely, to subdue motile prey species for ingestion. These chemical adaptations are generally defined as “secondary metabolites” and involve different classes of chemical compounds, which have evidenced great pharmacological potential (Simmons *et al.*, 2005). Therefore, marine organisms have revealed to be an exceptional reservoir of NPs, some of them with different structural features from those of terrestrial sources. Despite considerable challenges, some marine compounds arrived in the market and are currently used in therapeutics, providing a useful roadmap for future translational efforts (for details, please see section 1.4.1) (Arizza, 2013). Among the different illnesses, cancer is a growing threat to public health, particularly in developed countries, and it is expected that cancer occurrence and associated deaths will increase in the next years (American Cancer Society, 2015). This huge societal problem is directly associated with the growth and aging of the population and the adoption of

behaviors that contribute to increase cancer risk (American Cancer Society, 2015). Moreover, owing to the tumor cells resistance to drugs, significant toxicity, and undesirable side effects observed with synthetic drugs, there is an urgent need for new antitumor drugs development (Sawadogo *et al.*, 2015; Torre *et al.*, 2015)**.** Given that cancer is a multifactorial and multi-targeting disease that cannot be prevented by mono-targeted therapies, many researchers have focused their efforts towards NPs, especially those from marine environments, to identify novel anticancer compounds. Today, it is estimated that more than 60% of anticancer drugs in the market are of natural origin (Cragg and Newman, 2009). In addition, there are several NPs originated or derived from marine origin, which are presently undergoing clinical trials with oncological indications (AndisInsight, 2018; EMA, 2018a; b; FDA, 2018a; b).

1.2. Cancer biology – General overview

Cancer is one of the major human health problems worldwide, with high social and economic impacts. There is evidence of this disease in antiquity, dating back to the times of the Pharaohs in ancient Egypt and the classical world (Nobili *et al.*, 2009). Currently, worldwide, cancer is responsible for one in each seven deaths, causing more deaths than AIDS, tuberculosis and malaria combined (American Cancer Society, 2015). Only surpassed by cardiovascular diseases, cancer is the second leading cause of death in high-income countries while being the third leading cause of death in low- and middle-income countries, following cardiovascular, infectious and parasitic diseases (American Cancer Society, 2015). By 2030, it is estimated that the incidence of this illness will grow to over 21.7 million new cases and 13 million deaths (American Cancer Society, 2015). In specific case of Europe, despite it has been observed a decline of total cancer mortality rates between 2012 and 2018, the predictions of total number of deaths linked with this illness comparing 2012 to 2017 and 2018, it is expected to increase 3% and 3.6%, respectively (Malvezzi *et al.*, 2017; 2018). The most common cancers are prostate, lung, breast and colorectal. Amongst, lung cancer has the highest predicted number of deaths associated in the European Union for both sexes (Malvezzi *et al.*, 2018). Beyond the social impact, cancer is also associated with high financial costs for both the patient and for society. For example, in 2011, in the USA the direct medical costs (total of all health care expenditures) associated with hospital outpatient or office-based provider visits, patient hospital stays and medical prescriptions was estimated in $88.7 billion (American Cancer Society, 2015).

However, what we simply call “human cancer” comprises, in fact, more than 100 different diseases that result from the continuous uncontrolled proliferation of cancer cells (Urbano *et al.*, 2011), which have the capability to invade organs and normal tissues, as well as metastasizing through the body. These cells do not respond properly to the signals that regulate their normal behavior (Schulz, 2007; Cooper and Hausman, 2013). In line with this view, Hanahan and Weinberg (2000) published a review article that combined information about cancer biology and defined six hallmarks (sustaining proliferative signaling, resisting cell death, inducing angiogenesis, enabling replicative immortality, activating invasion and metastasis and evading growth suppressors) that all cancer cells have and that are responsible for their malignant properties. Subsequently, an upgrade of this list was done adding two new hallmarks, deregulating cellular energetics and evading immune destruction. Nevertheless, the occurrence of these hallmarks is directly associated with the genome instability, which is responsible by genetic diversity that stimulates hallmarks acquisition, and inflammation promoting multiple hallmark functions (Hanahan and Weinberg, 2011). Recently, case–control metagenomics studies suggest that dysbiosis in the commensal microbiota is also associated with various cancer types adding microbiome as an additional hallmark (Rajagopala *et al.*, 2017) (Figure 1.1). The complexity of tumors represents a great challenge for therapeutic approaches, as experimental evidence exists that each core hallmark capability is regulated by partially redundant signaling pathways (Hanahan and Weinberg, 2011). Consequently, targeted therapy mediated by drugs that only act on one key pathway in a tumor may not be enough to “switch off” a hallmark capability completely. In line with this view, some cancer cells can survive maintaining a basal function awaiting an adaptation of their progeny to the selective pressure imposed by the drug. This adaption can be accomplished by genetic changes, epigenetic reprogramming, or remodelling of the stromal microenvironment. All of these processes can contribute for restitution of the functional capability, allowing renewed tumor growth and consequently clinical relapse (Hanahan and Weinberg, 2011). Nevertheless, the drug resistance of tumor cell lines can also be mediated by other mechanisms, such as drug efflux, suppression of drug activity, changes in cellular targets, enhancement of DNA repair mechanisms, inability to induce cell death and the epithelial-to-mesenchymal transition (EMT) (Housman *et al.*, 2014). Among all the treatments currently used in cancer (surgery, radiotherapy, hormonal treatment and immunotherapy, adjunct therapy, and chemotherapy), chemotherapy continues to play an extremely important role. However, its effectiveness is limited in some cases by the existence of drug resistance, making it necessary to define optimal combinations for therapeutic strategies that ensure an efficient

elimination of the tumor. Moreover, in the last decades, with the continuous growth of cancer cases and concerns over toxicity, tumor cell resistance, the development of secondary cancers and the unwanted side effects observed with synthetic drugs, there has been an increased interest in exploiting NPs for cancer treatment (Newman and Cragg, 2012; Sawadogo *et al.*, 2015).

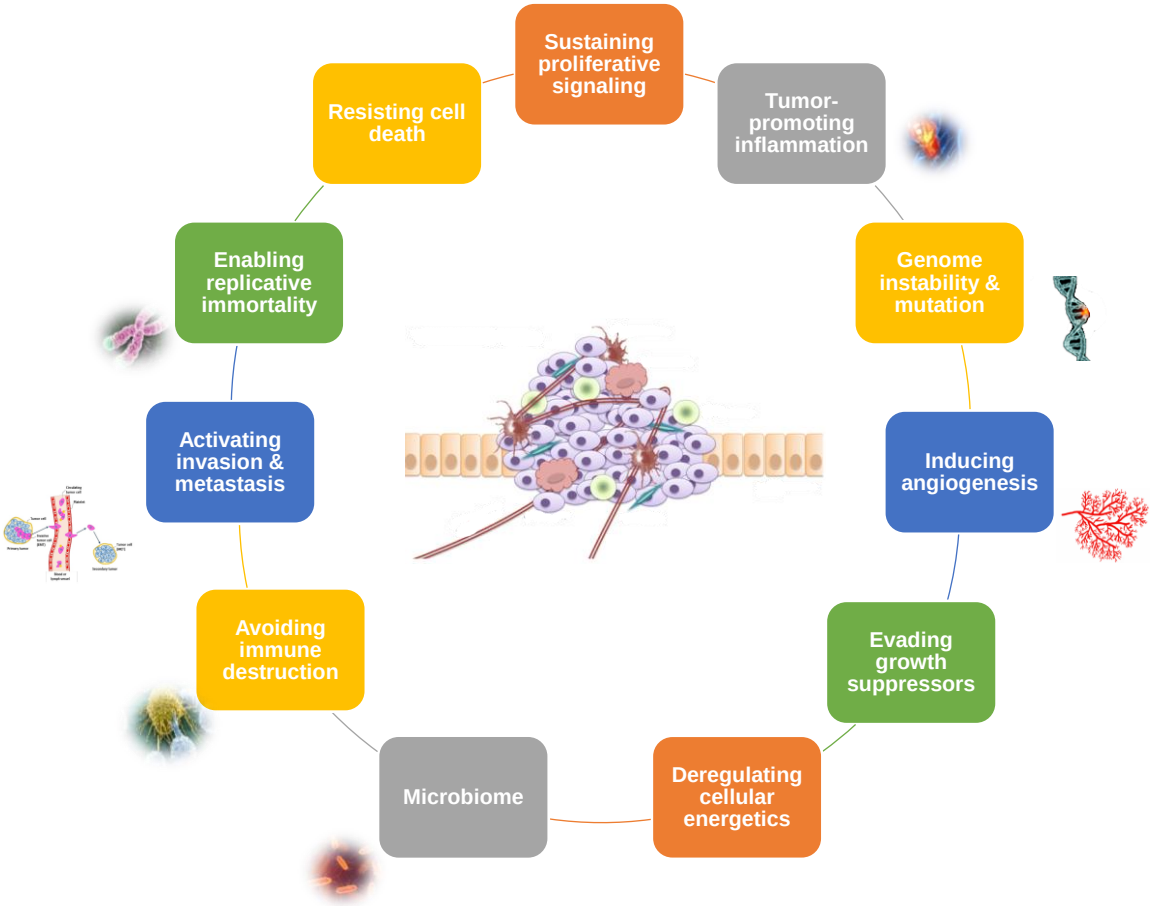

Figure 1.1. The hallmarks of cancer and their enabling characteristics (Adapted from Hanahan and Weinberg, 2000; 2011; Rajagopala *et al.*, 2017)

1.3. Role of the marine chemical ecology in the production of bioactive metabolites on algae

The oceans represent a vast area of the planet and play a fundamental role in its dynamic. Their physics, chemistry and biology are key elements in the functioning of the earth system, providing an interconnection between the different natural systems (terrestrial, freshwater, estuarine, coastal and oceanic) and a range of valuable ecosystem

services (Atkins *et al.*, 2011; Halpern *et al.*, 2012; Horta *et al*., 2015). Their essential role is further noted by the significant fraction of the Earth´s biodiversity that oceans harbour (Brahmachari, 2015). According to the 33 animal phyla listed by Margulis and Chapman (2009), 32 of them are represented in aquatic environments, with 15 exclusively marine, 17 found in marine and non-marine environments (with 5 of these having more than 95% of their species only in marine environments), and only one exclusively non-marine (Onychophora). A recent study predicted the existence of ~ 8.9 million eukaryotic species, of which ~ 2.2 million are marine organisms, suggesting that around 86% of the species on the earth, and 91% in the ocean, have not yet been described (Mora *et al.*, 2011; Cragg and Newman, 2013; Berkov *et al.*, 2014). The existence of a huge diversity of life forms in the oceans is associated with the very exigent, competitive and aggressive surrounding that promotes specific and complex interactions, both inter-species and intra-species. During the evolutionary period, many species share a common environment establishing well-balanced associations between them. Inside of these communities several organisms survive and live in close association with other species, both macro (e.g., algae, sponges, and ascidians) and micro (e.g., bacteria, fungi, and actinomycetes) in order to ensure their survival (Jebasingh *et al.*, 2011; da Cruz *et al.*, 2012; Graça *et al.*, 2013; Horta *et al.*, 2014; Smith *et al.*, 2018). Many of these complex interactions are mediated by chemical signals, which play a crucial role at the organizational level in the marine environment (Hay, 2009). These chemical cues constituting much of the language of sea life, are of the utmost importance for several marine species which have not some senses such as vision and hearing; nevertheless even species that see and hear rely on chemical cues (Horta *et al*., 2015). Interactions mediated by chemical signals play a crucial and decisive role in ecological processes. These signals influence population structure, community organization and ecosystem function. In addition, they are involved in the definition of escape strategies, commensal associations, partners and habitats, competitive interactions, feeding choices and energy and nutrients transfer within and among ecosystems (Hay, 2009).

Among marine organisms, algae are a clear example in which chemical signals play a fundamental role in ecological processes. These signs are involved in the growth and survival in extremely exigent conditions, giving them competitive advantages relative to other marine organisms including against predators and competitors. One such piece of evidence has recently been observed in coral reefs, which are in dramatic global decline, with algae commonly replacing them. Several studies have observed that algae damage corals directly or colonize opportunistically, suppressing coral recruitment through the production of specific chemical cues (Rasher and Hay, 2010; Dixson *et al.*, 2014; Rasher

and Hay, 2014). Recently, Rasher and co-workers (2011) identified four compounds (two loliolide derivatives and two acetylated diterpenes) from two algae as potent allelochemicals which directly damage corals. Marine organisms sometimes face the dilemma of how to allocate the limited resources available, having to strategically adapt and respond to ecosystem stress. Usually, these decisions may have consequences on their growth, reproduction, or ability to counteract biological (e.g., predators, maintenance of unfouled surfaces, paralyzing their prey, etc.) and/ or physical stress (e.g., UV light, temperature, nutrient availability, high pressure, salinity, oxygen content, etc.) (Winter *et al.*, 2010; Horta *et al*., 2015). The production of these types of compounds has also been revealed to be an important weapon for the successful invasion of non-indigenous species into new ranges. For instance, the invasive red alga *Bonnemaisonia hamifera* has become one of the most abundant species in Scandinavian waters. According to Svensson and co-workers (2013) the high capacity of this alga to colonize these waters seems to be linked with the presence of a specific chemical compound (1,1,3,3-tetrabromo-2-heptanone). The production of this metabolite inhibit the settlement of propagules on its thallus and on surrounding surfaces, achieving a competitive advantage over native algae (Svensson *et al.*, 2013). Chemical cues also play an important role in the symbiotic interactions established between algae and microorganisms. The cross-kingdom interactions between them are not restricted to the exchange of macronutrients, including vitamins and nutrients but also include the use of infochemicals with different functions, establishing a tight relationship and enabling them to interact as a unified functional entity (Egan *et al.*, 2013; Wichard, 2015). For instance, associated microorganisms are responsible to produce compounds of utmost importance which mediate essential ecological functions in the development and growth of algae species including quorum sensing signaling molecules, compounds with biological activities, substances that promote the growth and other effective molecules (Singh and Reddy, 2014). Some of these compounds, such as bacterial morphogenetic compounds, dimethylsulfoniopropionate (DMSP), the amino acids proline and alanine, halogenated furanones and fucoxanthin, play important roles in the ecological function, interfering with the surface fouling of others organisms as well as with the vital functions performance of the algae. These compounds prevent the attachment of certain bacteria (e.g., *Cytophaga* sp.) and support the fixation of others (e.g., *Rheinheimera baltica*), controlling the community composition and abundance of the algae-associated bacteria (Saha *et al.*, 2011; Saha *et al.*, 2012; Spoerner *et al.*, 2012; Egan *et al.*, 2013). Marine organism interactions promote the production of a high diversity of marine NPs with quite specific and potent

activities, representing an enormous source of new compounds with potential for biotechnology applications providing economic and human benefits.

1.4. Marine natural products as a source of new drugs

Along evolution, marine organisms developed exceptional metabolic capacities through the production of compounds with quite specific and potent activities (Murray *et al.*, 2013; Martins *et al.*, 2014). These compounds, often defined as secondary metabolites, are generally limited to a particular taxonomic family, genus, species or even organism, characterized by their wide heterogeneity, and often constitute a very small fraction of the total biomass of the organism (Ianora *et al.*, 2006; Avila *et al.*, 2008; Martins *et al.*, 2014). Predominantly, their production occurs in sessile or slow-moving organisms (e.g., algae, sponges, cnidarians, tunicates and bryozoans) that, without effective escape mechanisms or structural protection, ensure their protection through chemical defense (Noyer *et al.*, 2011; Horta *et al*., 2015). Nevertheless, many organisms have the capacity to sequester secondary metabolites from their diet and then derivatize them to more or less toxic forms, which can be used for different functions from their roles in the original producer (Ianora *et al.*, 2006; Kicklighter *et al.*, 2011; Horta *et al*., 2015; Gotsbacher and Karuso, 2015). Moreover, since natural compounds released into the water are rapidly diluted, they need to be highly potent to retain their efficacy (Haefner, 2003). For these reasons, it is widely accepted that a huge number of NPs and novel chemical entities that exist in the oceans could be useful for providing sustainable economic and human benefits.

To date, different types of secondary metabolites (e.g., terpenoids, alkaloids, polyketides, peptides, shikimic acid derivatives, sugars, steroids and a large mixture of biogenesis metabolites) were isolated from marine organisms and found to exhibit many biological activities (antimicrobial, antitumor, antidiabetic, anticoagulant, antioxidant, anti-inflammatory, antiviral, antimalarial, antitubercular, anti-aging, antifouling, and antiprotozoal) with huge industrial and therapeutic potential (Blunt *et al.*, 2013; Mayer *et al.*, 2013; Blunt *et al.*, 2014; Mayer *et al.*, 2017; Agrawal *et al.*, 2018). Marine NPs have exhibited rare and unique chemical structures, upon which the molecular modeling and chemical synthesis of new drugs can be based on (Dias *et al.*, 2012; Horta *et al*., 2015). Since this kind of compounds are originated from nature, they present several advantages when compared with synthetic compounds such as chemical diversity, biochemical

specificity, binding efficiency and affinity to interact with biological systems, making them interesting structures for the development of new drugs (Martins *et al.*, 2014).

The marine biodiscovery and vision of marine-derived drugs on the market had their beginning in the early 1950s with Bergmann, who isolated and identified two nucleosides, spongouridine and spongothymidine, from the Caribbean sponge *Cryptotethya crypta* (previously known as *Tethya crypta*). These discoveries led researchers to synthesize analogues, *Ara-A* (*Vidarabine*®, *Vidarabin Thilo*®, *Vira-A*®) and *Ara-C* (*Cytarabine*, *Alexan*®, *Udicil*®, Cytosar-U®), the first marine derived compounds that have reached the market as antiviral and antitumor drugs, respectively (Newman *et al.*, 2009; Horta *et al*., 2015). Over the last 50 years it was reported the isolation of more than 30,000 new compounds of marine origin (Figure 1.2) and the approval of more than 300 patents (Horta *et al*., 2015).

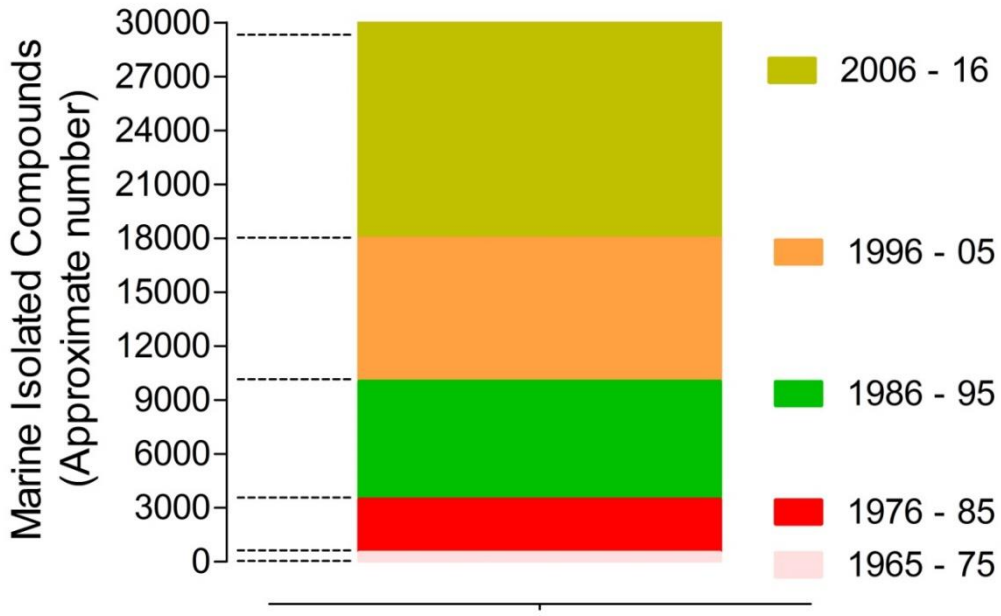

Figure 1.2. Marine compounds isolated in the last 50 years (approximate number/ 10 years) (Adapted from Faulkner, 1984; 1986; 1987; 1988; 1990; 1991; 1992; 1993; 1994; 1995; 1996; 1997; 1998; 1999; 2000; 2001; 2002; Blunt *et al.*, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013, 2014, 2015, 2016, 2017, 2018).

Nevertheless, despite of the remarkable potential of marine NPs as a source of new drugs, their role have undergone several changes, having had an evident decline in the pharmaceutical R&D activities by the mid-1990s. After that decline, the larger research and development efforts in the exploration of this niche were essentially assured by enterprising

academics, mainly partnered with industry. In the last decade, this area seems to have benefited from a renaissance, since the number of new isolated marine compounds has increased when compared with the previous similar period (Horta *et al*., 2015). This rebirth is directly associated with recent technological advances in analytical technology, spectroscopy, and high-throughput screening. Advances in "omics" techniques (genomics, metagenomics, proteomics), combinatorial biosynthesis, synthetic biology, selection methods, expression systems, and bioinformatics have contributed as powerful tools to discover new chemical entities with pharmaceutical potential (Molinski *et al.*, 2009; Bucar *et al.*, 2013).

1.4.1. Currently clinical pipeline of marine-derived drugs

Over the last 30 years, great efforts have been made, showing productive and promising results, since it has been defined the major tendencies in secondary metabolism of several classes of marine organisms. Only in the last 20 years, more than 18,000 new marine compounds were described and six out of the nine marine-derived drugs currently used in clinical therapy were approved, as well as one over-the-counter drug (OTC) (Figure 1.3). Cytarabine (*Cytosar-U*®), Vidarabine (*Vira-A*®) (US discontinued), Ziconotide (*Prialt*®), Brentuximab Vedotin (*Adcetris*®), Eribulin Mesylate (*Halaven*®), Omega-3-acid ethyl esters (*Lovaza*®), Trabectedin (*Yondelis*®), Fludarabine Phosphate (*Fludara*®), and Nelarabine (*Arranon*®) were approved by the Food and Drug Administration (FDA) in the US Pharmacopeia and/ or by the European Medicines Agency (EMA). Iota-carrageenan (Carragelose®), one OTC, was approved by EMA. However, it is expected that the number of newly approved drugs from marine origin will continue to increase, since 28 marine or marine-derived drugs are currently in clinical trials (six marine molecules – phase III; fourteen marine molecules – phase II; eight marine molecules – phase I) (Figure 1.3) (AndisInsight, 2018; EMA, 2018a; b; FDA, 2018a; b; Mayer, 2018).

Considering all the marine drugs available in the market, it is particularly interesting to see that six are used in cancer therapies (Figure 1.4), and the majority of the compounds that are in clinical trials are also for cancer therapy, which reveals the great potential of marine compounds as anticancer drugs (Figure 1.3).

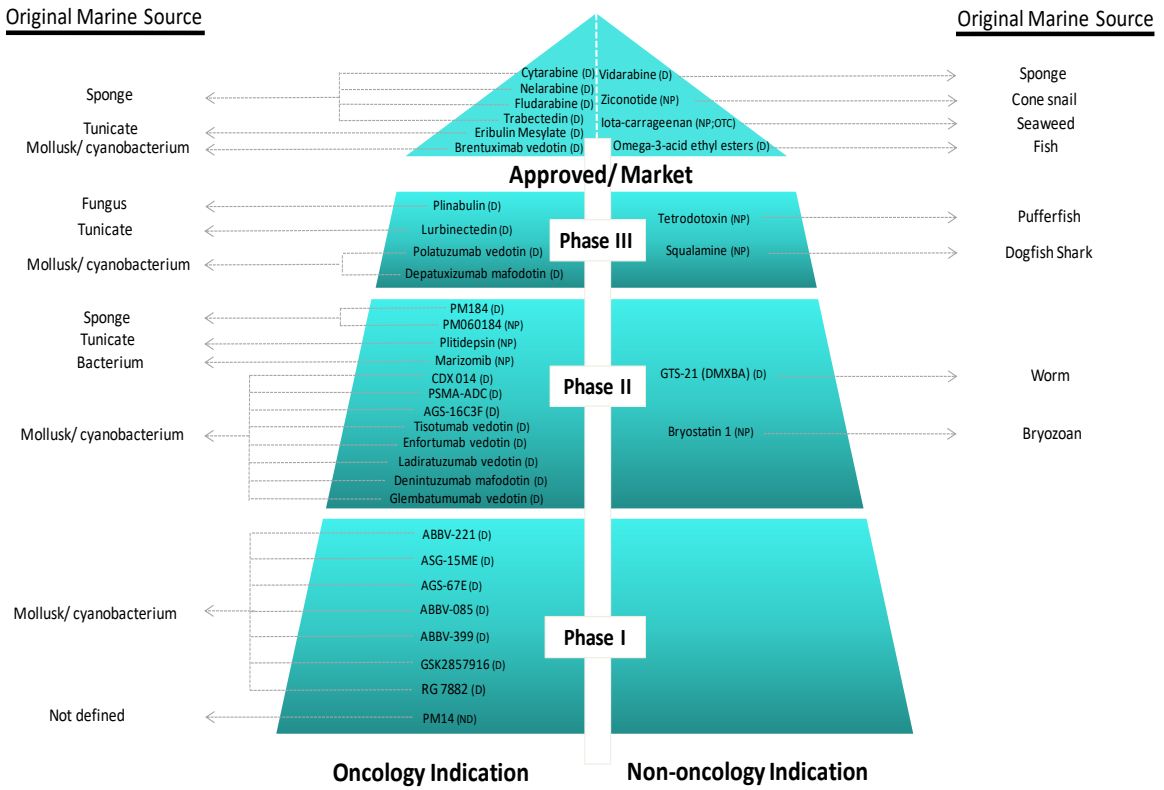

Figure 1.3. Current clinical pipeline of marine-derived drugs and their original marine source. NP, Natural product; D, Derivate; OTC, Over-the-counter (Adapted from AndisInsight, 2018; EMA, 2018a; b; FDA, 2018a; b; Mayer, 2018).

Arabinosyl cytosine (Ara-C; Cytarabine; Cytosar-U®) received FDA approval in 1969 for therapeutics of acute myeloid leukaemia and it is also used against other haematological malignancies, including acute lymphoblastic leukaemia and non-Hodgkin's lymphoma. The development of this drug was based on a nucleoside previously isolated from the Caribbean sponge, *Cryptotheca crypta*. Its mechanism of action is associated with the inhibition of DNA polymerase and DNA synthesis (Mayer *et al.*, 2010; Momparler, 2013).

Trabectedin (ecteneinascidin-143 (ET-143); Yondelis®) was approved by EMA in 2007 for the treatment of soft tissue sarcoma and, in 2009, for ovarian carcinoma. More recently, it was approved by FDA (2016). ET-143 is an alkaloid firstly isolated from a marine tunicate (*Ecteinascidia turbinata*) and currently prepared by chemical synthesis. Its mechanism of action is not fully clear. However, ET-143 showed to interact with several targets demonstrating ability to act on both cancer cells and the tumor microenvironment representing a new class of anti-neoplasic drugs. Moreover, it is the first drug from marine origin approved for therapeutics in advanced soft tissue sarcoma and in patients with

relapsed platinum-sensitive ovarian cancer when administered in combination with pegylated liposomal doxorubicin (Bai *et al.*, 1990; Mayer *et al.*, 2010; Vaklavas and Forero-Torres, 2012; Newman and Cragg, 2014).

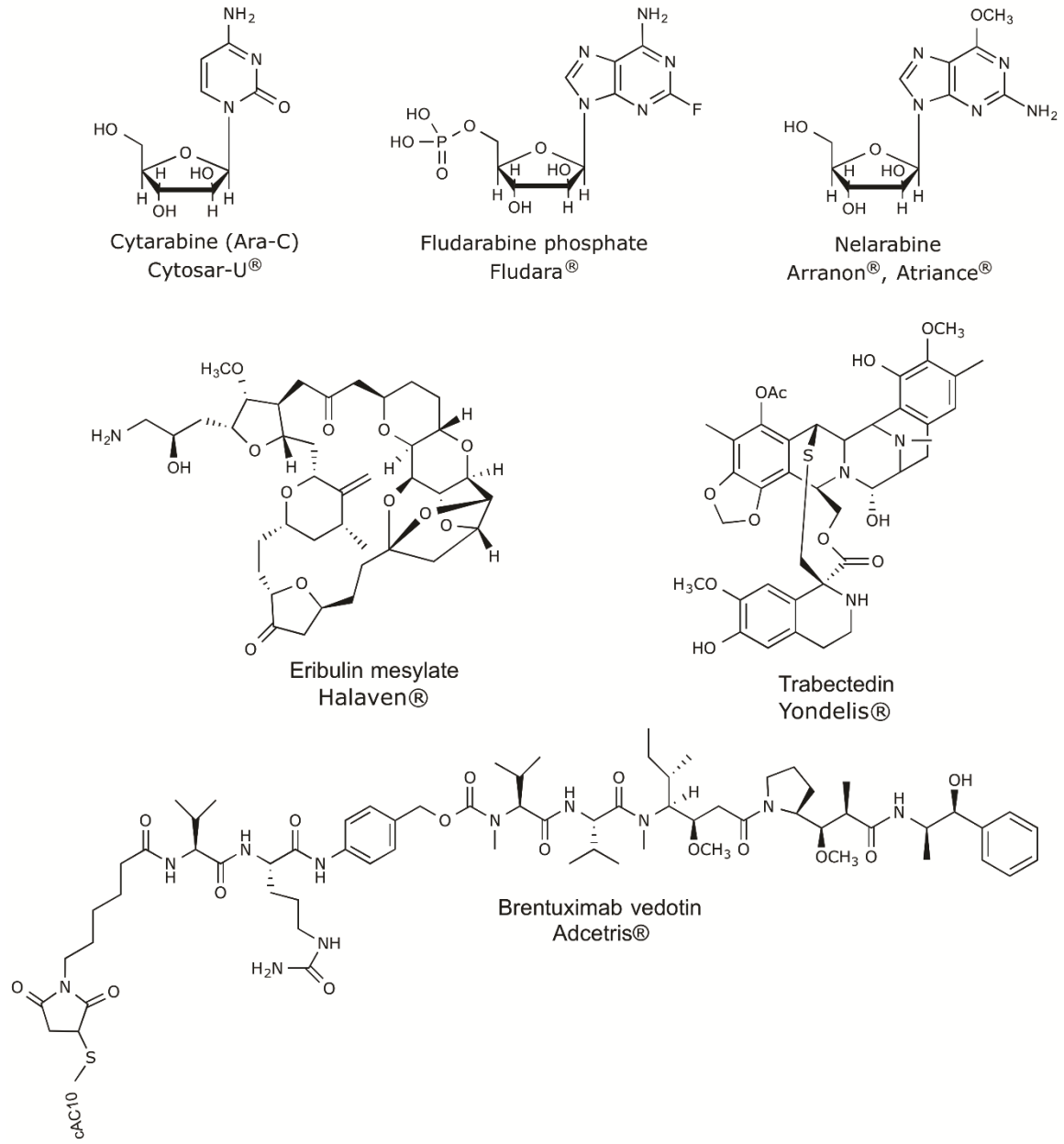

Figure 1.4. Chemical structures of anticancer marine-derived drugs in the market.

Eribulin mesylate (EM; Halaven®) was approved by the FDA in 2010, and by EMA in 2011, as second- and later-line chemotherapy for patients with metastatic breast cancer, who were previously treated with anthracycline and taxane chemotherapy regimens.

Development of EM was based on halichondrin B, a macrolide isolated from a marine sponge (*Halichondria okadai*). Its mechanism of action has tubulin as target, a protein component of the cytoskeleton and important for proper cell division. Compared with other microtubule-inhibiting-drugs, EM binds to tubulin, at a site distinct from the bindind site of the other drugs, blocking the cell cycle at G2/M and consequently, disrupting the mitotic spindles and inducing apoptosis on cancer cells (Mayer *et al.*, 2010; Donoghue *et al.*, 2012; Martins *et al.*, 2014).

Brentuximab vedotin (BV; Adcetris®) was approved by the FDA in 2011, and by the EMA in 2012, for the treatment of Hodgkin's and systemic anaplastic large cell lymphoma. BV is an antibody–drug conjugate (ADC) that results from the combination of the antibody cAC10 and the compound monomethyl auristatin E (MMAE). MMAE is a cytostatic compound, analogue to the marine compound dolastatin 10, previously isolated from the sea hare *Dolabella auricularia*. Its mechanism of action is related with inhibition of microtubule assembly, tubulin-dependent GTP hydrolysis and polymerization, inducing a potent cytostatic effect. The use of ADC results in a highly effective and well-tolerated drug, since it has capacity to recognize and bind to a protein antigen expressed on the surface of specific tumor cells, allowing selectively deliver of antitumor drug (Bai *et al.*, 1990; Mayer *et al.*, 2010; Vaklavas and Forero-Torres, 2012; Newman and Cragg, 2014; Calado *et al.*, 2018). Moreover, there are, presently, in clinical trials seventeen drugs for cancer therapeutics that use monomethyl auristatin E or F associated to specific monoclonal antibodies (Calado *et al.*, 2018).

Fludarabine (F-Ara-A), approved by the FDA in 1991, and by the EMA, has been used in the treatment of hematological malignancies, namely chronic lymphocytic leukemia and indolent B-cell malignancies (Lapponi *et al.*, 2016; Berdis, 2017). F-Ara-A is a fluorinated purine nucleoside analogue to the antiviral agent vidarabine (Ara-A), which was obtained by the addition of a fluorine atom at the C-2 position (Liu *et al.*, 2008). This modification allowed to avoid the problem related with deamination process keeping its antineoplasic activity. Moreover, the limited solubility and difficulties in its formulation led to the synthesis of fludarabine 5-monophosphate (FM-Ara-A; Fludara®) which is administered to patients as a soluble pro-drug (Robak and Robak, 2013). FM-Ara-A is transported into cells and then converted by intracellular kinases in its triphosphate ester, the active metabolite that induces the cytotoxic activity. Its mechanism of action seems to inhibit the activity of key enzymes linked to DNA synthesis, including DNA polymerase, ribonucleotide

reductase, DNA ligase and DNA primase promoting the inhibition of tumor cell growth (Gandhi *et al.*, 1994; Gomes *et al.*, 2018).

Nelarabine (Arranon®) approved by the FDA in 2005, and by the EMA in 2007, is used in the treatment of acute T-cell lymphoblastic leukemia and T-cell lymphoblastic lymphoma in patients with refractory or relapsed disease who failed two preceding therapeutic regimens (Cohen *et al.*, 2008). Its development was inspired on the structure of the guanosine nucleoside (Ara-G), previously synthesized in 1964, on which the hydrogen at the 6-position of the ring was substituted by a methoxy group (Lapponi *et al.*, 2016). This modification allowed to overcome the poor solubility and difficulty of Ara-G synthesis rendering it possible to be used in patient´s treatment (DeAngelo *et al.*, 2007). Nelarabine is administrated to pacients as pro-drug and transported into cells, where it is transformed by adenosine deaminase to Ara-G, the nucleoside with cytotoxic properties. After that, Ara-G is phosphorylated and converted to the active nucleotide, ara-GTP which is incorporated into DNA, promoting the inhibition of its synthesis that results in DNA chain termination and leukemic cell apoptosis (Reilly and Kisor, 2009; Calado *et al.*, 2018).

Although not used in cancer treatment, iota-carrageenan (*Carragelose*®) was the first product developed from algae in the market. It is a type of carrageenan, isolated from a red edible alga belonging to a family of linear sulfated polysaccharides. *Carragelose*® has the ability to block the attachment of the virus to the host cells being effective against a broad spectrum of respiratory viruses (Ludwig *et al.*, 2013; Calado *et al.*, 2018).

1.5. Antitumor potential of marine algae-derived compounds

Among marine organisms, algae are one of the most important resources of the ocean, both economically and ecologically (Kim, 2014). Their inclusion in the Asian diet has been associated with health benefits, where there has been observed a lower incidence of chronic diseases, such as hyperlipidaemia, coronary heart disease, diabetes and cancer, according to epidemiological studies comparing Japanese and Western diets (Brown *et al.*, 2014; Bouga and Combet, 2015). Algae are valuable sources of protein, fibre, vitamins, polyunsaturated fatty acids, and macro- and trace elements. More recently, they have also revealed to be an interesting source of useful bioactive components such as antioxidants, phycocolloids, proteins, vitamins, minerals, carotenoids, soluble dietary fibres,

polyunsaturated fatty acids, phycobilins, polysaccharides, sterols, tocopherols, terpenes, and phycocyanins. These compounds demonstrated to possess nutritional and functional value apart from their potential use as therapeutic agents in biomedical area (Chandini *et al.*, 2008; Lordan *et al.*, 2011; Mohamed *et al.*, 2012; Alves *et al.*, 2016b). Due to their unique structures and biochemical characteristics, the multifunctional properties of algae should be exploited in their fullness. In addition, the idea that algae are a promising prolific source of structurally unique NPs with biomedical potential is even more supported by the discovery that the number of algae species identified around the world is more than 30,000 (Plouguerné *et al.*, 2014). Moreover, algae have been revealed to be one of the major source of new compounds of marine origin, after sponges, microorganisms and phytoplankton (Figure 1.5).

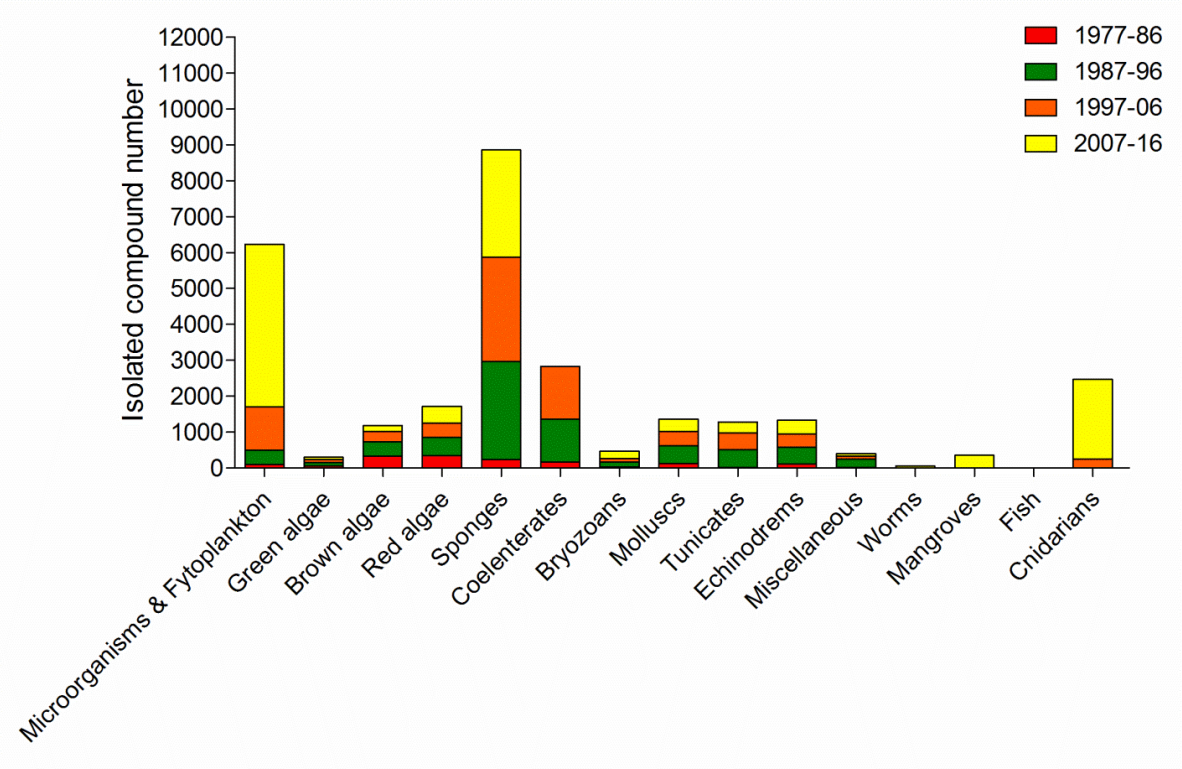

Figure 1.5. Approximate numbers of new compounds isolated from different marine organism sources between 1977 and 2016 (Adapted from Faulkner, 1984; 1986; 1987; 1988; 1990; 1991; 1992; 1993; 1994; 1995; 1996; 1997; 1998; 1999; 2000; 2001; 2002; Blunt *et al.*, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013, 2014, 2015, 2016, 2017, 2018).

Many of these algae-derived compounds are reported for their therapeutic properties associated with numerous health-promoting effects, including anti-obesity (Maeda *et al.*, 2005; Kim *et al.*, 2018), antidiabetic (Mayer *et al.*, 2013), antihypertensive (Sivagnanam *et al.*, 2015), antihyperlipidemic (Sathivel *et al.*, 2008), antioxidant (Magalhaes *et al.*, 2011; Pinteus *et al.*, 2017), anticoagulant (Magalhaes *et al.*, 2011), anti-inflammatory (De Souza *et al.*, 2009), immunomodulatory (Pérez-Recalde *et al.*, 2014), anti-estrogenic (Skibola, 2004), thyroid-stimulating (Teas *et al.*, 2007), neuroprotective (Pangestuti and Kim, 2011) , anti-osteoarthritic (Moon *et al.*, 2018), anti-osteoporosis (Deng *et al.*, 2018), antiviral (Aguilar-Briseño *et al.*, 2015), antimicrobial (Pinteus *et al.*, 2015; Rodrigues *et al.*, 2015) and antineoplastic (Moussavou *et al.*, 2014; Rodrigues *et al.*, 2015; Alves *et al.*, 2016a). Among the well-documented bioactive compounds are brominated phenols, polysaccharides, and carotenoids, but especially a large diversity of terpenoids, several of them being halogenated compounds (Gribble, 2015; Rodrigues *et al.*, 2015). Although many works have attempted to identify marine-derived compounds, detailed chemical characterization and identification of bioactive components are still largely lacking (Santos *et al.*, 2015).

Along the last five decades, it is estimated that more than 3,000 NPs have been discovered from algae (Leal *et al.*, 2013) and, among all of the biological activities observed, the antitumor activity is one of the most promising. Despite several studies have shown the high cytotoxic potential of the compounds isolated from algae on different tumor cell lines, there are few studies that have characterized the intracellular signaling pathways involved in the process (Annex I – Table 1). Currently, according to the National Cancer Institute (USA), different targeted therapies have been approved for use in cancer treatment, including hormonal therapies, inhibitors of signal transduction and angiogenesis, modulators of gene expression, inducers of apoptosis, immunotherapies and toxin delivery molecules.

1.5.1. *In vitro* antitumor activities of algae-derived compounds and intracellular signaling pathways activated

Analysing the compounds isolated from algae with antitumor activity (Annex I – Table 1), it is possible to observe that several of them exhibit ability to act in the same targets used in driven therapies already approved by the National Cancer Institute. For example, dioxinodehydroeckol (Kong *et al.*, 2009), sargachromanol E (Heo *et al.*, 2011), EI-

SP (Wang *et al.*, 2014), siphonaxanthin (Ganesan *et al.*, 2011), sulfated carrageenan (Murad *et al.*, 2015), TDB (Lee *et al.*, 2007), GLP (Thangam *et al.*, 2014), mertensene (Tarhouni-Jabberi *et al.*, 2017), TTB (Choi *et al.*, 2017), DDSD (Velatooru *et al.*, 2016) and clerosterol (Kim *et al.*, 2013) induced apoptosis in different cell lines by similar intracellular signaling pathways, regulated by Caspase (-3, -9 or both) activation, downregulation of Bcl-xL or Bcl-2, upregulation of Bax and cleavage of PARP. Moreover, some of these compounds, such as EI-SP and clerosterol, also caused the loss of the mitochondrial membrane potential. The treatment of colon26 cells with *Eucheuma serra* agglutinin (Fukuda *et al.*, 2006) also promoted an increase of Caspase-3 expression and translocation of phosphatidylserine in lectin-treated cells, suggesting that cell death was mediated by apoptosis. On the other hand, algae-derived compounds such as lophocladines B (Gross *et al.*, 2006), hexadecyl-1-*O*-α-L-arabinopyranoside (Du *et al.*, 2010) and caulerpenyne (Fischel *et al.*, 1994) affected the intracellular signaling pathways linked with regulation of the cell cycle. Lophocladines B showed a marked reduction of MDA-MB-435 cells at the G1 and S phases, with an accumulation of cells at G2/M, indicating a G2/M cell cycle arrest. This compound also induced microtubule depolymerization on A-10 cells. Du and co-workers (2010) isolated, from the alga *Laurencia majuscula*, a new arabinopyranoside compound designed as hexadecyl-1-*O*-α-L-arabinopyranoside, which exhibited significant antitumor activity in different cancer cell lines. The active compound arrested cell lines at G2/M phase of the cell cycle by decreasing the expression of CDK1 and Cyclin A proteins, which are critical for the G2/M-phase transition. Caulerpenyne induced cell cycle arrest in colorectal cancer cells that exhibited an early shift into the S phase followed by a blockade at the G2/M phase (Fischel *et al.*, 1994).

Nevertheless, most of the intracellular signaling pathways activated by these compounds are simultaneously linked with the regulation of cell cycle and apoptosis. Park and co-workers (2013) demonstrated that laminarin, extracted from the brown alga *Laminaria digita*, induced apoptosis on HT-29 colon cancer cells and increased the percentage of cells in the sub-G1 and G2/M phases. The observed decrease in cellular proliferation was found to be dependent on ErbB, followed by subsequent activation of c-Jun N-terminal kinase. In the same way, Liu and collaborators (2012) verified that a bromophenol compound, bis(2,3-dibromo-4,5- dihydroxybenzyl) ether, induced apoptosis on K562 cells by a mitochondria-mediated pathway, as well as the arrest of the cell cycle at the S phase. Additionally, this compound interacted with the minor groove of DNA and inhibited Topoisomerase I activity. On A2780 human ovarian cells, bromophycolide A induced the arrest at the G1 phase of the cell cycle and a consequent and consistent loss

of cells from the S and G2/M phases, while simultaneously induced apoptosis (Kubanek *et al.*, 2005). Similar effects were induced by elatol, a compound isolated from the alga *Laurencia microcladia*, which induced cell cycle arrest in the G1 and sub-G1 phases, leading the cells to undergo apoptosis. It influenced the expression of several proteins (cyclins, Bax, Bcl-xl, caspases, p53) that play important roles in these biological processes (Campos *et al.*, 2012). Diphlorethohydroxycarmalol, isolated by Kang and co-workers (2012), induced apoptosis by the accumulation of the sub-G1 cell population and nuclear condensation, depletion of mitochondrial membrane potential (ΔΨm) and regulation of the expression of the pro-survival and pro-apoptotic Bcl-2 family members. HFGP (Ryu *et al.*, 2012) and LJGP (Go *et al.*, 2010) glycoproteins induced apoptosis on HepG2 and HT-29 cells, which was mediated by Fas signaling and mitochondrial pathway, and cell cycle arrest. On the other hand, Cf-PS polysaccharide (Kwon and Nam, 2007) inhibited the cell proliferation and induced apoptosis by inhibiting IGF-IR signaling and the PI3K/Akt pathway, which are involved in the regulation of cell growth, proliferation, differentiation, motility, survival, metabolism and protein synthesis (Chen *et al.*, 2014).

Other examples such as dieckol (Oh *et al.*, 2011; Park and Jeon, 2012), 6,6'-bieckol (Zhang *et al.*, 2010), ascophyllan (Abu *et al.*, 2015), 9'-cis-(6'*R*) fucoxanthin (FcA) (Nguyen *et al.*, 2014), fucoxanthinol (Rokkaku *et al.*, 2013), and 13'-cis-(6'*R*) fucoxanthin complex (FcB) (Nguyen *et al.*, 2014), SargA (Dias *et al.*, 2005), MSP (Tang *et al.*, 2006) and Cf-GP (Boo *et al.*, 2013) showed capacity to inhibit the motility, migration, adhesion and invasion on different *in vitro* and *in vivo* models using distinct intracellular signaling pathways, as described in Annex I - Table 1.

Currently, one of the targets of cancer treatment, especially in solid tumors, is angiogenesis, which is responsible for the formation of blood vessels from pre existing ones (sprouting) and it is a requirement for the sustained growth and proliferation of solid tumors. Accordingly, the search for inhibitors of this process has become a leading line of investigation in anticancer research, with the consequent release of several drugs on the market that have clearly improved outcomes in patients with different tumor types and metastatic disease (Marín-Ramos *et al.*, 2015). The compounds PSV1 (Guerra Dore *et al.*, 2013), SargA (Dias *et al.*, 2005), BDDE (Qi *et al.*, 2015), GFP08 (Yu *et al.*, 2012), GLP (Zhang *et al.*, 2006), fucodiphloroethol G (Li *et al.*, 2011), C10 (Furuno *et al.*, 2011) and BDDPM (Wang *et al.*, 2015b) also demonstrated interesting anti-angiogenic activities. For example, the sulfated polysaccharide, PSV1, inhibited tubulogenesis in RAEC cells in Matrigel and VEGF secretion (Guerra Dore *et al.*, 2013); SargA induced a marked dose-

dependent inhibition of capillary networks development (Dias *et al.*, 2005). Like fucodiphloroethol G, this compound inhibits VEGF induced angiogenesis on ECV-304 and EA.hy926 cells. Simultaneously, SargA by inhibiting MAPK and Akt signaling pathways activates the transcriptional factor c-Fos and its downstream targets AP-N, MMP-2 (Li *et al.*, 2011). However, one of the most interesting and promising compounds is BDDPM, reported to inhibit various biological processes associated with angiogenesis, including endothelial cell sprouting, migration, proliferation, and tube formation (Wang *et al.*, 2015b). Kinase assays revealed that BDDPM is a potent selective but multi-target receptor tyrosine kinase (RTKs) inhibitor (VEGFR, PDGFR, FGFR, and EGFR). Other compounds have not only evidenced *in vitro* but also *in vivo* activities, as described in the section Preclinical and Clinical Evidence of Antitumor of Algae-Derived Compounds (1.5.2).

Compounds such as laurenditerpenol (Mohammed *et al.*, 2004), caulerpin (Liu *et al.*, 2009), thyrsiferol (Mahdi *et al.*, 2011), phlorofucofuroeckol-A (Lee *et al.*, 2012), SQDG (Eitsuka *et al.*, 2004) and DAEB (Jiao *et al.*, 2009) showed antitumor activity by activating other intracellular signaling pathways. For example, laurenditerpenol, thyrsiferol and caulerpin showed the capacity to inhibit the transcription factor HIF-1 by blocking the induction of the oxygen-regulated HIF-1α protein, which promotes tumor cell adaptation and survival under hypoxic conditions (Ke and Costa, 2006). Lee and co-workers (2012) observed that phlorofucofuroeckol-A, a compound isolated from the edible brown alga *Eisenia bicyclis*, is a potent inhibitor of the aldo-keto reductase family 1 B10 (AKR1B10), a member of the NADPH-dependent aldo-keto reductase (AKR) superfamily, considered to be a potential cancer therapeutic target. In same way, the compound SQDG showed a marked inhibition of the telomerase activity, an enzyme with a key role in the replicative immortality characteristic of cancer cells (Eitsuka *et al.*, 2004).

On the other hand, some of the compounds isolated from algae can also be used as co-adjuvants to improve the efficiency of the drugs currently used as therapeutics. For instance, the pre-treatment of HepG2 cells with fucoxanthin allowed to improve the therapeutic effect of cisplatin (Yang *et al.*, 2013). According to Liu and co-workers (2013), these effects were associated with NFκB downregulation and an increase in the Bax/Bcl-2 mRNA ratios regulated by NFκB. Moreover, inhibition of the DNA repair systems regulated by ERK, p38 and PI3K/AKT seems also to be associated with these effects. Additionally, the conjugation of λ-carrageenan (Zhou *et al.*, 2006), Cf-PLS (Lins *et al.*, 2009) and porphyran (Wang and Zhang, 2014) compounds with 5-fluorouracil (5-FU) drug enhanced its antitumor activity. According to previous studies, the conjugation of these compounds

with 5-FU increased the antitumor activities of the drug and mitigated the immunocompetence damage induced by 5-FU (Zhou *et al.*, 2006; Lins *et al.*, 2009; Wang and Zhang, 2014).

Many of the studies discussed above have identified compounds, such as polysaccharides, polyphenols, carotenoids, alkaloids, terpenes and others, that mediate specific inhibitory activity on a number of key cellular processes, including apoptosis pathways, angiogenesis, migration and invasion processes, in different *in vitro* models revealing their potential use as anticancer drugs (Figure 1.6).

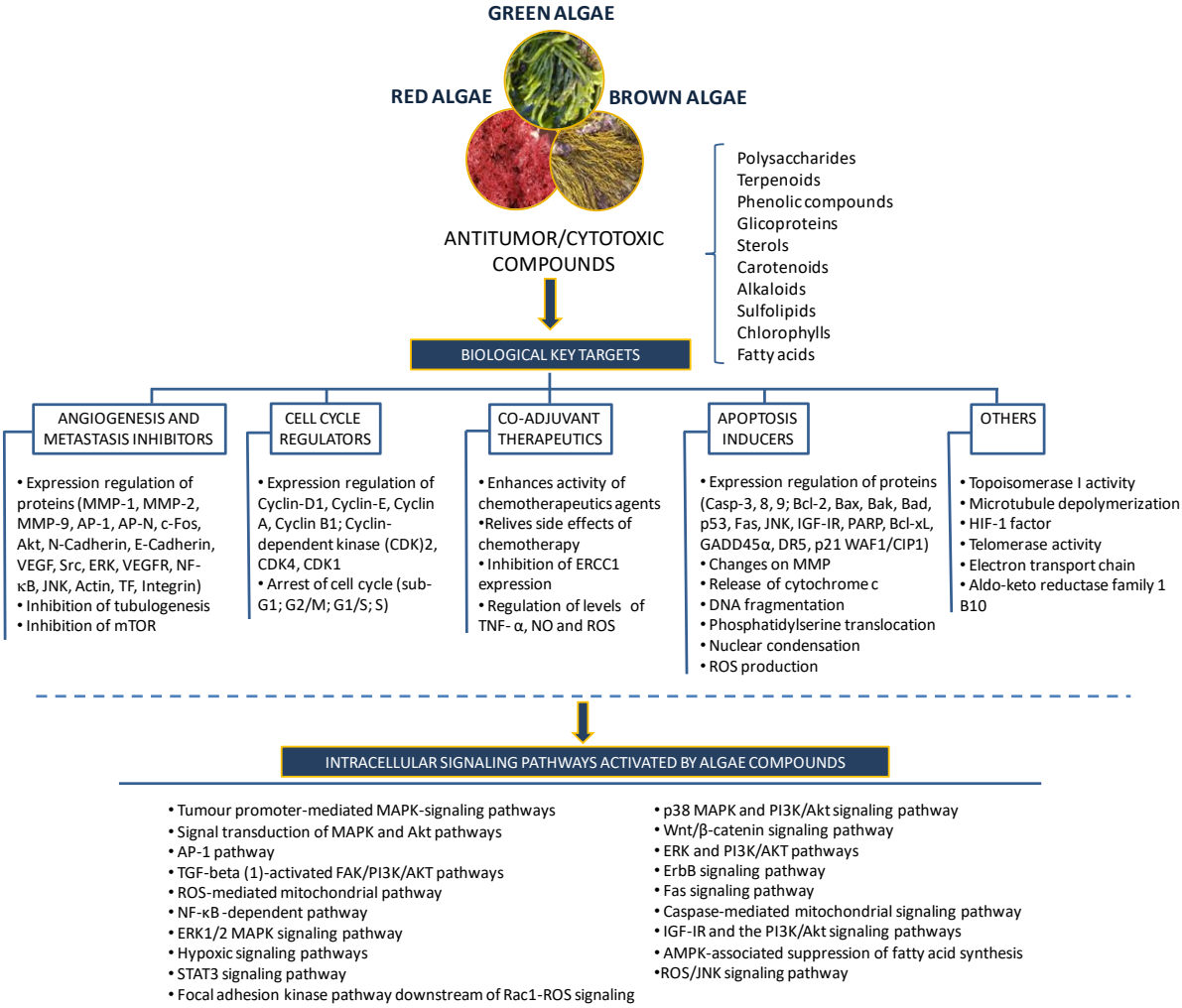

Figure 1.6. Overview of the antitumor/ cytotoxic compounds isolated from algae, biological targets and intracellular signaling pathways activated (Adapted from the references present in Table 1 – Annex I).

1.5.2. Preclinical and clinical evidence of antitumor activities of algae-derived compounds

Despite the antitumor activities of algae-derived compounds have mainly been studied on *in vitro* human tumor cell models, there are several studies in preclinical and clinical trials demonstrating the potential of these compounds as antitumor and/or co-adjuvant drugs with capacity to act in distinct intracellular signaling pathways.

Among the chemical structures derived from algae, the highest number of *in vivo* studies were conducted with sulfated polysaccharides, revealing their potential to be used in antitumor therapies. For instance, the sulfated polysaccharides H3-a1 (Wang *et al.*, 2010) and DAEB (Jiao *et al.*, 2009) revealed antitumor activity by immune system enhancement, through the increase of the tumor necrosis factor-alpha (TNF-α) and reactive oxygen species (ROS) levels and by the activation of peritoneal macrophages leading to the secretion of TNF-α and NO. In addition, when administrated in tumor-bearing BALB/c mice at doses of 20 and 50 mg/Kg, the sulfated polysaccharide H3-a1 exhibited capacity to suppress the ascitic sarcoma 180 tumor growth, increasing the lifespan of the tumor-bearing mice in ~30 - 40%. On the other hand, the treatment with the sulfated polysaccharide DAEB reduced the thymus and spleen tumor growth in mice promoting the increase of immune organs weight.

Due to increasing evidences of the antitumor potential of algae derived sulfated polysaccharides, several studies have been conducted to deepen their antitumor potential, including studies addressing the possibility to be used as co-adjuvant drugs, to increase treatments efficiency, and also, as an attempt to reduce undesirable side-effects. As an example, the sulfated polyssacharide Cf-PLS extracted from the red alga *C. feldmannii* exhibited capacity to reduce the tumor growth on mice transplanted with sarcoma 180 tumor cells, and to increase the leukocytes number in the peritoneal cavity and neutrophil migration. In addition, its co-administration with the chemotherapeutic agent 5-FU on tumor-bearing animals allowed to significantly increase the tumor growth inhibition, comparing with single administration, also preventing the immunocompetence hindered by 5-FU (Lins *et al.*, 2009).

As described above, one of the targets for cancer treatment is angiogenesis. In these studies, several compounds have exhibited promising activities on *in vivo* models. The treatment of mice bearing sarcoma-180 cells with GLP polysaccharide promoted the

reduction of tumor weight around 52% and its vascular density (Zhang *et al.*, 2006). In the same model, the GFP08 compound decreased the tumor weight by 68.9% and inhibited the formation of new vessels in the chicken chorioallantoic membrane assay (*ex vivo*) (Yu *et al.*, 2012). On the other side, the bromophenol BDDE also exhibited anti-angiogenesis properties, by inhibiting sub-intestinal vessel formation in zebrafish embryos *in vivo* (Qi *et al.*, 2015). Similarly to the bromophenol BDDE, the compound C10 inhibited markedly the neovascularization induced by VEGF in the mouse cornea (Furuno *et al.*, 2011). On the other hand, the compound SargA inhibited the vascularization of Gelfoam skin implants and exhibited antitumor properties on melanoma cells with doses of 1.5 and 150 µg/animal without inducing deaths or body weight loss (Dias *et al.*, 2005).

The MSP sulfated polysaccharide mediated a potent inhibitory effect on the metastasis of Lewis lung carcinoma reducing significantly the number of pulmonary metastatic colonies. The reduction of colony-formation rate was between 70% and 93.6%. Moreover, when compared with cisplatin treatment, this compound promoted a significant amelioration of alveolar structures without inducing weight loss (Tang *et al.*, 2006). DDSD (4, 10, and 15 mg/Kg) and elatol [oral (3, 10, 30 mg/Kg) or intraperitoneal (1, 3, 10 mg/Kg)] demonstrated significant reduction of the tumor growth without evident toxic effects (Campos *et al.*, 2012; Velatooru *et al.*, 2016). The lectin ESA extracted from the red alga *Eucheuma serra*, injected in the tail vein of BALB/c mice with colon26 cells, promoted a significant delay of the tumors growth inducing the cells death by apoptosis as observed *in vitro*. Additionally, the treatment with ESA compound did not promoted a weight loss or animal death (Fukuda *et al.*, 2006). These studies become more relevant since the antitumor activities mediated by many of these compounds are not linked with toxic effects. Another compound widely studied on *in vivo* models, including clinical trials, is the sulfated polyssacharide fucoidan. This compound exhibits multi-target capacity, acting in different signaling pathways, including the activation of the intrinsic and extrinsic pathways of apoptosis, suppression of angiogenesis, increase immune response, and mobilization of haematopoietic progenitor cells (Moghadamtousi *et al.*, 2014). The co-administration of low-molecular –weight fucoidan with standard drugs in patients with metastatic colorectal cancer improved the disease control rate, suggesting its potential application as additional therapy (Tsai *et al.*, 2017). In addition, when administrated in conjugation with the standard hormonal drugs letrozole and tamoxifen, in patients with breast cancer, it revealed to be well tolerated and without influence in the steady-state plasma concentrations of these drugs (Tocaciu *et al.*, 2018). Despite the great potential demonstrated by fucoidans on clinical trials, to be used as suplementary therapy, its use as anticancer drugs is being

studied, since fucoidan preparations obtained from different sources have induced different anticancer activities *in vivo*. These differential responses seem to be associated with their different structural properties (Kwak, 2014). The marine-derived cyclic depsipeptide kahalalide F was the first compound found in algae that achieved the phase II of clinical trials (Murphy *et al.*, 2014). Kahalalide F is a potent cytotoxic compound produced by the green alga *Bryopsis pennata* and found in the mollusk *Elysia rufescens* (Miguel-Lillo *et al.*, 2015; Sable *et al.*, 2017). It advanced through five clinical trials and completed the safety evaluation in phase I in patients with distinct advanced solid tumors. Nevertheless, kahalalide F dropped in phase II due to lack of efficacy, short half-life, limited spectrum of activity and a poor response in patients. However, due to the high potential of this compound as cytotoxic, it inspired the development of several synthetic analogues to overcome its limitations increasing its potency and half-life time (Wang *et al.*, 2015a).

In the last years, nanotechnology has emerged as a promising solution to be used in drug delivery systems to suppress some of these limitations, being considered as one of the next-generation platform for cancer therapy (Sun *et al.*, 2014; Xin *et al.*, 2017). Therefore, the production of nano-formulations of drugs derived from algae-derived compounds can be an interesting approach to potentiate anticancer properties. In addition, the use of nano-formulations can also be useful to overcome some limitations that arise from specific characteristics of each compound, such as nonspecific biodistribution, low water solubility, lack of targeting capability, systemic toxicity, weak therapeutic effect, and limited bioavailability (Sun *et al.*, 2014; Blanco *et al.*, 2015). For instance, one of the most promising compounds isolated from algae with interesting antitumor proprieties is fucoxanthin. However, the poor solubility, chemical instability, and low bioavailability of this carotenoid limit its use in cancer therapeutics. To overcome these drawbacks several approaches have been assessed such as the inclusion of fucoxanthin in nano-emulsions (Huang *et al.*, 2017), nano-suspensions (Muthuirulappan and Francis, 2013) and nano-gels (Ravi and Baskaran, 2015). However, as described by Bajpai and co-workers (2018), for the microalgae, the published data on nano-formulations using compounds derived from macroalgae is also scarce suggesting a new area to be explored that can potentiate the capacity of these compounds in cancer therapeutics.

According to the interesting activities mediated by algae-derived compounds, it is expected that, in the next few years, some of them may reach the clinical trials stages or inspire the development of new compounds allowing their translation into clinically useful drugs in the future. Moreover, the use of some of those compounds as co-adjuvants in pre-

existent therapeutics regimens appears to be a valid approach to improve the therapeutic effects of the antitumor drugs and decrease their side-effects.

1.6. Therapeutic potential of *Sphaerococcus coronopifolius* compounds

S. coronopifolius is a red alga belonging to the Rhodophyta phylum, which is narrow, compressed, two-edged, cartilaginous, scarlet fronds and main axes that are dark brownish-red. The habitat of this species is rarely on rocks on the lower littoral, but it is common in the shallow sublittoral to a 15 meters depth. It is distributed in the East Atlantic (Ireland and Britain to Canary Islands), Mediterranean and Black Seas (Guiry and Guiry, 2015). Since its first chemical analysis in 1976, *S. coronopifolius* has demonstrated to be an interesting source of brominated cyclic diterpenes, most of them containing one or two bromine atoms (Etahiri *et al.*, 2001; Smyrniotopoulos *et al.*, 2010a; Piazza *et al.*, 2011). Although more than 40 compounds have been isolated and described from *S. coronopifolius*, in the last four decades, few studies characterized their biological activities. According to previous studies, some of the isolated compounds evidenced great biological activities, including antifouling (Piazza *et al.*, 2011), antimalarial (Etahiri *et al.*, 2001), and antimicrobial (Etahiri *et al.*, 2001). Another interesting bioactivity exhibited by these compounds is their antitumor potential. Several compounds revealed interesting cytotoxic activities in different *in vitro* models (Table 1.1). For example, 14*R*-hydroxy-13,14-dihydro-sphaerococcenol-A decreased significantly the viability of NSCLC-N6-L16 and A549 human lung cancer cell lines with an IC_{50} of 5 and 4 µg/mL, respectively (Smyrniotopoulos *et al*., 2008). Moreover, spirosphaerol and corfusphaeroxide showed moderate cytotoxic activity against the malignant cell lines A549, Hs683 and MCF-7 (Smyrniotopoulos *et al*., 2015). Smyrniotopoulos and co-workers (2010a) isolated several metabolites and evaluated their cytotoxic activity towards four human apoptosis-resistant (U373, A549, SK-MEL-28, OE21) and two human apoptosis-sensitive (PC-3, LoVo) cancer cell lines with IC_{50} values ranging from 3 to 100 µM. Although some compounds isolated from *S. coronopifolius* have exhibited interesting biological activities, its potential remains understudied since the intracellular mechanisms associated with the observed effects are yet uncovered. Therefore, the still underexplored *S. coronopifolius* is a clearly example of the potential of algae as a source of cytotoxic compounds, suggesting that algae potential as valuable source of new compounds for the development of new antitumor drugs is probably much higher than previously thought.

Table 1.1. Cytotoxic compounds isolated from *Sphaerococcus coronopifolius*.

Compound name*	First report	Local of collection	Cytotoxic activities (IC_{50} in µM or µg/mL)	References
Sphaerococcenol A	1976	La Escala, Spain	U373 (IC_{50}:3.2 µM)**; A549 (IC_{50}:3.7 µM); SK-MEL-28 (IC_{50}:5.2 µM); OE21 (IC_{50}:3 µM); PC-3 (IC_{50}:3.7 µM); LoVo (IC_{50}:2.8 µM)	(Fenical *et al.*, 1976; Smyrniotopoulos *et al.*, 2010a)
Bromosphaerol	1976	Italy	U373 (IC_{50}:30 µM); A549 (IC_{50}:35 µM); SK-MEL-28 (IC_{50}:34 µM); OE21 (IC_{50}:28 µM); PC-3 (IC_{50}:30 µM); LoVo (IC_{50}:23 µM)	(Fattorusso *et al.*, 1976; Smyrniotopoulos *et al.*, 2010a)
Bromosphaerodiol	1977	Portopalo, Sicily, Italy	U373 (IC_{50}:22 µM); A549 (IC_{50}:24 µM); SK-MEL-28 (IC_{50}:31 µM); OE21 (IC_{50}:15 µM); PC-3 (IC_{50}:26 µM); LoVo (IC_{50}:20 µM)	(Smyrniotopoulos *et al.*, 2010a)
12*S*-hydroxy-bromosphaerol	1982	Bay of Salerno, Italy	U373 (IC_{50}:16 µM)***; A549 (IC_{50}:19 µM), SK-MEL-28 (IC_{50}:22 µM); OE21 (IC_{50}:19 µM); PC-3 (IC_{50}:12 µM); LoVo (IC_{50}:9 µM)	(Cafieri *et al.*, 1982; Smyrniotopoulos *et al.*, 2010a)
1*S*-hydroxy-1,2-dihydro-bromosphaerol	1982	Bay of Salerno, Italy	U373 (IC_{50}:25 µM); A549 (IC_{50}:28.6 µM); OE21 (IC_{50}:20 µM); SK-MEL-28 (IC_{50}:26 µM); PC-3 (IC_{50}: 25 µM); LoVo (IC_{50}: 23 µM)	(Cafieri *et al.*, 1982; Smyrniotopoulos *et al.*, 2010a)
Bromotetrasphaerol	1986	Bay of Napoles, Massalubrense, Italy	U373 (IC_{50}:34 µM); A549 (IC_{50}:38 µM); OE21 (IC_{50}:33 µM); SK-MEL-28 (IC_{50}:43 µM); PC-3 (IC_{50}:43 µM); LoVo (IC_{50}:56 µM)	(Cafieri *et al.*, 1986; Smyrniotopoulos *et al.*, 2010a)
12*R*-hydroxy-bromosphaerol	1987	Bay of Napoles, Massalubrense, Italy	U373 (IC_{50}:25 µM); A549 (IC_{50}:28 µM); OE21 (IC_{50}:25 µM); SK-MEL-28 (IC_{50}:29); PC-3 (IC_{50}: 26 µM); LoVo (IC_{50}: 26 µM)	(Cafieri *et al.*, 1987; Smyrniotopoulos *et al.*, 2010a)
Alloaromadendrene	1988	Plomin, Croatia	U373 (IC_{50}:71 µM); A549 (IC_{50}:79 µM); OE21 (IC_{50}:83 µM); PC-3 (IC_{50}: 35 µM); LoVo (IC_{50}: 63 µM)	(Rosa *et al.*, 1988; Smyrniotopoulos *et al.*, 2010a)
1*S*-hydroperoxy- 12*R*-hydroxy-bromosphaerol-B	2008	Palaiokastritsa bay, Corfu Island, Greece	NSCLC-N6-L16 (IC_{50}: 9.5 µg/mL); A549 (IC_{50}:12 µg/mL); U373 (IC_{50}:32 µM); A549 (IC_{50}:40 µM); OE21 (IC_{50}:25 µM); SK-MEL-28 (IC_{50}:31 µM); PC-3 (IC_{50}: 30 µM); LoVo (IC_{50}: 22 µM)	(Smyrniotopoulos *et al.*, 2008; Smyrniotopoulos *et al.*, 2010a)

1*S*-hydroperoxy-12*S*-hydroxy-bromosphaerol-B	2008	Palaiokastritsa bay, Corfu Island, Greece	NSCLC-N6-L16 (IC_{50}: 6 µg/mL); A549 (IC_{50}:5 µg/mL); U373 (IC_{50}:22 µM); A549 (IC_{50}:26 µM); OE21 (IC_{50}:27 µM); SK-MEL-28 (IC_{50}:28 µM); PC-3 (IC_{50}: 28 µM); LoVo (IC_{50}: 28 µM)	(Smyrniotopoulos *et al.*, 2008; Smyrniotopoulos *et al.*, 2010a)
14*R*-hydroxy-13,14-dihydro-sphaerococcenol-A	2008	Palaiokastritsa bay, Corfu Island, Greece	NSCLC-N6-L16 (IC_{50}: 5 µg/mL); A549 (IC_{50}: 4 µg/mL); U373 (IC_{50}:7.2 µM)**** ; A549 (IC_{50}:18 µM); OE21 (IC_{50}:8.4 µM); SK-MEL-28 (IC_{50}:21 µM); PC-3 (IC_{50}: 8.1 µM); LoVo (IC_{50}: 5.3 µM)	(Smyrniotopoulos *et al.*, 2008; Smyrniotopoulos *et al.*, 2010a)
4*R*-hydroxy-1-deoxy-bromotetrasphaerol	2010	Palaiokastritsa bay, Corfu Island, Greece	U373 (IC_{50}:75 µM); A549 (IC_{50}:63 µM); OE21 (IC_{50}:64 µM); PC-3 (IC_{50}: 43 µM); LoVo (IC_{50}: 56 µM)	(Smyrniotopoulos *et al.*, 2010a)
Coronone	2010	Palaiokastritsa bay, Corfu Island, Greece	U373 (IC_{50}:31 µM); A549 (IC_{50}:42 µM); SK-MEL-28 (IC_{50}:38 µM); OE21 (IC_{50}:30 µM); PC-3 (IC_{50}:30 µM); LoVo (IC_{50}:28 µM)	(Smyrniotopoulos *et al.*, 2010a)
Sphaerollane-I	2009	Palaiokastritsa bay, Corfu Island, Greece	U373 (IC_{50}:20 µM); A549 (IC_{50}:44 µM); SK-MEL-28 (IC_{50}:57 µM); OE21 (IC_{50}:34 µM); PC-3 (IC_{50}:34 µM); LoVo (IC_{50}:23 µM)	(Smyrniotopoulos *et al.*, 2009; Smyrniotopoulos *et al.*, 2010a)
Sphaerostanol	2010	Palaiokastritsa bay, Corfu Island, Greece	U373 (IC_{50}:85 µM); A549 (IC_{50}:97 µM); SK-MEL-28 (IC_{50}:96 µM); OE21 (IC_{50}:60 µM); PC-3 (IC_{50}:74 µM); LoVo (IC_{50}:64 µM)	(Smyrniotopoulos *et al.*, 2010a)
10*R*-hydroxy-bromocorodienol	2010	Palaiokastritsa bay, Corfu Island, Greece	U373 (IC_{50}:60 µM); A549 (IC_{50}:64 µM); SK-MEL-28 (IC_{50}:62 µM); OE21 (IC_{50}:33 µM); PC-3 (IC_{50}:48 µM); LoVo (IC_{50}:24 µM)	(Smyrniotopoulos *et al.*, 2010a)
Spirosphaerol	2015	Liapades Bay, Corfu, Greece	A549 (IC_{50}:69 µM); Hs683 (IC_{50}:56 µM); MCF-7 (IC_{50}:67 µM); B16F10 (IC_{50}:65 µM)	(Smyrniotopoulos *et al.*, 2015)
Anthrasphaerol	2015	Liapades Bay, Corfu, Greece	A549 (IC_{50}:90 µM); Hs683 (IC_{50}:93 µM); MCF-7 (IC_{50}:85 µM); B16F10 (IC_{50}:63 µM)	(Smyrniotopoulos *et al.*, 2015)
Corfusphaeroxide	2015	Liapades Bay, Corfu, Greece	A549 (IC_{50}:67 µM); Hs683 (IC_{50}:63 µM); MCF-7 (IC_{50}:60 µM); U373 (IC_{50}:81 µM); SK-MEL-28 (IC_{50}:75 µM); B16F10 (IC_{50}:46 µM)	(Smyrniotopoulos *et al.*, 2015)

*Chemical structures are presented in the Annex I (Table 2).

Induces cytostatic activity on U373 cells inhibiting cell entrance into mitosis (3 µM). * Induces cytostatic activity on U373 cells through marked increases in mitosis length (16 µM). **** Induces marked vacuolization processes that delay U373 cells proliferation (7 µM). For the other compounds, the possible intracellular signaling pathways were not characterized.

1.7. References

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Chapter 2

Goals and outline of the thesis

2.1. General goals

Cancer diseases represent one of the major threats to human health having an enormous impact on society across the world. Additionally, the incidence of this disease is expected to increase in the next decades. Thus, it is of utmost importance to develop strategies to prevent, diagnose and treat cancer, improving life quality and survival rate of oncologic patients. Along the last decades, NPs have played an important role in cancer therapeutics as a source of new anticancer drugs. In this area, marine natural compounds have demonstrated to possess interesting antitumor activities through distinct mechanisms of action when compared to metabolites attained from terrestrial source.

In line with this view, the main goal of this thesis was to study the chemical profile of the red alga *Sphaerococcus coronopifolius* collected in the Berlenga Nature Reserve, Atlantic coast (Peniche, Portugal) and to evaluate the antitumor activities of its major metabolites. This purpose is important as most of the studies performed so far were with compounds isolated from *S*. *coronopifolius* collected in the Mediterranen sea. In addition, although many of these compounds have shown great cytotoxic activities, their potential remains understudied, since the intracellular signaling pathways underlying their effects is yet uncover.

2.2. Specific goals

Emerging from the general goals, specific questions urged to be answered:

1) *Sphaerococcus coronopifolius* specimens collected in Atlantic coast (Berlenga Nature Reserve) exhibit or not the same chemical profile of the specimens collected from the Mediterranean sea? Can the distinct conditions of the Mediterranean Sea and the Atlantic Ocean promote the production of different secondary metabolites?

2) What is the antitumor potential of the major metabolites of *Sphaerococcus coronopifolius* collected in the Berlenga Nature Reserve? Can they mediate selective antitumor activities?

3) What kind of intracellular signaling pathways are underlying their antitumor activities?

4) Do these compounds possess the potential to overcome the challenges imposed by tumors resistance to chemotherapeutic drugs? What is their ability to prevent the development of cancer stem cells?

The answers to these questions will be an essential tool for the development of new drugs to treat cancer, as this study will provide the knowledge of the mechanisms of action of the compounds isolated from the red alga *Sphaerococcus coronopifolius*.

2.3. Outline of the thesis

The present thesis is divided into seven chapters. Chapter 1 includes the general introduction; Chapter 2 comprises the goals and outline of this thesis; Chapter 3 describes the experimental procedures to isolate the compounds from the red alga *Sphaerococcus coronopifolius*; Chapters 4 and 5 describe the laboratory experiments conducted to assess the antitumor activities and intracellular signaling pathways activated by the compounds. Chapter 6 discusses, summarizes and integrates the general conclusions of this thesis. Chapter 7 described the future approaches that can be outline to reinforce the results obtained during the development of this thesis.

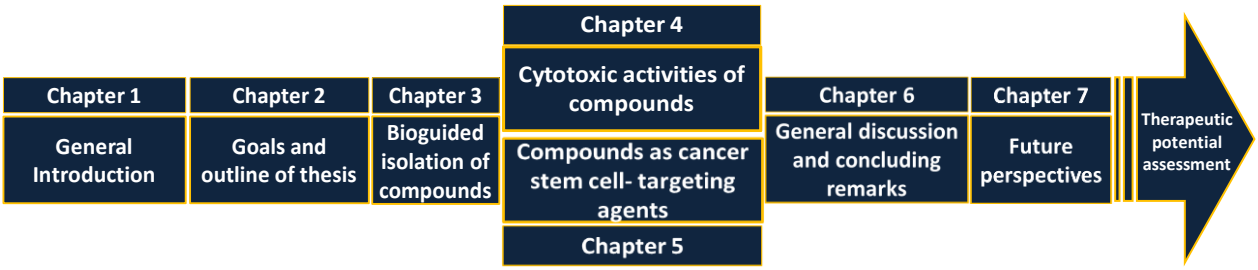

Figure 2.1. Outline of the thesis to assess the antitumor potential of *Sphaerococcus coronopifolius* compounds.

In detail:

Chapter 3, entitled "Bioguided isolation of *Sphaerococcus coronopifolius* compounds" describes all procedures accomplished, from the samples collection to compounds

isolation, which were driven through cytotoxic assays on HepG2 cells to assess the antitumor potential of each sample attained along the process.

Chapter 4, entitled "Cytotoxic activities of *Sphaerococcus coronopifolius* compounds" describes the experiments performed on several cancer cells lines (A549; CACO-2; HCT-15; MCF-7; NCI-H226; PC-3; SH-SY5Y; SK-MEL-28) and non-tumor cells (3T3; L929) to study the selectivity and the potency of the compounds as well as the activation of hallmarks linked to oxidative stress, apoptosis, and DNA damage.

Chapter 5, entitled "*Sphaerococcus coronopifolius compounds as cancer stem cell – targeting agents*" describes the experiments performed on monoculture systems with different cells lines (BEAS-2B; HBF; RenG2; SC-DRenG2) to define the most promissing compounds to be used on a co-culture system. Also, a co-culture system was implemented to better understand what is observed *in vivo* in lung cancer and, thus, to better evaluate the potential of *S. coronopifolius* compounds to inhibit the development of cancer cells with stem-like phenotype.

Chapter 6, entitled "General discussion and concluding remarks" discusses the main findings of Chapters 3, 4, and 5, and summarizes the results attained.

Chapter 7, entitled "Future perspectives" discusses the future approaches to reinforce the results here obtained, contributing to the better understanding of the effective pharmacological potential of the compounds isolated from the red alga *Sphaerococcus coronopifolius* in cancer therapeutics.

Chapter 3

Bioguided isolation of *Sphaerococcus coronopifolius* compounds

This chapter is partially based on the following manuscript:

Rodrigues*, D., Alves, C*., Horta, A., Pinteus, S., Silva, J., Culioli, G., Thomas, O.P., Pedrosa, R. (2015). Antitumor and Antimicrobial Potential of Bromoditerpenes Isolated from the Red Alga, *Sphaerococcus coronopifolius*. *Marine Drugs* 13 (2), 713-726 (doi:10.3390/md13020713). *These authors contributed equally to this work.

3.1. Introduction

Cancer continues to be one of the major threat to public health; consequently, there is a perpetual need for the development of new chemotherapeutic drugs that can be used on cancer treatment (Sawadogo *et al.*, 2013; Xiong *et al.*, 2013). In the last decades, nature has played a significant role as a source of new medicines and recent trends in drug research emphasize that marine environment has a high potential for the discovery of new pharmaceuticals (Simmons *et al.*, 2005; Sawadogo *et al.*, 2013). Marine ecosystems are amongst the richest and most complex ones in terms of biodiversity. Original chemical and physical conditions in such environment provide a production of quite specific and potent active molecules. Among other reasons, marine organisms have been found to produce original and bioactive substances, since they are living in competitive, and aggressive environment (Aneiros and Garateix, 2004; Rocha *et al.*, 2011). These characteristics render marine organisms ideal candidates as novel sources of both preexisting and unrecognized high value added biomolecules with potential for providing sustainable economic and human benefits (Murray *et al.*, 2013). Marine algae have been one of the richest and promising sources of bioactive specialized metabolites that probably have diverse simultaneous functions for their producer and can act for example as antimicrobial, antifouling, and herbivore deterrents, or as ultraviolet-screening agents (Paul and Puglisi, 2004; Ianora *et al.*, 2006; De Almeida *et al.*, 2011). These defensive strategies can result in a large number of chemical metabolites with structural diversity, originated from different metabolic pathways with great pharmaceutical and biomedical potential (Pangestuti and Kim, 2011; Joana Gil-Chávez *et al.*, 2013). Marine algae natural compounds have been associated to numerous health-promoting effects, in particular anti-oxidative, anti-inflammatory, antiviral, antimicrobial, or anticancer effects (Pangestuti and Kim, 2011; Thomas and Kim, 2013). Well-documented bioactive metabolites of marine algae include mainly brominated phenols, polysaccharides, but especially a large variety of terpenoids, several of them halogenated (Cabrita *et al.*, 2010; Viano *et al.*, 2011; Lee *et al.*, 2013; Li *et al.*, 2013).

Since its first chemical analysis in 1976, more than 40 compounds, mostly containing one or two bromine atoms, were isolated from *Sphaerococcus coronopifolius* (Fattorusso *et al.*, 1976; Fenical *et al.*, 1976; Cafieri *et al.*, 1977; Cafieri *et al.*, 1979; Cafieri *et al.*, 1981; Cafieri *et al.*, 1982a; Cafieri *et al.*, 1982b; 1983; Cafieri *et al.*, 1984; Cafieri *et al.*, 1985; 1986; Bavoso *et al.*, 1987; Cafieri *et al.*, 1987; 1988; De Rosa *et al.*, 1988; Cafieri *et al.*,

1990; Etahiri *et al.*, 2001; Smyrniotopoulos *et al.*, 2008a; Smyrniotopoulos *et al.*, 2008b; Smyrniotopoulos *et al.*, 2009; Smyrniotopoulos *et al.*, 2010a; Smyrniotopoulos *et al.*, 2010b; c; Piazza *et al.*, 2011). Concerning the biological activities of such compounds, some of them have demonstrated antibacterial activity against Gram positive bacteria (Etahiri *et al.*, 2001), and others have been assayed for their cytotoxicity against human cancer cell lines (Smyrniotopoulos *et al.*, 2010a) or their antibacterial activity against multidrug-resistant and methicillin-resistant *Staphylococcus aureus* strains (Smyrniotopoulos *et al.*, 2010b;c). Several bromoditerpenes have also been screened against the model organism *Amphibalanus amphitrite* to evaluate their antifouling properties (Piazza *et al.*, 2011).

The aim of this chapter was to address the chemical and antitumor bioactivity characterization of the major compounds isolated from the red alga *Sphaerococcus coronopifolius* collected in the Atlantic coast (Berlenga Nature Reserve, Peniche, Portugal), since most of the chemically studied specimens of this alga were collected from the Mediterranean sea.

3.2. Materials and methods

The procedures accomplished to isolate *S. coronopifolius* compounds with antitumor potential are described in the following sections (3.2.1 – 3.2.6).

3.2.1. General experimental procedures

Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker Avance 500-MHz spectrometer or on a Bruker Avance 400-MHz spectrometer. Chemical shift values (δ) were reported in parts per million (ppm) and coupling constants (*J*) were given in Hertz, and referenced to residual solvent signals ($CDCl_3$, Sigma, Switzerland) at δ^H 7.26 and δ^C 77.16. High Performance Liquid Chromatography (HPLC) purifications were carried out on a Jasco LC-2000 system equipped with a PU-2087 Plus preparative pumping system and a UV-2075 Plus detector. HRESIMS analysis was conducted on a LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) or on a QqTOF Impact II™ mass spectrometer (Bruker Daltonics), both in ESI positive mode.

3.2.2. Collection of *Sphaerococcus coronopifolius* samples

Specimens of *Sphaerococcus coronopifolius* were collected in Berlenga Nature Reserve, Peniche, Portugal (Flandres Bay) (39°24'44.8"N 9°30'29.5"W) (Figure 3.1), in June 2012 and June 2013, according to the previous authorization of ICNF - Instituto da Conservação da Natureza e das Florestas. The collection of samples was performed in Flandres bay, by scuba diving, between 10 and 14 meters deep, and immediately transported to MARE-IPLeiria facilities. Samples were then washed and cleaned using seawater to remove detritus, epiphytes and encrusting matter. The clean alga (19.180 Kg) was freeze - dried (Scanvac Cool Safe, LaboGene, Lynge, Denmark) and the dry material was ground and stored at room temperature until further use.

Figure 3.1. Collection of *Sphaerococcus coronopifolius* specimens in Flandres bay, Berlenga Nature Reserve, Peniche, Portugal (Images adapted from tourism media).

3.2.3. Extraction and fractionation of *Sphaerococcus coronopifolius* samples

Freeze - dried algae samples (2.777 Kg) were sequential extracted with methanol (MeOH, Fisher Scientific, UK) and dichloromethane (CH_2Cl_2, VWR, France) solvents in a 1:40 (w/v) ratio, at room temperature with constant stirring during 12 hours. The CH_2Cl_2 extract was further concentrated using a rotary evaporator. This extract (23.637 g) was then

fractionated by normal phase vacuum liquid chromatography (VLC) on silica gel 60 (0.06–0.2 mm) (Scharlau, Spain). The mobile phase was composed by cyclohexane (VWR, France) with increasing amounts (25%) of ethyl acetate (EtOAc, VWR, Belgium) resulting on five fractions (F1 – 2.779 g; F2 – 6.006 g; F3 – 4.115 g; F4 – 0.595 g; F5 – 0.326 g) each one with 400 mL of eluent. Therefore, fractions were concentrated under vacuum in a rotary evaporator and stored at 4ºC until further use. The extracts and fractions tested on antitumor activity screening assays were dissolved in dimethyl sulfoxide (DMSO, Sigma, France) and stored at -20ºC. The process of extraction and fractionation of *Sphaerococcus coronopifolius* samples is represented in Figure 3.2.

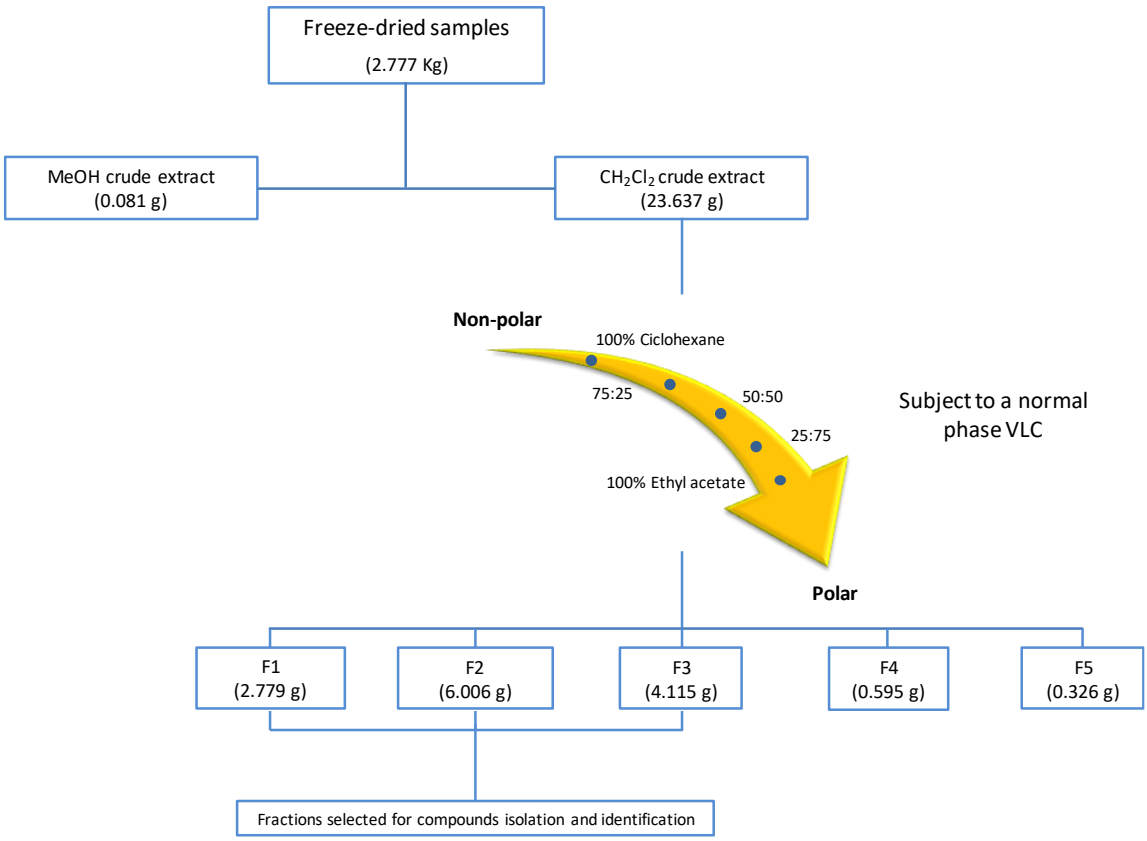

Figure 3.2. Overview of *Sphaerococcus coronopifolius* samples extraction and fractionation processes.

3.2.4. Isolation and structure elucidation of *Sphaerococcus coronopifolius* compounds

Based on the preliminary antitumor activity screening assays, F1, F2, and F3 fractions were selected to isolate compounds. Thus, in order to establish the greatest

procedure for *S. coronopifolius* compounds isolation, these fractions were analyzed by HPLC. F1 fraction was purified by silica gel 60 column (column height: 25 cm; diameter: 4 cm; collection volume: 10 mL) using as mobile phase mixtures of *n*-Hexane (*n*-Hex, Panreac, AppliChem, Germany) and EtOAc of increasing polarity. From 100% *n*-Hex to *n*-Hex/EtOAc (1:1) 291 fractions were attained (f1-f291) while *n*-Hex/EtOAc (3:7) afforded 18 fractions (f292 – f307). The column was then eluted sequentially with 100% EtOAc and 100% MeOH (solutions volume: 500 mL). This approach afforded one compound (**1**) (f5 to f7 fraction; 220.8 mg). On the other hand, F2 and F3 fractions analyzed by HPLC revealed a similar profile (Figure 3.3). For this reason, the purification approach applied to both fractions was the same in order to ensure sufficient amount of biomass to supply the antitumor screening assays.

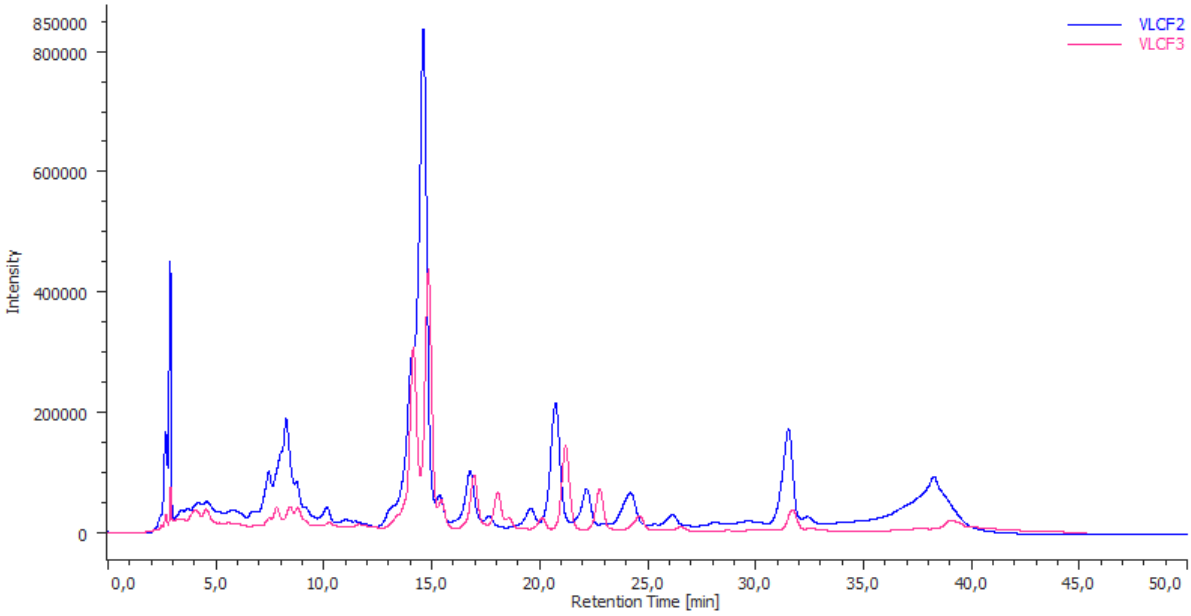

Figure 3.3. Profile of F2 and F3 fractions analyzed by HPLC.

F2 and F3 fractions were purified by semi-preparative reversed-phase HPLC column (Synergi Fusion-RP 80ª, Phenomenex, 10 x 250 mm, 4 µm semi-preparative column) at a flow rate of 5.19 mL/min (25ºC) using as mobile phase a mixture of H_2O (water): CH_3CN (acetonitrile) (Fisher Scientific, U.K.) in isocratic conditions from 0 to 5 min (25:75), a linear gradient from 5 to 25 min (from 25:75 to 15:85), and isocratic after 25 min (15: 85). This first purification step afforded 12 sub-fractions (P1–P12) from which only compound **2** (P5; tR 20.0 min; 263.2 mg) and compound **3** (P7; tR 23.4 min; 267.8 mg) were isolated (Figure 3.4). The sub-fraction P1 was purified by silica gel 60 column (Sharlau, 0.04-0.06 mm;

column height: 19.5 cm; diameter: 2 cm; collection volume: 10 mL) using as mobile phase a mixture of EtOAc/*n*-Hex (1:9), affording two isolated metabolites, compound **4** (P1.1 , f12 to f15; 597.9 mg) and compound **5** (P1.2, f34 to f60; 101.2 mg). The sub-fraction P2 was purified by repeating chromatographic steps: first, by semi-preparative reversed-phase HPLC column (Synergi Fusion-RP 80ª, Phenomenex, 10 x 250 mm, 4 µm) using isocratic conditions (60% CH_3CN: 40% H_2O), at a flow rate of 5.0 mL/min (25ºC) affording 7 sub-fractions (P2.1 - P2.7). P2.5 fraction was subjected to silica gel 60 preparative column (Sharlau, 0.04-0.06 mm; column height: 19.0 cm; diameter: 2 cm; collection volume: 10 mL) eluted with a mixture of *n*-Hex/ EtOAc (8:2) along the first 20 fractions, followed by elution with *n*-Hex/ EtOAc (3:7). This step allowed the isolation of two metabolites, compound **6** (f2 fraction, 7.0 mg) and compound **7** (f5 to f9 fraction, 118.1 mg).

Structural elucidation of *Sphaerococcus coronopifolius* compounds was performed by extensive spectroscopic analysis, including NMR spectroscopy and mass spectrometry (MS) techniques, and comparison with literature data. An overview of the *Sphaerococcus coronopifolius* compounds isolation process is presented in Figure 3.4

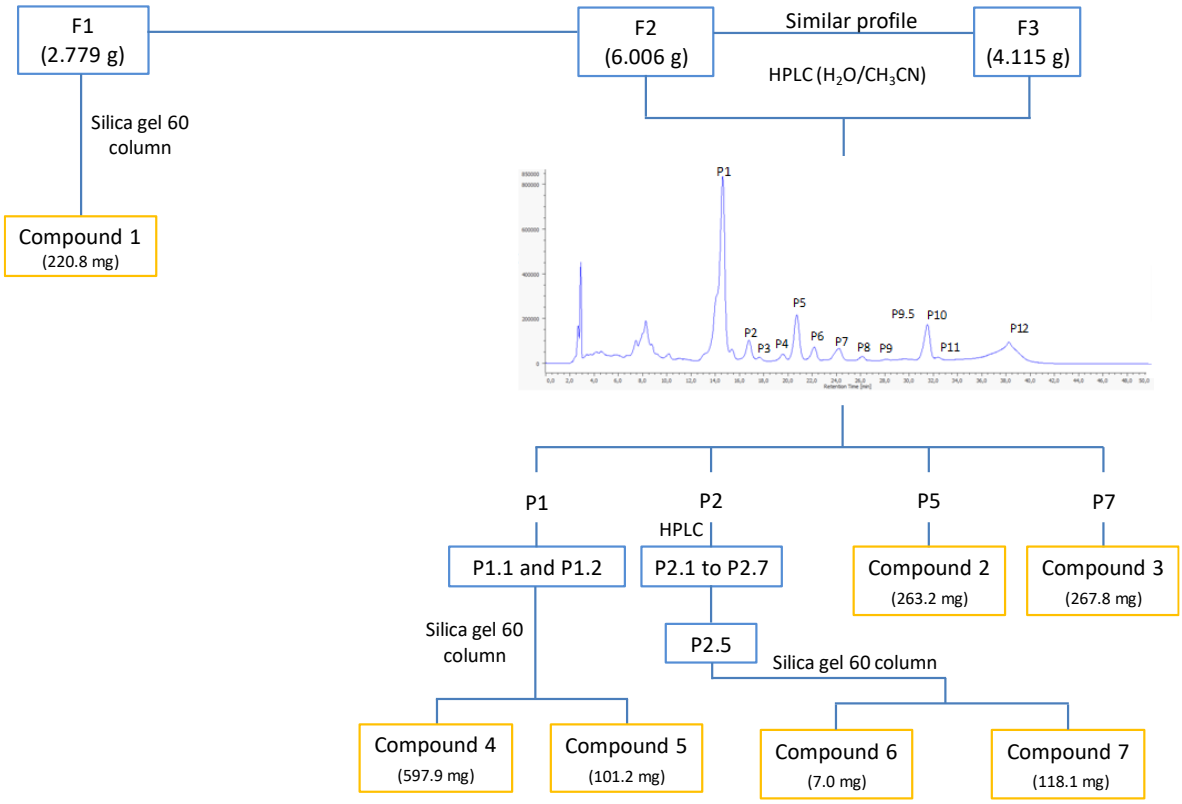

Figure 3.4. Overview of the isolation process of *Sphaerococcus coronopifolius* compounds.

3.2.5. Bioguided screening of cytotoxic activities on HepG2 cells

Cytotoxicity of *Sphaerococcus coronopifolius* crude extracts, fractions and isolated compounds were performed using a hepatocarcinoma cell line (HepG2).

3.2.5.1. Maintenance of cell culture

Human hepatocellular carcinoma model (HepG2) was acquired from the American Type Culture Collection (ATCC - HB-8065). HepG2 cells were cultured in RPMI 1640 medium (Sigma, Saint-Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and supplemented with 100 U/mL penicillin G, 0.25 µg/mL amphotericin B and 100 µg/mL streptomycin (Sigma, USA). The cells' medium was changed every 3 days, and the cells reached confluence after 5–6 days of initial seeding in plates of 25 cm^2 growth area, at a density of 4.22×10^6 cells/ plate. For the subculture, cells were dissociated with trypsin-EDTA (Sigma, Saint-Louis, MO, USA), split into a 1:3 ratio and subcultured in Petri dishes with 25 cm^2 growth area. Cells were maintained in controlled conditions: 95% of humidified atmosphere, 5% CO_2 and 37°C.

3.2.5.2. Evaluation of cytotoxic and antiproliferative activities

HepG2 cells were seeded in 96-well plates at a density of 4.4×10^4 cells/ well for both experiments. However, cytotoxic activity was evaluated after the cells reached total confluence 5 – 6 days after the initial seeding (medium without FBS), and antiproliferative activity after 36 hours of initial seeding (medium with FBS, essential to cells' growth). Cells were treated with 1 mg/mL of crude extracts, fractions or isolated compounds filtered prior to their use to ensure sterilization (0.2 µm, Whatman, Little Chalfont, UK) during 24 hours. Dose-response effects (10 - 1000 µg/mL or 10 - 1000 µM; 24 hours) were determined for the samples with highest activity (> 50%). Cisplatin (Sigma, St. Louis, MO, USA) and tamoxifen (Sigma, Shanghai, China) standard anticancer drugs were used as positive controls. The effects were estimated using the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) (Sigma, Seelze, Germany) colorimetric assay, as described by Mosmann, 1983. This assay is based on the enzymatic activity of mitochondrial dehydrogenases, which have the capacity to cleave the yellow tetrazolium salt MTT to purple formazan crystals. The amount of formazan products are proportional to

the number of living cells and can be measured colorimetrically. After the treatment, cells' medium was removed, and cells were washed with Hank's medium (medium composition, in mM: NaCl 137, KCl 5, $MgSO_4$ 0.8, Na_2HPO_4 0.33, KH_2PO_4 0.44, $CaCl_2$ 0.25, $MgCl_2$ 1.0, Tris HCl 0.15 and sodium butyrate 1.0, pH = 7.4). Cells were then incubated with MTT (1.2 mM), previously dissolved in Hank's medium, during 4 hours at 37°C. The formazan products were dissolved in isopropanol (Panreac, Barcelona, Spain), containing 0.04 N HCl, and cellular viability were determined by the absorbance at 570 nm.

3.2.6. Data analysis

Results were expressed as IC_{50}, that is defined as the concentration causing a 50% reduction or inhibition of cell viability and cell proliferation, respectively. IC_{50} values were expressed as the mean of eight independent experiments with 95% confidence intervals. The calculation of IC_{50} was performed by the analysis of non-linear regression by means of the equation:

$$y = \frac{100}{[1+10(X-log(IC_{50}))]}$$

All the calculations were performed using GraphPad v5.1 (GraphPad Software, La Jolla, CA, USA) software.

3.3. Results

Bioguided antitumor screening on HepG2 cells was accomplished along the isolation process of *Sphaerococcus coronopifolius* compounds. The attained results are presented in the following sections (3.3.1 – 3.3.3).

3.3.1. *In vitro* bioguided extraction and fractionation

S. coronopifolius samples were sequentially extracted with MeOH and CH_2Cl_2 solvents producing two crude extracts, and their anticarcinogenic potential was evaluated on HepG2 cells. CH_2Cl_2 extract exhibited the highest cytotoxic and antiproliferative activities on HepG2 cells (Table 3.1). Therefore, it was selected for further studies regarding the isolation and identification of compounds responsible for the observed antitumor activities. This extract was fractionated by VLC on a silica gel column eluted with a mixture of solvents of increasing polarity (from cyclohexane to EtOAc), yielding five fractions (F1–F5 through a 25% step). F2 fraction exhibited the strongest cytotoxic (IC_{50}: 104.3 µg/mL) and antiproliferative (IC_{50}: 19.8 µg/mL) activities. On the other hand, fractions F1, F3, F4, and F5 were far more efficient in decreasing the cells' proliferation than their viability (Table 3.1).

Table 3.1. Cytotoxic and antiproliferative (IC_{50} values; µg/mL) effects of crude extracts and fractions derived from the red alga *Sphaerococcus coronopifolius* on HepG2 cells (10 – 1000 µg/mL; 24 h). The values in parentheses represent the confidence intervals for 95%.

		IC_{50} (µg/mL)	
		Cytotoxicity	**Antiproliferative activity**
Crude extract	MeOH	470.6 (310.7-712.6)	646.5 (398.4-1049.0)
	CH_2Cl_2	14.13 (8.12-24.60)	32.32 (22.37-46.70)
Fraction	F1	> 1000	102.5 (68.08-154.2)
	F2	104.3 (81.82-132.9)	19.78 (13.79-28.38)
	F3	> 1000	70.17 (38.78-127.0)
	F4	> 1000	36.68 (23.37-57.55)
	F5	> 1000	39.32 (25.89-59.71)

The yields obtained along the extraction and fractionation processes of *Sphaerococcus coronopifolius* samples were registered and are presented in Table 3.2.

Table 3.2. Yield (%) calculated for crude extracts and fractions derived from the red alga *Sphaerococcus coronopifolius* in relation to freeze - dried biomass.

	Initial mass (g)	Crude extract mass (g)	Yield (%)	Fraction	Fraction mass (g)	Yield (%)
MeOH	470	0.081	0.017	-	**-**	-
CH_2Cl_2	2,777	23.738	0.855	**F1**	2.779	0.100
				F2	6.006	0.216
				F3	4.115	0.148
				F4	0.442	0.094*
				F5	0.159	0.034*

* The yield of F4 and F5 fractions was calculated from biomass (470 g) used in the first approach performed to isolated *Sphaerococcus coronopifolius* compounds (Annex II – Chapter II article).

Based on the yield, HPLC profile and the cytotoxic activities of *Sphaerococcus coronopifolious* fractions, it was decided to proceed with the purification and structurally characterization of the main components of F1, F2 and F3 fractions. Amongst the selected fractions (F1, F2 and F3) for purification process, F2 exhibited the highest yield, with a value of 0.216%, followed by F3 (0.148%), and F1 (0.100%).

3.3.2. Isolation and structure elucidation of the major compounds from bioactive fractions

The isolation of the major compounds from the bioactive fractions (F1, F2 and F3) was accomplished by liquid chromatographic methodologies (preparative column chromatography, and HPLC). The structural characterization of isolated metabolites was performed by NMR and MS techniques. These combined methodologies led to the isolation and identification of seven terpenes (**1**-**7**) from the red alga *Sphaerococcus coronopifolius* (Figure 3.5). The analysis of NMR spectra of compounds **1**, **3**, **4**, **5,** and **7,** and comparison of the attributed ^{1}H and ^{13}C chemical shifts with literature data allowed to identify, unambiguously, five known terpenes: the non-halogenated sesquiterpene alloaromadendrene (**1**) (Annex III – Table 1) (De Rosa *et al.*, 1988), and four bromoditerpenes: bromosphaerol (**3**) (De Rosa *et al.*, 1988) (Annex III –Table 2), sphaerococcenol A (**4**) (Fenical *et al.*, 1976; De Rosa *et al.*, 1988) (Annex III –Table 3), 12*S*-hydroxy-bromosphaerol (**5**) (Cafieri *et al.*, 1982a; Smyrniotopoulos *et al.*, 2008a) (Annex III –Table 4) and 12*R*-hydroxy-bromospaherol (**7**) (Cafieri *et al.*, 1987; Smyrniotopoulos *et al.*, 2008a) (Annex III –Table 5). Additionally, two new natural terpenes

were also identified: a brominated diterpene belonging to the rare dactylomelane family, and named sphaerodactylomelol (**2**), and a brominated 7-*epi*-eudesmane sesquiterpene, named 6-acetyl-sphaeroeudesmanol (**6**). The chemical structures of compounds are presented in Figure 3.5.

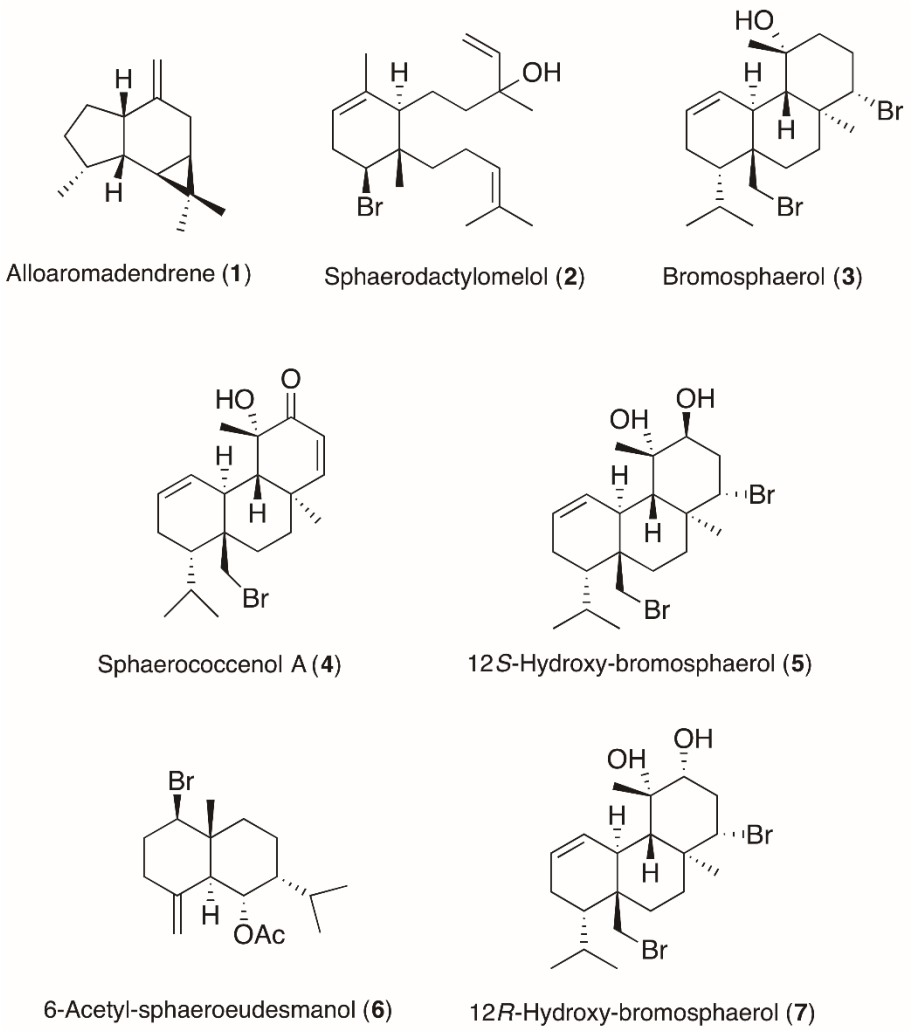

Figure 3.5. Chemical structures of terpenes isolated from *Sphaerococcus coronopifolius* collected in the Berlenga Nature Reserve, Peniche, Portugal (Atlantic coast).

The new natural terpenes, sphaerodactylomelol (**2**) (Figure 3.6) and 6-acetyl-sphaeroeudesmanol (**7**) (Figure 3.9) were isolated as optically-active oils, and their (+)HRESIMS spectra revealed an isotopic pattern, $[M+H]^+/[M+2+H]^+$ (intensities 1:1), characteristic of the presence of one bromine atom in both compounds (for **2,** at *m*/*z* 351.17117/353.16891and for **6**, at *m*/*z* 343.12469/345.1247), which is consistent with the molecular formulas $C_{20}H_{32}Br^+$ and $C_{17}H_{28}BrO_2^+$. The ^1H NMR spectra of

sphaerodactylomelol (**2**) (Annex IV – Figure 1) confirmed the presence of a diterpene with five characteristic methyl signals at δ_H 0.89 (*s*, H3-19), 1.30 (*s*, H3-17), 1.63 (*s*, H3-16), 1.69 (*s*, H3-20) and 1.70 (*s*, H3-18), and the chemical shifts of the last three revealing three methyls substituted on olefinic double bonds (Table 3.3).

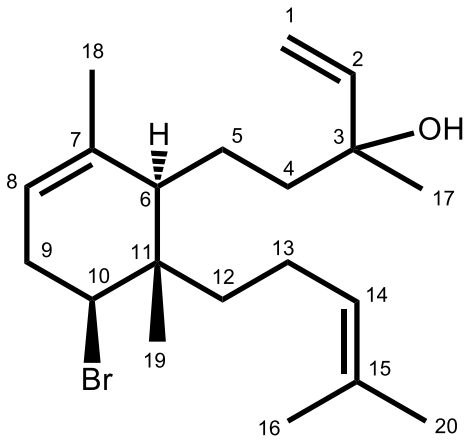

Figure 3.6. Chemical structure of sphaerodactylomelol (**2**) isolated from the red alga *Sphaerococcus coronopifolius*.

A terminal monosubstituted double bond was evidenced by the vinylic signals at δ_H 5.92 (*dd*, *J* = 17.2 and 10.8 Hz, H-2), 5.22 (*dd*, *J* = 17.2 and 1.2 Hz, H-1a) and 5.09 (*dd*, *J* = 10.8 and 1.2 Hz, H-1b). Two additional trisubstituted double bonds were inferred from the NMR signals at δ_H 5.22–5.17 (*m*, H-8), δ_C 120.5 (CH, C-8) and 137.2 (qC, C-7) and at δ_H 5.12–5.08 (*m*, H-14), δ_C 124.3 (CH, C-14) and 131.8 (qC, C-15). It appeared that the terminal double bond was connected to a quaternary carbon, as evidenced by the absence of further scalar coupling from the vinylic protons. The H-1 and H-2/C-3 HMBC correlations indicated that the chemical shift of the quaternary carbon was δ_C 73.6 (qC, C-3), which was characteristic of an allylic alcohol (Figure 3.7a). A terminal monosubstituted double bond was evidenced by the vinylic signals at δ_H 5.92 (*dd*, *J* = 17.2 and 10.8 Hz, H-2), 5.22 (*dd*, *J* = 17.2 and 1.2 Hz, H-1a) and 5.09 (*dd*, *J* = 10.8 and 1.2 Hz, H-1b). Two additional

Table 3.3. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of sphaerodactylomelol (**2**).

	Sphaerodactylomelol (2)		
Atom n°	**δ_H (ppm), *m*, *J* (Hz)**	**δ_C (ppm)**	**Mult.**
1	5.09 *dd* (10.8, 1.2) 5.22 *dd* (17.2, 1.2)	112.3	CH_2
2	5.92 *dd* (17.2, 10.8)	144.9	CH
3	-	73.6	C
4	1.83 *td* (13.1, 4.7) 1.46 *m*	44.2	CH_2
5	1.63 *tdd* (13.1, 4.7, 2.0) 1.38-1.29 *m*	23.3	CH_2
6	2.11-2.06 *m*	45.0	CH
7	-	137.2	C
8	5.22 – 5.17 *m*	120.5	CH
9	2.62-2.49 *m*	35.3	CH_2
10	4.31 *dd* (10.2, 6.2)	61.5	CH
11	-	41.4	C
12	1.57 *ddd* (14.4, 12.4, 5.1) 1.43 *m*	38.6	CH_2
13	2.03 *dd* (12.9, 5.8) 1.90 – 1.81 *m*	21.5	CH_2
14	5.12-5.08 *m*	124.3	CH
15	-	131.8	C
16	1.63 *s*	17.9	CH_3
17	1.30 *s*	27.9	CH_3
18	1.70 *s*	22.4	CH_3
19	0.89 *s*	16.8	CH_3
20	1.69 *s*	25.9	CH_3

trisubstituted double bonds were inferred from the NMR signals at δ_H 5.22–5.17 (*m*, H-8), δ_C 120.5 (CH, C-8) and 137.2 (qC, C-7) and at δ_H 5.12–5.08 (*m*, H-14), δ_C 124.3 (CH, C-14) and 131.8 (qC, C-15). The conclusion on the presence of an alcohol, and not a bromine atom, at this position was also given by the observation of a second heteroatom-substituted carbon at δ_H 4.31 (*dd*, *J* = 10.2 and 6.2 Hz, H-10), δ_C 61.5 (CH, C-10), with chemical shifts fully consistent with a brominated methine. Consequently, the isotopic pattern obtained by HRESIMS was the result of dehydration of the allylic and tertiary alcohol at C-3 $[M - H_2O + H]^+$, and the molecular formula of compound **2** was then determined to be $C_{20}H_{33}BrO$. In view of the NMR data, from the four unsaturations corresponding to this molecular formula, the last one was assigned to a cycle. Full analysis of ^{1}H- ^{1}H COSY and HMBC spectra (Annex IV – Figures 3 and 5, respectively) allowed the establishment of the carbon skeleton of compound **2**. More precisely, the closing of the precursor geranylgeranyl chain through the C-6/C-11 bond was as certained by the key H3-19, H-10, H2-12/C-6 and H3-19, H-10, H-9/C-11 HMBC correlations, thus leading to the six-membered ring. The key H2-12/C-6,

C-11, C-19 HMBC correlations placed the last H-12/H-13/H-14 spin coupled system at C-11, thus revealing the full planar structure of compound **2**. We relied on the NOESY spectrum (Annex IV – Figure 6) for the elucidation of the relative configuration of compound **2**. H-10/H-6 nOe placed the bromine on the same side as the linear alkyl chain at C-6, while the second alkyl chain was placed on the other side, thanks to H3-18/H2-4 and H-10/H2-13 nOes (Figure 3.7b). The absolute configuration was not determined, due to the lack of simple assessment. However, we assume the same stereochemistry as the one determined for the known analogues **3**, **5** and **7**.

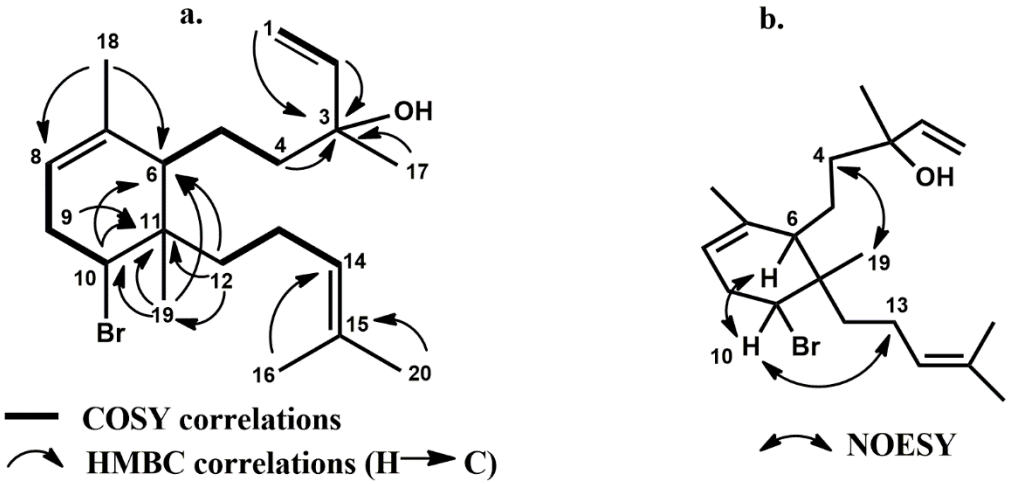

Figure 3.7. Key COSY, HMBC (a) and NOESY (b) correlations of sphaerodactylomelol (**2**).

The analysis of NMR spectra of 6-acetyl-sphaeroeudesmanol (**6**) reveals the structure of an acetylated sesquiterpene (Annex V – Figure 1). The presence of an acetoxy group is confirmed by the NMR signals at δ_C 170.08, characteristic of a carbonyl carbon, that correlates in the HMBC with a methyl group at δ_H 1.98s (δ_C 21.33) and with the geminal methine proton at δ_H 5.21 (*dd*, *J* =5.0 and 11.7 Hz, H-6; δ_C 73.05). In addition to the carbon signals of the acetoxy group, the carbon spectrum has other 15 signals. The bicyclic sesquiterpene skeleton of compound **6** is supported by the presence of these 15 carbon signals together with the existence, in the proton spectrum, of the signals characteristic of an isopropyl [two methyl groups at δ_H 0.94*d* and δ_H 0.99*d* coupled by 6.5 Hz to the methine proton at 1.99*m* geminal to the methine group at δ_H 1.81*m* (the corresponding carbon at δ_C 42.7 correlates, by HMBC, with the proton signals of the two geminal methyl)], an exocyclic double bond (two methylenic protons at δ_H 4.34*brs* and δ_H 4.87*brs* that correlate by HMBC with the other carbon of the double bond at δ_C 142.94), four methylenic groups with diastereotopic protons (δ_H 2.12*m*/2.23*m* coupled with δ_H 2.10*m*/2.29*m*, and 1.61*m*/1.77*m*

coupled with 1.27*m*/1.68*m*), a methine proton (δ_H 2.40*d* , *J*=11.7 Hz) vicinal to the methyl group at δ_H 0.95s (δ_C 13.61), and to the acetoxy group (due the HMBC correlations of δ_H 0.95*s* with δ_C 13.61, and δ_C 170.08), and a bromomethine at δ_H 4.09*m* (δ_C 66.40). Key ^{1}H-^{1}H COSY and HMBC correlations allowed to establish the skeleton of compound **6** present in Figure 3.8.

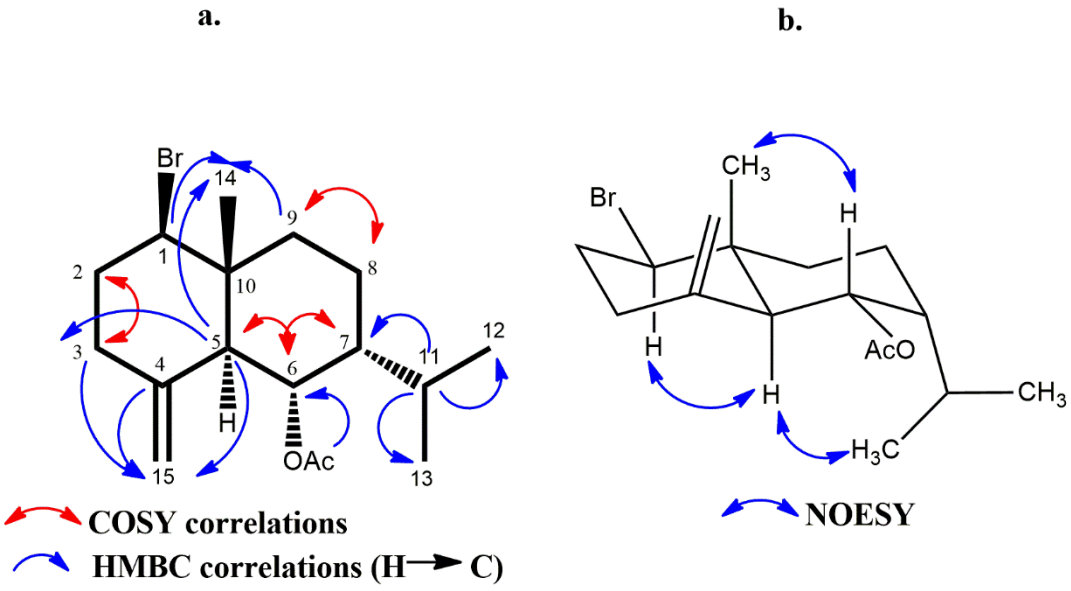

Figure 3.8. Key COSY, HMBC (a) and NOESY (b) correlations of 6-acetyl-sphaeroeudesmanol (**6**).

The relative stereochemistry was determined by the values of the coupling constants of the spin sytem H5/H6/H7 and by the analysis of the NOESY spectrum (Annex V – Figure 6). The value of 11.7 Hz of the signal of H-5, which is characteristic of a *trans-diaxial* coupling constant, indicates that both H-5 and H-6 are in *axial* position and, consequently, the acetoxy group at the C-6 position is *equatorial*. In addition, the coupling constant between H-6/H-7 of 4 Hz indicates that proton H-7 is in *equatorial* position and the isopropyl group is *axial*. Me-14/H-6 nOe placed the acetoxy group and the methyl group at C-10 on the oposite side of the skeleton, but at the same side as H-5, consequently the ring fusion of the sesquiterpene is *trans*. The H-1/H-5 nOe placed these protons in the same side and, consequently, the bromine at C-1 in the same side of the Me-4, but in *equatorial* position. Full analysis of NMR spectra allowed to make the assignments of the proton (Annex V – Figure 1), and carbon (Annex V – Figure 2) signals presented in Table 3.4 of compound 6 (Figure 3.9).

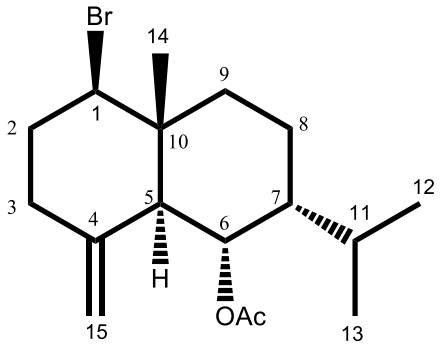

Figure 3.9. Chemical structure of 6-acetyl-sphaeroeudesmanol (**6**) isolated from the red alga *Sphaerococcus coronopifolius*.

Table 3.4. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of 6-acetyl-sphaeroeudesmanol (**6**).

6-Acetyl-sphaeroeudesmanol (6)			
Atom n°	**δ_H (ppm), *m*, *J* (Hz)**	**δ_C (ppm)**	**Mult.**
1	4.09 *m ax*	66.40	CH
2	2.12 *m*, 2.23 *m*	35.45	CH_2
3	2.10 *m*, 2.29 *m*	38.09	CH_2
4	-	142.94	C
5	2.40 *d* (11.7)	48.92	CH
6	5.21 *d* (5.0, 11.7) *ax*	73.05	CH
7	1.81 *m*	42.66	CH
8	1.61 *m*, 1.77 *m*	22.31	CH_2
9	1.27 *m*, 1.68 *m*	34.14	CH_2
10	-	43.01	C
11	1.99 *m*	25.16	CH
12	0.94 *d* (6.6)	22.11	CH_3
13	0.99 *d* (6.6)	23.86	CH_3
14	0.95 s	13.61	CH_3
15	4.34 *brs*	108.56	CH_2
	4.87 *brs*		
OAc	1.98 *s*	21.33	CH_3
	-	170.80	C

These data are in agreement with the published NMR results of austradiol acetate, a compound previously isolated from the Australian red alga *Laurencia* sp., and its synthetic derivatives that have the same relative stereochemistry (Brennan and Erickson, 1982).

The yields obtained along of the isolation process for each compound, and also respective chemical formula and molecular weight are shown in Table 3.5.

Table 3.5. Chemical formula, molecular weight and yield (%) calculated of isolated compounds derived from the red alga *Sphaerococcus coronopifolius* in relation to initial freeze - dried biomass (2.777 Kg).

Compound	Chemical formula	Molecular weight (g/mol)	Mass (mg)	Yield (%)
Alloaromadendrene (**1**)	$C_{15}H_{24}$	204.36	220.8	0.0080
Sphaerodactylomelol (**2**)	$C_{20}H_{33}BrO$	369.38	263.2	0.0095
Bromosphaerol (**3**)	$C_{20}H_{32}Br_2O$	448.28	267.8	0.0096
Sphaerococcenol A (**4**)	$C_{20}H_{29}BrO_2$	381.35	597.9	0.0215
12*S*-Hydroxy-bromosphaerol (**5**)	$C_{20}H_{32}Br_2O_2$	464.28	101.2	0.0036
6-Acetyl-sphaeroeudesmanol (**6**)	$C_{17}H_{27}BrO_2$	343.07	7.0	0.0003
12*R*-Hydroxy-bromosphaerol (**7**)	$C_{20}H_{32}Br_2O_2$	464.28	118.1	0.0043

Amongst the terpenes isolated from the red alga *Sphaerococcus coronopifolius*, the new compound sphaerodactylomelol (**2**) and the know compounds bromosphaerol (**3**) and sphaerococcenol A (**4**), revealed the highest yield exhibiting a value of 0.0095%, 0.0096% and 0.0215%, respectively. However, it is expected that these values increase in the future since still there is available freeze - dried biomass for to be used in further fractionation and isolation processes.

3.3.3. Antitumor activities of compounds isolated from *Sphaerococcus coronopifolius*

Antitumor activities of isolated compounds and chemotherapeutic standard drugs were accomplished by cell viability and cell proliferation studies on HepG2 cells. The results obtained by the MTT assay are presented in Table 3.6.

Table 3.6. Cytotoxic and antiproliferative effects (IC_{50}) induced on HepG2 cells by compounds (**1-5, 7**) isolated from *Sphaerococcus coronopifolius* and chemotherapeutic drugs (10 - 1000 µM; 24h). IC_{50} (µM) values are expressed as the means of eight independent experiments. The values in parentheses represent the confidence intervals for 95%.

		IC_{50} (µM)	
		Cytotoxicity	**Antiproliferative activity**
Compounds	1	> 1000	> 1000
	2	719.85 (519.79 - 996.81)	279.93 (206.78 - 378.74)
	3	> 1000	203.33 (90.65 - 456.18)
	4	> 1000	42.87 (22.76 - 78.88)
	5	> 1000	291.42 (206.22 - 411.83)
	7	> 1000	104.83 (55.27 - 198.89)
Drugs	Cisplatin	454.6 (388.9 - 531.3)	75.41 (61.78 - 92.05)
	Tamoxifen	>1000	45.68 (31.84 - 65.57)

The terpenes isolated from *Sphaerococcus coronopifolius* exhibited great antiproliferative activities on HepG2 cells at sub-toxic concentrations. The highest effect on HepG2 cells' proliferation was mediated by sphaerococcenol A (**4**) and 12*R*-hydroxy-bromosphaerol (**7**), which exhibited an IC_{50} of 42.87 and 104.83 µM, respectively. On the other hand, the major reduction of HepG2 cell' viability was induced by the new compound sphaerodactylomelol (**2**) with an IC_{50} of 719.85 µM, which also exhibited antiproliferative activity (IC_{50}: 279.93 µM). On the other side, alloaromadendrene (**1**) did not induced effects on HepG2 cells. Due to the limited amount of 6-acetyl-sphaeroeudesmanol (**6**) it was not possible to evaluate its antitumor potential.

3.4. Discussion and main remarks

The red alga *Sphaerococcus coronopifolius* has revealed to be an interesting source of brominated cyclic diterpenes with a significant number of compounds isolated in the last four decades. The studies were carried out mostly with *Sphaerococcus coronopifolius* collected from the Mediterranean sea with few exceptions from the Atlantic Ocean. The present work, accomplished with samples from Berlenga Nature Reserve (Portugal), led to the identification of seven terpenes belonging to distinct classes: two sesquiterpenes (**1** and **6**), including a new brominated 7-*epi*-eudesmane sesquiterpene (**6**), and five bromoditerpenes (**2-5**, **7**), including a new dactylomelane (**2**).

Sphaerodactylomelol (**2**) does not belong to the usual sphaerane family found in this species, but it is another rare example of the dactylomelane family (Fernandez *et al.*, 2005). To date, there are nine compounds of this class of diterpenes described in the literature. Eight of them are characterized by a bridged oxide between C-7 and C-10 thus leading to a 7-oxabicyclo[2.2.1]heptanes. These compounds were isolated from sea hares of the genus *Aplysia*, and also from a red alga of the genus *Laurencia*, thus confirming the diet of the sea hare (Estrada *et al.*, 1989; Findlay and Li, 2002; Fernandez *et al.*, 2005). The last compound of the dactylomelane family was isolated from the same species *S. coronopifolius* harvested also along the Atlantic coast (Etahiri *et al.*, 2001). Its chemical structure is very similar to those of compound **2**, differing from the presence of an allylic alcohol at C-14 instead of a dimethylsubstituted double bond. Interestingly, all diterpenes isolated from samples of *S. coronopifolius* collected in the Mediterranean sea belong to the sphaerane family, suggesting that the presence of dactylomelanes would be restricted to Atlantic specimens. Regarding the new sesquiterpene metabolite, compound **6**, although it has the same relative stereochemistry than a synthetic derivative the austradiol acetate, present in the extracts of the Australian red alga *Laurencia* sp. (Brennan and Erickson, 1982), this was the first report of its isolation from a natural source.

The presence of compounds belonging to the sphaerane class in our extracts, namely the bromoditerpenes sphaerococcenol A (**4**), bromosphaerol (**3**), and 12*R*-hydroxy-bromosphaerol (**7**) was not surprising since they represent the major constituents of the red alga *S. coronopifolius* (Piazza *et al.*, 2011). In addition, many of the coexisting minor compounds are structurally related to the main metabolites, suggesting a common biosynthetic origin from genanyl-geranylpyrophosphate (Piazza *et al.*, 2011). Unlike what happen with some macro-organisms, the biosynthesis of the main constituents of *S.*

coronopifolius seems not to be linked with microbial biogenetic origin, since they are present in high quantities in the alga biomass (Piazza *et al.*, 2011).

Concerning the antitumor activities of those compounds, comparing the results attained with extracts, fractions and isolated compounds, it is possible to observe that along the isolation process their efficiency on HepG-2 cells' viability decreased. In fact, it was expected that, throughout the isolation process, the cytotoxic potential would increase. A possible explanation for the unexpected results is a possible synergistic effect between the different compounds, some of which yet unknown, that exist in the extracts and fractions. This happens often and, in many cases crude extracts appear to be more active than the corresponding purified compounds (Ginsburg and Deharo, 2011). Amongst all compounds isolated herein, sphaerococcenol A (**4**) exhibited the highest antiproliferative activity on HepG2 cells with an IC_{50} around 45 µM revealing an efficacy similar to the standard antineoplastic drugs, cisplatin and tamoxifen. In fact, sphaerococcenol A has already shown high cytotoxic activities against A549, OE21, PC-3 and LoVo cell lines, with LC_{50} of 3.7, 3.0, 3.7 and 2.8 µM, respectively (Smyrniotopoulos *et al.*, 2010a). In the same report, 12*R*-hydroxy-bromosphaerol (**7**) also showed cytotoxic activity towards four cell lines studied with LC_{50} values ranging from 26 to 28 µM. However, when tested in the lung cancer cell lines NSCLC-N6 and A549, the IC_{50} values were higher than 30 µM (Smyrniotopoulos *et al.*, 2008a). The drugs used in chemotherapy are usually hepathotoxic, which requires the evaluation of the liver function in patients undergoing chemotherapy before and after treatments (King and Perry, 2001; Vincenzi *et al.*, 2016). It is interesting to observe that, in the present work, most part of the isolated compounds induced antiproliferative activities on HepG2 cells at sub-toxic concentrations, suggesting low hepatotoxicity. The only exception was verified with the new compound sphaerodactylomelol (**2**) that mediate cytotoxic effects on HepG2 cells. However, to corroborate this hypothesis it will be necessary to accomplish additional cytotoxic studies on other cells lines derived from hepatic tissues. Regarding the new compound 6-acetyl-sphaeroeudesmanol (**7**), due the limited mass attained, it was not possible to evaluate its antitumor activity.

Summarizing, the work developed in this chapter allowed to chemical characterize the major constituents derived from the red alga *S. coronopifolius* collected in the Berlenga Nature Reserve (Atlantic coast). Seven isolated compounds, including a new brominated dactylomelane diterpene, named sphaerodactylomelol (**2**), a new brominated 7-*epi*-eudesmane sesquiterpene, named 6-acetyl-sphaeroeudesmanol (**6**) and five known terpenes (**1**, **3**–**5, 7**) were isolated and characterized. These compounds exhibited

interesting antitumor activities on HepG2 cells, suggesting their therapeutic potential. Nonetheless, Chapter 4 will be focused on the study of the antitumor activities and on the intracellular signaling pathways involved in the effects mediated by those compounds with the exception of alloaromadendrene and 6-acetyl-sphaeroeudesmanol, which were not studied due to the absence of cytotoxicity and to reduced amount of isolated compound, respectively.

3.5. References

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Chapter 4

Cytotoxic activities of *Sphaerococcus coronopifolius* compounds

4.1. Introduction

Due to demographic alterations, such as aging, world population growth, and societal, economic and lifestyle changes it is expected that the number of new cancer cases diagnosed each year will increase in the next decades (Atun and Cavalli, 2018; Fidler *et al.*, 2018). Overall, prostate, lung, colorectal and breast cancers remain associated with the higher number of estimated new cancer cases as well as estimated deaths (Malvezzi *et al.*, 2018; Siegel *et al.*, 2018). Despite the advances in prevention, diagnosis and treatments achieved in the last years, just a combined effort between research and healthcare communities will modify this reality (Rahib *et al.*, 2014). Therefore, the implementation of new strategies to fight this burden is of utmost importance, including the development of new drugs.

Several therapies have been approved for cancer treatments, including the use of apoptosis inducers. Apoptosis plays a crucial role in the development and maintenance of normal tissues homeostasis, tightly regulating programmed cell death by several biochemical and genetic pathways (Hassan *et al.*, 2014; Baig *et al.*, 2016)**.** However, in many cancers, the expression of apoptosis inhibitors promotes cancer cells escape from the apoptotic process (Baig *et al.*, 2016)**.** To avoid this problem one possible approach is to develop selective drugs that modulate the components of apoptotic pathways. However, this strategy has significant challenges because many of these targets involve protein-protein interactions and are difficult to modulate. Despite these facts, several apoptosis inducers are under investigation, as single agents or in combination with other anticancer drugs (Khan *et al.*, 2014). To overcome this challenge, new strategies involving the generation of reactive oxygen species (ROS) are being implemented (Banerjee *et al.*, 2017; Habtetsion *et al.*, 2018; Jing *et al.*, 2018). In fact, ROS play a dual role in cancer, since these species are involved in its development, mediating protumorigenic signaling pathways linked with cell proliferation, survival, and adaptation to hypoxia. Conversely, these species can induce antitumorigenic signaling pathways, and consequently, by triggering oxidative stress, inducing cell death (Reczek and Chandel, 2017). Several anticancer drugs mediate their activities through the generation of ROS. This strategy leads to an increase of oxidative stress in tumor tissues, which when exceding the antioxidant capacity of the tissue, will promote tumorigenic cells death by necrosis or apoptosis (Teppo *et al.*, 2017; Yokoyama *et al.*, 2017). Various mechanisms have been proposed for ROS-induced apoptosis (Raza *et al.*, 2017). For instance, several studies have demonstrated that the excessive production of ROS mediates the opening of the mitochondrial membrane permeability transition pore

complex, stimulating the activity of caspases, upregulating death receptor 5, as well as signaling cascades linked to MAPK and ERK pathways triggering apoptosis (Izeradjene *et al.*, 2005; Lee *et al.*, 2012; Chen, 2014; Raza *et al.*, 2017; Pritchard *et al.*, 2018).

Among ROS, hydrogen peroxide (H_2O_2) has demonstrated to be a mediator of apoptotic cell death (Marie-Véronique *et al.*, 1998; Xiang *et al.*, 2016; Mizutani *et al.*, 2017). For example, Mizutani and co-workers (2017) observed that the treatment of HL-60 cells with the anticancer agent pirarubicin induced apoptosis through generation of H_2O_2. Similarly, sesquiterpenoids isolated from soft coral, *Sinularia* sp. induced HCT116 cells death mediated by via caspase activation, which seems to be triggered by H_2O_2 generation (Miyazato *et al.*, 2016). In fact, marine natural products (MNP) have exhibited potent and selective activities on different pharmacological targets by inducing ROS generation and, thus, apoptosis (Yang *et al.*, 2013; Choi *et al.*, 2017; Huang *et al.*, 2018; Salucci *et al.*, 2018). Among MNP, terpenes have the ability to induce cytotoxic activities, and the intracellular signaling pathways linked with ROS production and apoptosis by mitochondrial mediated caspase dependent pathway. For instance, prostate cancer cells treated with heteronemin, a marine sesterterpenoid, induced mitochondrial dysfunction and oxidative stress leading to cell death by apoptosis (Lee *et al.*, 2018). Likewise, the diterpenoid spatane and meroterpenoid tuberatolide B, both algae metabolites, portrayed the ability to induce cytotoxicity promoting intracellular signaling pathways linked with ROS generation and apoptosis (Velatooru *et al.*, 2016; Choi *et al.*, 2017).

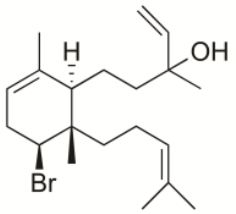
Therefore, the aim of the work presented in this chapter was to evaluate the antitumor potential of *Sphaerococcus coronopifolius* compounds on several *in vitro* human cancer cells to define their selectivity and antineoplastic capacity. In addition, to the best of our knowledge, it was the first study that characterizes the intracellular signaling pathways linked with *Sphaerococcus coronopifolius* compounds cytotoxic activities, namely hallmarks associated to ROS production and apoptosis.

4.2. Materials and methods

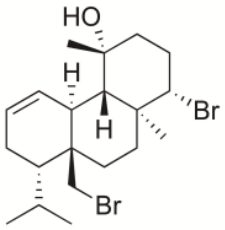
The procedures performed to evaluate the antitumor potential of *S. coronopifolius* isolated compounds are described in the following sections (4.2.1 – 4.2.6).

4.2.1. Compounds isolated from the red alga *Sphaerococcus coronopifolius*

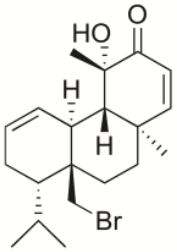
Compounds were isolated from the red alga *S. coronopifolius*, collected in Berlenga Nature Reserve (Peniche, Portugal), according to the procedures described in Chapter 3. Isolation of compounds was achieved by chromatographic methods and their structure was characterized by NMR and MS techniques. Chemical information and the structures of each compound are presented in Figure 4.1.



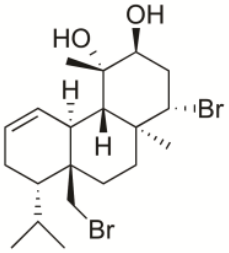
Sphaerodactylomelol (**2**)
$C_{20}H_{33}BrO$
(369.38 g/mol)



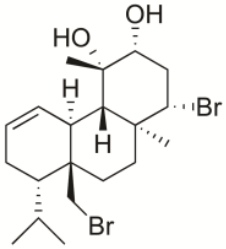
Bromosphaerol (**3**)
$C_{20}H_{32}Br_2O$
(448.28 g/mol)



Sphaerococcenol A (**4**)
$C_{20}H_{29}BrO_2$
(381.35 g/mol)



12*S*-Hydroxy-bromosphaerol (**5**)
$C_{20}H_{32}Br_2O_2$
(464.27 g/mol)



12*R*-Hydroxy-bromosphaerol (**6**)
$C_{20}H_{32}Br_2O_2$
(464.27 g/mol)

Figure 4.1. Compounds isolated from the red alga *Sphaerococcus coronopifolius* collected in Berlenga Nature Reserve, Peniche, Portugal.

For biological assays, compounds were dissolved in DMSO. To avoid DMSO toxicity its concentration along all the experiments was lower than 0.2%. The control situation was always treated with the highest concentration of DMSO as vehicle.

4.2.2. Cytotoxic activities of compounds on malignant cell lines viability and normal fibroblasts

Studies were accomplished on various human cancer cells lines derived from distinct tissues: breast carcinoma (MCF-7; DSMZ: ACC 115), colon adenocarcinoma (HCT-15, DSMZ: ACC 357; CACO-2, DSMZ: ACC 169), lung carcinoma (A549; ATCC: CCL-185), lung squamous cell carcinoma (NCI-H226; ATCC: CRL-5826), malignant melanoma (SK-MEL-28; ATCC: HTB-72), neuroblastoma (SH-SY5Y; ATCC: CRL-2266), and prostate adenocarcinoma (PC-3; ATCC: CRL-1435). As normal cell lines, fibroblasts derived from murine tissues were used (3T3 - DSMZ: ACC173; L929 – DSMZ: ACC 2). Cells lines were acquired from the Americam Type Culture Collection (ATCC) or Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures (DSMZ) biobanks.

4.2.2.1. Maintenance of cell cultures

Cell lines were cultured according to the information supplied by ATCC and DSMZ biobanks. 3T3, A549, and SH-SY5Y cells were cultured in Dulbecco´s Modified Eagle´s medium: Nutrient Mix F-12 (DMEM/F-12) (Merck, Germany) supplemented with 10% FBS (Gibco, Grand Island, NY, USA), GlutaMAX™ (Gibco, Grand Island, NY, USA), 100 UI/mL penicillin, and 100 µg/mL streptomycin (Sigma, USA). CACO-2, HCT-15, L929, MCF-7, NCI-H226, and PC-3 cells were maintained in RPMI medium supplemented with 10% FBS, 100 UI/ mL penicillin, and 100 µg/mL streptomycin. SK-MEL-28 cells were grown in Eagle's Minimum Essential Medium (Sigma, USA) supplemented with 10% FBS, 100 UI/mL penicillin, and 100 µg/mL streptomycin. At the end of each sub-culture, cells were dissociated with trypsin-ethylenediaminetetraacetic acid (Sigma-Aldrich, USA), which was neutralized with medium. Then, to remove trypsin residues, cells were centrifuged at 290 *g* during 5 min at room temperature. Cells were then resuspendend in new medium, performing a split 1 to10 dilution and cultured in T-Flask´s with 25 cm^2 of growth area. Cells were maintained in 5% CO_2 and humidified atmosphere at 37°C.

4.2.2.2. Evaluation of the cytotoxic activities of compounds

The cytotoxic activities of compounds were evaluated on cell lines (A549: 5×10^4 cells/ well; CACO-2: 5×10^4 cells/ well; HCT-15: 5×10^4 cells/ well; MCF-7: 5×10^4 cells/ well; NCI-H226: 1.5×10^4 cells/ well; PC-3: 2.5×10^4 cells/ well; SH-SY5Y: 5×10^4 cells/ well; SK-ML-28: 5×10^4 cells/ well; 3T3: 1.5×10^4 cells/ well) after to be seeded in 96-well plates and incubated overnight. Cells were then treated with the aformentioned *Sphaerococcus coronopifolius* compounds (0.1-100 µM) for 24, 48 and 72 hours. Untreated cells were used as control. Saponin (Sigma, Germany) was used as a cellular death positive control (100% of cell death). The drugs cisplatin, tamoxifen and 5-fluorouracil (Sigma, China) were used as antitumor standards (0.1-500 µM; 24h). The effects mediated by the compounds on cell lines were evaluated by the MTT assay already described in Chapter 3 and lactate dehydrogenase (LDH) assay. After the treatment, medium was removed and cells were washed with phosphate-buffered saline buffer (PBS) and incubated at 37ºC, for 1 hour, with a MTT solution (1.2 mM) previously dissolved in PBS buffer. After washing off excess MTT, cells were disaggregated with DMSO and the absorbance of the crystals of formazan measured at 570 nm using a spectrophotometer plate reader (Bio-Tek Synergy plate reader, Bedfordshire, UK). The cell death was measured by the LDH cytotoxicity assay kit (Pierce™ LDH Cytotoxicity Assay Kit; ThermoScientifc, Rockford, USA) according to the manufacturer instructions. Briefly, this assay allows to measure the LDH released into the media from damaged cells through a coupled enzymatic reaction. Firstly, LDH transforms the lactate to pyruvate, via reduction of NAD^+ to NADH. Thus, the NADH produced is used by diaphorase to reduce a tetrazolium salt (INT) to a red formazan product, which can be measured spectrophotometrically. Fifty µL of treated cells' medium was transferred into a new microplate, and 50 µL of the reaction solution was added. Then, the solution was incubated during 30 min at room temperature. After that, a sttoped solution, supplied by the Pierce™ LDH Cytotoxicity Assay Kit, was added and LDH activity measured by spectrophotometry at 490 nm. The experiments were carried out in triplicate and, at least three independent assays were performed, and results expressed in percentage of control untreated cells.

4.2.3. Hallmarks of oxidative stress – H_2O_2 production

Production of H_2O_2 was measured in real-time using "Amplex ™ Red hydrogen peroxide Assay" Kit (Life Tecnologies, A22188, Camarillo, USA) according to the manufacturer instructions. This assay is based on the use of a fluorescent probe Amplex red, which has low basal fluorescence and reacts with H_2O_2 (1:1). This reaction is initiated in the presence of horseradish peroxidase leading to the production of a highly fluorescent product designated by resofurin. Cells (5×10^4 cells/ well) were treated in 96-well plates with each compound IC_{50} concentration, previously determined (4.2.2. section) and presented in Table 4.1, for 1, 3, and 6 hours. H_2O_2 (200 µM) was used as positive control. Untreated cells were used as control. Cells' medium was then removed, and cells were washed twice with PBS buffer and the reaction mixture solution was added. The real-time H_2O_2 production was accompanied along 60 min at room temperature. The fluorescence intensity was measured using a wavelength of excitation and emission of 590 nm and 530 nm, respectively (Bio-Tek Synergy plate reader, Bedfordshire, UK). The levels of H_2O_2 were calculated by the slope of the linear phase of fluorescence curve, and results expressed in percentage of control untreated cells. The experiments were carried out in triplicate, and at least three independent assays were accomplished.

4.2.4. Hallmarks of cell death mediated by apoptosis

To understand if the decrease on cells' viability was mediated by apoptosis, different hallmarks were evaluated, including alterations in mitochondrial membrane potential, membrane translocation of phosphatidylserine, Caspase-9 activity, chromatin condensation, and/ or DNA fragmentation.

4.2.4.1. Mitochondrial membrane potential (MMP)

MMP was measured using a fluorescent probe, JC-1 (Molecular Probes, T3168, Eugene, Oregon, USA) according to Silva and co-workers (2018). Cells (5×10^4 cells/ well) were treated with each compound IC_{50}, previously determined (4.2.2. section) and presented in Table 4.1, in 96-well plates for 15, 30, and 60 min. Untreated cells were used as control. After the treatment, the cells' medium was removed. Cells were then washed with PBS buffer and incubated with JC-1 probe (3 µM) for 15 min at 37°C. After that, JC-1

solution was removed, and cells were washed twice with PBS buffer. Therefore, the formation of JC-1 aggregates (490 nm of excitation and 590 nm of emission) and JC-1 monomers (490 nm of excitation and 530 nm of emission) were evaluated simultaneously in the plate reader during 30 min (Bio-Tek Synergy plate reader, Bedfordshire, UK). FCCP (2.5 µM) (Sigma, Israel) and oligomycin A (1 µg/mL) (Sigma, USA) conjugate solution was used as positive control. Results were expressed as the ratio of the monomers/aggregates of JC-1 in percentage of control untreated cells. The experiments were carried out in triplicate, and at least three independent assays were performed.

4.2.4.2. Annexin V and propidium iodide staining

The translocation of membrane phosphatidylserine from the inner side of the plasma membrane to the surface (Annexin V), as well as the membrane integrity (Propidium iodide) was evaluated using Apoptosis Detection Kit (Immunostep, Salamanca, Spain) according to the manufacturer's instructions. Cells were seeded in 6-well plates at a density of $1x10^6$ cells/well and incubated overnight. Cells were then treated with each compound IC_{50} concentration, previously determined (4.2.2. section) and presented in Table 4.1, for 24 hours and stained with probes before being analyzed by flow cytometry. Untreated cells were used as control. Staurosporine (1 µg/mL) (Sigma, Israel) was used as positive control. Ten thousand events were attained with AMNIS imaging flow cytometer using the AMNIS INSPIRE™ software. Data analysis was performed with the AMNIS IDEAS™ software. The experiments were carried out in quadruplicate and results expressed as percentage of events defined as viable, apoptosis, late apoptosis, and necrosis.

4.2.4.3. Caspase-9 activity

Caspase-9 activity was determined by the Caspase 9 Fluorimetric Assay Kit (Biovision, Milpitas, CA, USA) according to the manufacturer's instructions. Cells were cultured in 6-well plates ($1x10^6$ cells/ well) and treated with each compound IC_{50}, previously determined (4.2.2. section) and presented in Table 4.1, for 3, 6, and 9 hours. Untreated cells were used as control. Staurosporine (1 µg/mL) was used as positive control as apoptosis inducer. After the treatment, the cells' medium was removed. The cells were then washed twice with PBS buffer and collected by centrifugation at 2655 *g* for 10 min at 4°C. After that, 50 µL of lysis buffer was added to pellets and cells incubated on ice for 20 min.

Thereafter, 50 µL of supernatant was placed into a 96-well plate, to which 50 µL of reaction buffer containing DTT (10 mM) and 5 µL of substrate was added. Caspase-9 activity was then followed at wavelengths of 400 nm (excitation) and 505 nm (emission), along 90 min, at room temperature. Its activity was calculated by the slope of the fluorescence resulting from 7-amino-4-(trifluoromethyl) coumarin accumulation. The experiments were carried out in quadruplicate and are expressed in percentage of control untreated cells (∆fluorescence (u.a)/ mg of protein/ min).

4.2.4.4. Nuclear condensation and/ or DNA fragmentation by DAPI staining

Nuclear condensation and/ or DNA fragmentation were determined by DAPI staining according to the method described by Brizi and co-workers (2016) with slight modifications. This dye intercalates with nuclear DNA, allowing the observation of changes in the nucleus, such as the abnormity in its margin, chromatin condensation and DNA fragmentation, which are characteristic events of apoptosis (Tounekti *et al.*, 1995). Cells were seeded in 6-well plates at a density of $1x10^6$ cells/ well and incubated overnight. Cells were then treated with each compound IC_{50}, previously determined (4.2.2. section) and presented in Table 4.1, during 18, 24, 36, 48, and 72 hours. Untreated cells were used as control. After the treatment, the cells' medium was removed, cells were washed twice with PBS buffer, and incubated for 30 min with paraformaldehyde (4%) (Fisher Scientif, U.K.) solution for fixation. Paraformaldehyde solution was then removed, the cells were washed twice with PBS buffer and incubated with SDS (0.1%) (Sigma, Japan) solution for 30 min. Thereafter, SDS solution was removed, cells were washed twice with PBS buffer and incubated with DAPI (1 µM) (AppliChem, Germany) solution for 30 min. DAPI solution was then removed, PBS buffer was added, and cells were observed in fluorescence inverted microscope (ZEISS Axio, VERT. A1, equipped with a AxioCam MRC-ZEISS camera, München, Germany). Staurosporine (1 µg/mL) was used as positive control of apoptosis. The images presented are representative of one well of each situation tested.

4.2.5. Genotoxicity of compounds on L929 fibroblasts

The study of genotoxic effects of compounds was also studied on L929 mouse fibroblasts according to the method described by Singh and co-workers (1988) with slight modifications. Cells were cultivated on T-flask´s, and posteriorly in 12-well plates at a

density of 2×10^4 cells/ well for 12 hours at 37°C. L929 cells were then treated with compounds at a concentration of 0 (control), 25 and/ or 50 µM for 3 hours. Ethyl methanesulfonate (200 µg/mL) (Sigma, USA) was used as positive control. Briefly, 15 µL of the cells' suspension was suspended in 90 µL of low-melting-point agarose (37ºC). After that, the suspension was spread on a previously prepared thin layer of UltraPureTM agarose slide, covered with coverslip, and maintained at 4ºC for 10 min. Thereafter, the slides were immersed into a lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% Triton×100, and 10% DMSO; pH 10.0) at 4°C for 24 hours. The slides were then subjected to electrophoresis and stained with silver nitrate. Samples were then analyzed by optical microscopy (400 X). The DNA damage induced by the compounds on L929 cells was quantified as the amount of DNA released from the nucleolus. Analysis of 100 cells randomly chosen and non-overlapping was performed. Cells were visually scored and classified into five levels, according to tail size formed by breaks in the DNA: Level 0 – intact, no tail; Level 1 – short tail, smaller than the diameter of the head (nucleus); Level 2 – medium tail, 1 to 2 times the diameter of the head (nucleus); Level 3 – long tail, more than twice the diameter of the head (nucleus); Level 4 – very hide tail, comets without head, maximum DNA damage. The different levels of DNA damage defined are illustrated in Figure 4.2.

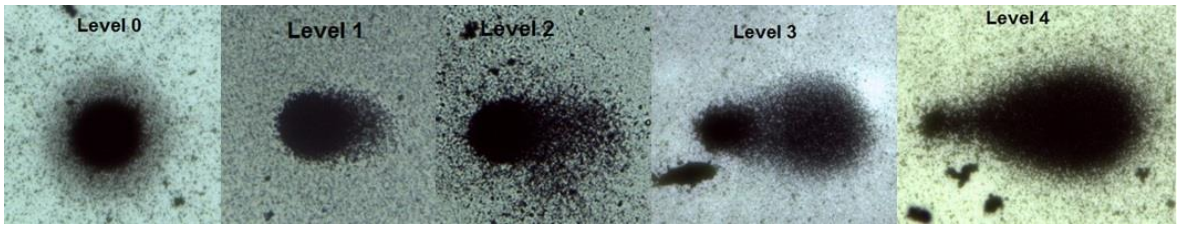


Figure 4.2. Levels of DNA damage defined for classification purposes.

4.2.6. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) with Dunnett's multiple comparison of group means to determine significant differences relatively to control treatment. In addition, for the remaining multiple comparisons the Tukey's test was applied. Additionally, a two-way ANOVA was performed in order to evaluate the simultaneous effects of the compounds' concentrations on the decrease on cells' viability. When applicable, Bonferroni multiple comparison tests were used for the pairwise

comparison of the groups. The analysis was performed and evaluated separately for each cell line studied, i.e., MCF-7, CACO-2, HCT-15, SH-SY5Y, A549, NCI-H226, SK-MEL-28, PC-3, and 3T3 cells. When applicable, the Student's *t* test was used to verify the differences between the means of control and compounds treatment in specific situation. For all analyzes, all data were checked for normality and homoscedasticity. Results are presented as mean ± standard error of the mean (SEM). Differences were considered statistically significant at a level of 0.05 (p - value < 0.05). The determination of IC_{50} was performed by the analysis of non-linear regression by means of the equation:

$$y = \frac{100}{[1+10(X-log(IC_{50}))]}$$

Calculations were performed using IBM SPSS Statistics 24 (IBM Corporation, Armonk, NY, USA) and GraphPad v5.1 (GraphPad Software, La Jolla, CA, USA) softwares.

The overview of experiments accomplished into the Chapter 4 to evaluate the antitumor potential of *Sphaerococcus coronopifolius* compounds are presented in Figure 4.3.

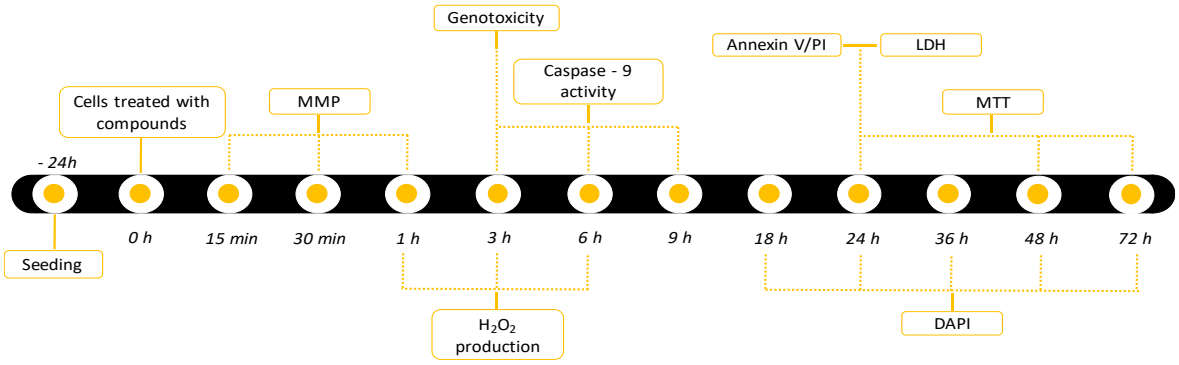


Figure 4.3. Overview of experiments accomplished in Chapter 4 to evaluate the antitumor potential of *Sphaerococcus coronopifolius* compounds.

4.3. Results

Cytotoxic activities of *Sphaerococcus coronopifolius* compounds were evaluated on several cancer cell lines models and hallmarks related with oxidative stress, apoptosis and genotoxicity. The results are presented in the following sections (4.3.1 – 4.3.6).

4.3.1. Cytotoxic activities of compounds on malignant cell lines viability and normal fibroblasts

Cytotoxicity of *Sphaerococcus coronopifolius* compounds was evaluated on several human cancer cell lines derived from distinct tissues, namely breast (MCF-7), colon (CACO-2; HCT-15), CNS (SH-SY5Y), lung (A549; NCI-H226), melanoma (SK-MEL-28), prostate (PC-3), as well as on non-tumor cells (3T3). Cells were treated with different concentrations of the compounds isolated from *Sphaerococcus coronopifolius* (0.1- 100 µM) and standard drugs (0.1- 500 µM) for 24 hours. The effects were revealed by the MTT assay. Results are presented in Figures 4.4 to 4.12, and the IC_{50} values summarized in Table 4.1.

- The effects on MCF-7 cells' viability induced by the compounds isolated from *S. coronopifolius* and by tamoxifen are presented in Figure 4.4.

The results revealed that *S. coronopifolius* compounds reduced the MCF-7 cells' viability in a concentration-dependent manner (Figure 4.4). In addition, the results obtained revealed also that: 25, 50, and 100 µM 12*R*-hydroxy-bromosphaerol, 50 µM sphaerococcenol A, 50 and 100 µM 12*S*-hydroxy-bromosphaerol as well as 50 and 100 µM tamoxifen are more cytotoxic than sphaerodactylomelol exhibiting significant statistical differences (Two-way ANOVA, Bonferroni test; p - value < 0.05) (Figure 4.4A). Moreover, the analysis of the dose–response curves (Figure 4.4B) demonstrated that sphaerococcenol A exhibited the smallest IC_{50} (9.40 µM), followed by 12*R*-hydroxy-bromosphaerol (18.28 µM), 12*S*-hydroxy-bromosphaerol (19.06 µM), bromosphaerol (27.76 µM), and sphaerodactylomelol (47.19 µM). The standard drug tamoxifen showed an IC_{50} of 27.19 µM.

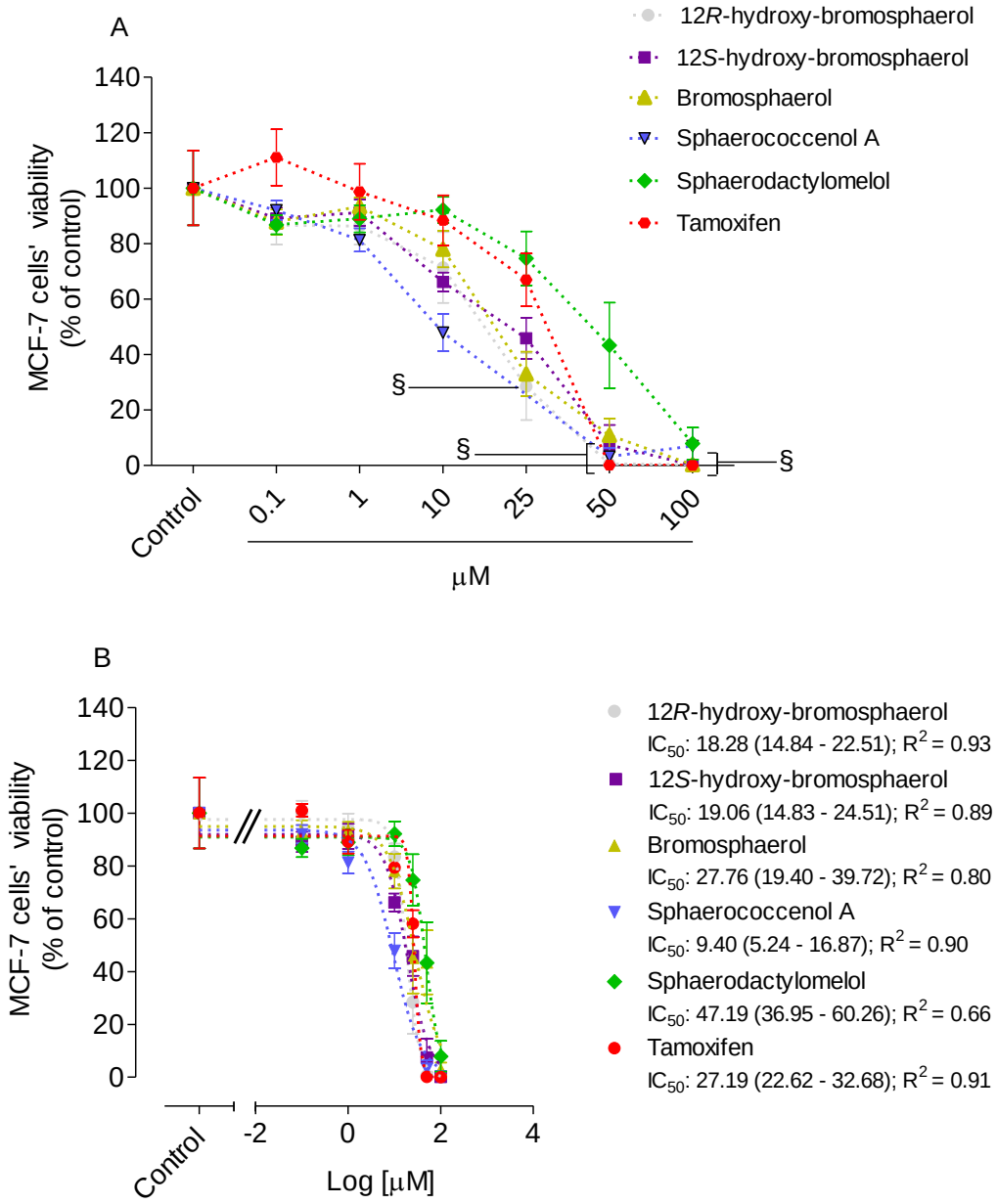


Figure 4.4. Cytotoxic effects of *Sphaerococcus coronopifolius* isolated compounds (0.1-100 µM) and tamoxifen (0.1 - 100 µM) on MCF-7 cells' viability (% of control) after treatment for 24 hours (A). Dose-response curves of *S. coronopifolius* compounds (0.1 - 100 µM; 24h) and tamoxifen (0.1 - 100 µM; 24h) for IC_{50} determination (B). Symbols represent statistically significant differences (Two-way ANOVA, Bonferroni test; *p* - value < 0.05) when compared to: [§] sphaerodactylomelol. MTT assay was used to evaluate the compounds effects and the values represent mean ± SEM of at least three independent experiments carried out in triplicate.

- The effects of the *S. coronopifolius* isolated compounds as well as 5-fluorouracil (5-FU) on CACO-2 cells' viability are displayed in Figure 4.5.

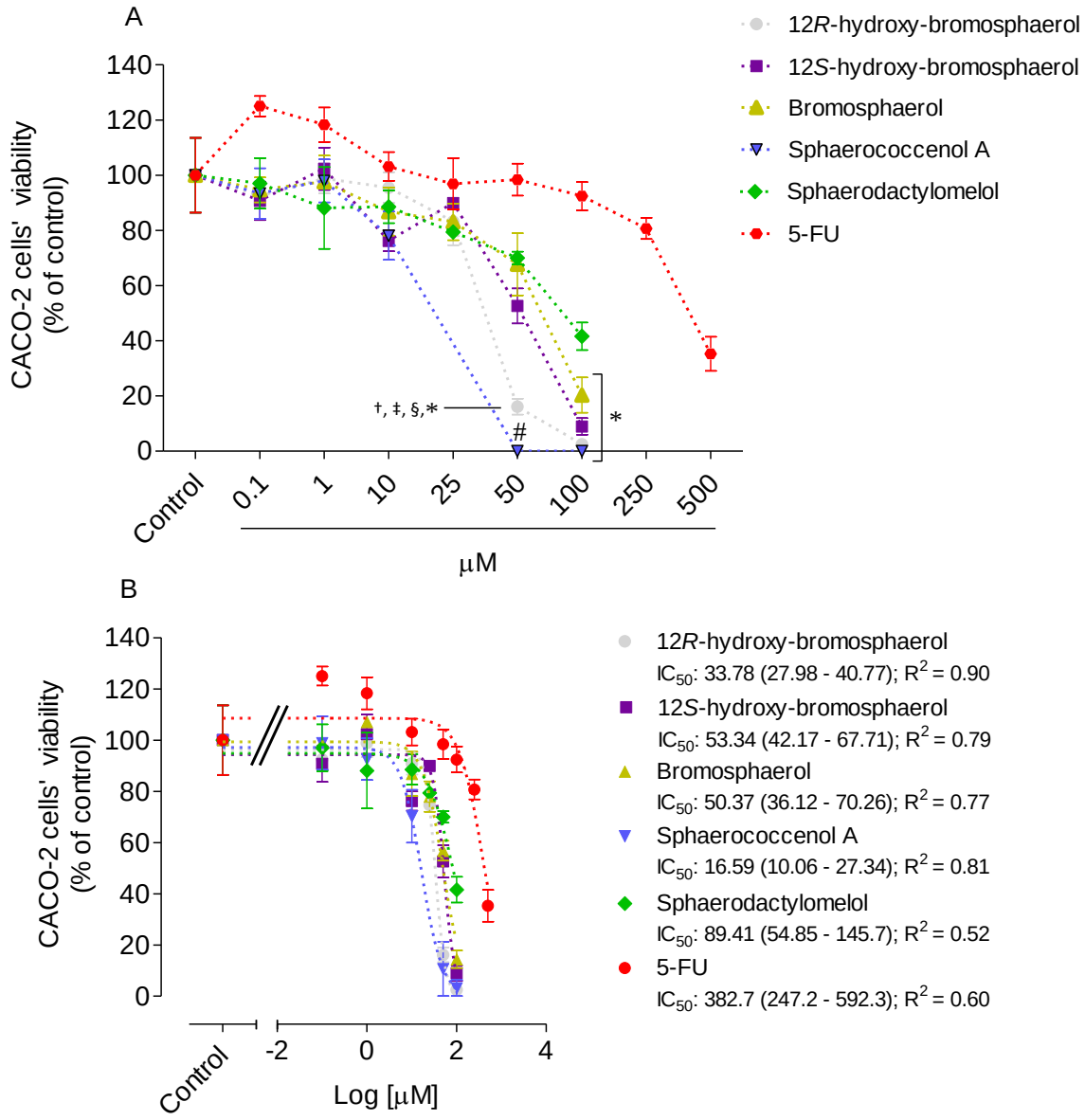


Figure 4.5. Cytotoxic effects of *Sphaerococcus coronopifolius* isolated compounds (0.1 - 100 µM) and 5-FU (0.1 - 500 µM) on CACO-2 cells' viability (% of control) after treatment for 24 hours (A). Dose-response curves of *S. coronopifolius* compounds (0.1 - 100 µM; 24h) and 5-FU (0.1 - 500 µM; 24h) for IC_{50} determination (B). Symbols represent statistically significant differences (Two-way ANOVA, Bonferroni test; *p* - value < 0.05), when compared to: # all compounds; † 12*S*-hydroxy-bromosphaerol; ‡ bromosphaerol; § sphaerodactylomelol; * 5-FU. MTT assay was used to evaluate the compounds effects and the values represent mean ± SEM of at least three independent experiments carried out in triplicate.

The results revealed that most of the compounds tested on CACO-2 cells exhibited a concentration-dependent effect (Figure 4.5). A concentration of 50 µM sphaerococcenol A induced a significant decrease on cells' viability when compared with all other tested compounds (Two-way ANOVA, Bonferroni test; *p* - value < 0.05) (Figure 4.5A). Likewise, 12*R*-hydroxy-bromosphaerol also showed significant differences, when compared with other compounds (Two-way ANOVA, Bonferroni test; *p* - value < 0.05). However, when compared with sphaerococcenol A, it displayed less cytotoxicity. Furthermore, all other compounds but sphaerodactylomelol, at a concentration of 100 µM, induced significant effects on CACO-2 cells' viability when compared to 5-FU (Two-way ANOVA, Bonferroni test; *p* - value < 0.05). Concerning dose-response assays (Figure 4.5B), sphaerococcenol A displayed the highest efficiency exhibiting an IC_{50} of 16.59 µM followed by 12*R*-hydroxy-bromosphaerol (33.78 µM), bromosphaerol (50.37 µM), 12*S*-hydroxy-bromosphaerol (53.34 µM), and sphaerodactylomelol (89.41 µM). 5-FU was the drug with highest IC_{50} with a value of 382.7 µM.

- The effects on HCT-15 cells' viability induced by the *S. coronopifolius* isolated compounds and by 5-FU are presented in Figure 4.6.

The treatment of the HCT-15 cells with the aforementioned compounds decreased their viability in a concentration-dependent manner (Figure 4.6). 10 µM sphaerococcenol A was the most effective agent to reduce HCT-15 cells' viability as illustrated in Figure 4.6A (Two-way ANOVA, Bonferroni test; *p* - value < 0.05). Similar to sphaerococcenol A, 25 µM of 12*R*-hydroxy-bromosphaerol significantly decreased HCT-15 cells' viability. Less pronounced decreases were observed with 12*S*-hydroxy-bromosphaerol and sphaerodactylomelol. When tested at a concentration of 50 µM, sphaerococcenol A and 12*R*-hydroxy-bromosphaerol were the most efficient at decreasing the cells' viability in contrast to sphaerodactylomelol, and by far the less efficient of all the compounds isolated from *S. coronopifolius*. On the other hand, all compounds showed statistically significant differences when compared with 5-FU drug, at 100 µM (Two-way ANOVA, Bonferroni test; *p* - value < 0.05). Regarding dose-response experiments (Figure 4.6B), sphaerococcenol A exhibited the smallest IC_{50} (7.11 µM), followed by bromosphaerol (24.55 µM), 12*R*-hydroxy-bromosphaerol (25.08 µM), 12*S*-hydroxy-bromosphaerol (27.54 µM), and sphaerodactylomelol (46.25 µM). The anticancer drug 5-FU displayed an IC_{50} of 155.5 µM.

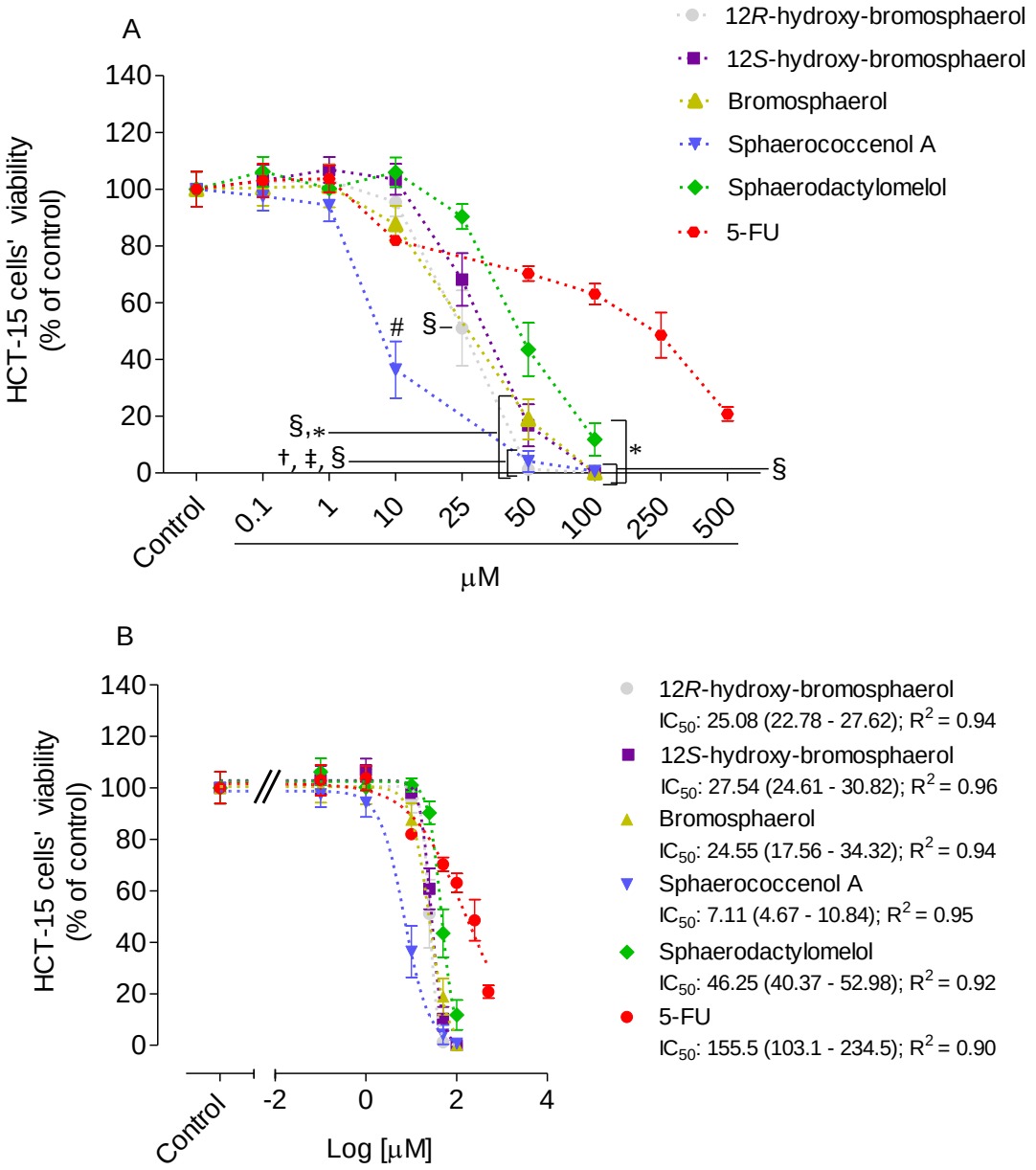


Figure 4.6. Cytotoxic effects of *Sphaerococcus coronopifolius* isolated compounds (0.1 - 100 μM) and 5-FU (0.1 - 500 μM) on HCT-15 cells' viability (% of control) after treatment for 24 hours (A). Dose-response curves of *S. coronopifolius* compounds (0.1 - 100 μM; 24h) and 5-FU (0.1 - 500 μM; 24h) for IC_{50} determination (B). Symbols represent statistically significant differences (Two-way ANOVA, Bonferroni test; *p* - value < 0.05) when compared to: # all compounds; † 12*S*-hydroxy-bromosphaerol; ‡ bromosphaerol; § sphaerodactylomelol; * 5-FU. MTT assay was used to evaluate the compounds effects and the values represent mean ± SEM of at least three independent experiments carried out in triplicate.

- The effects on SH-SY5Y cells' viability induced by *S. coronopifolius* isolated compounds and by cisplatin are shown in Figure 4.7.

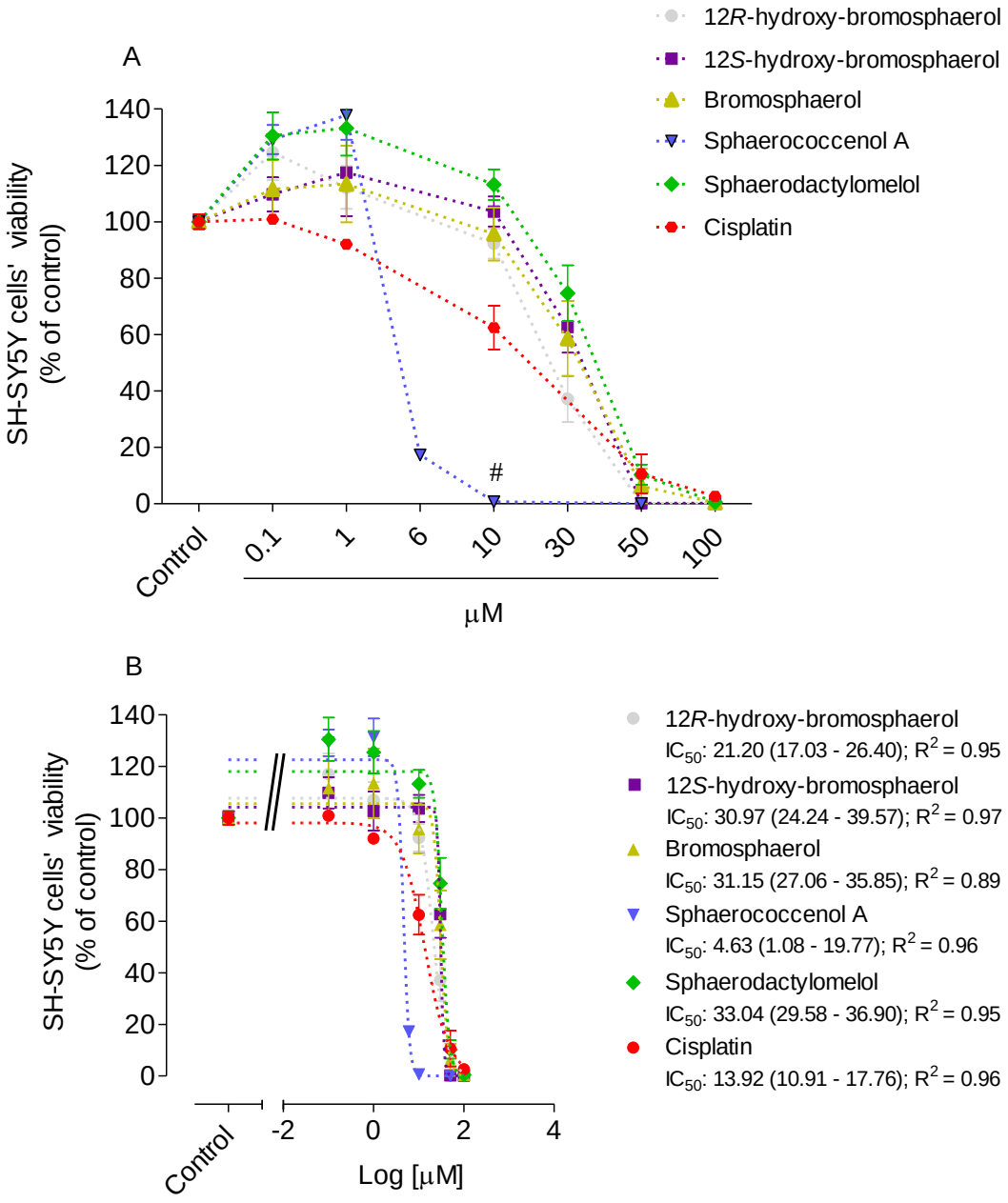


Figure 4.7. Cytotoxic effects of *Sphaerococcus coronopifolius* isolated compounds (0.1 - 100 μM) and cisplatin (0.1 - 100 μM) on SH-SY5Y cells' viability (% of control) after treatment for 24 hours (A). Dose-response curves of *S. coronopifolius* compounds (0.1 - 100 μM; 24h) and cisplatin (0.1 - 100 μM; 24h) for IC_{50} determination (B). Symbols represent statistically significant differences (Two-way ANOVA, Bonferroni test; *p* - value < 0.05) when compared to: # all compounds. MTT assay was used to evaluate the compounds effects and the values represent mean ± SEM of at least three independent experiments carried out in triplicate.

The results showed that the effects on SH-SY5Y cells' viability mediated by the compounds were also concentration-dependent (Figure 4.7). Comparing concentrations of all compounds it is possible to observe that only 10 µM sphaerococcenol A was significantly more cytotoxic towards SH-SY5Y than all other compounds tested (Two-way ANOVA, Bonferroni test; *p* - value < 0.05) (Figure 4.7A). In addition, dose-response study (Figure 4.7B) showed that sphaerococcenol A has the smallest IC_{50} (4.63 µM) followed by 12*R*-hydroxy-bromosphaerol (21.20 µM), 12*S*-hydroxy-bromosphaerol (30.97 µM), bromosphaerol (31.15 µM), and sphaerodactylomelol (33.04 µM). After sphaerococcenol A, the anticancer drug cisplatin exhibited the smallest IC_{50} (13.92 µM).

- The effects of *S. coronopifolius* isolated compounds and cisplatin on A549 cells' viability are presented in Figure 4.8.

As illustrated in Figure 4.8, both *S. coronopifolius* isolated compounds and cisplatin reduced A549 cells' viability in a concentration-dependent manner. When tested at a concentration of 50 µM, 12*R*-hydroxy-bromosphaerol, 12*S*-hydroxy-bromosphaerol, and sphaerococcenol A showed statistically significant differences, when compared to bromosphaerol, sphaerodactylomelol, and cisplatin (Two-way ANOVA, Bonferroni test; *p* - value < 0.05) (Figure 4.8A). Increasing the concentration of the isolated compounds to 100 µM, all compounds induced stronger effects significantly different from cisplatin. Additionally, 12*R*-hydroxy-bromosphaerol and 12*S*-hydroxy-bromosphaerol displayed significant differences compared with bromosphaerol and sphaerodactylomelol (Two-way ANOVA, Bonferroni test; *p* - value < 0.05). Sphaerococcenol A only demonstrated significant differences when compared to sphaerodactylomelol (Two-way ANOVA, Bonferroni test; *p* - value < 0.05). Concerning the dose-response curves (Figure 4.8B), the smallest IC_{50} was exhibited by sphaerococcenol A (11.29 µM). The second more potent compound was 12*R*-hydroxy-bromosphaerol (29.12 µM) followed by 12*S*-hydroxy-bromosphaerol (29.35 µM), bromosphaerol (46.12 µM), and sphaerodactylomelol (71.99 µM). Cisplatin displayed the largest IC_{50} value (271.1 µM).

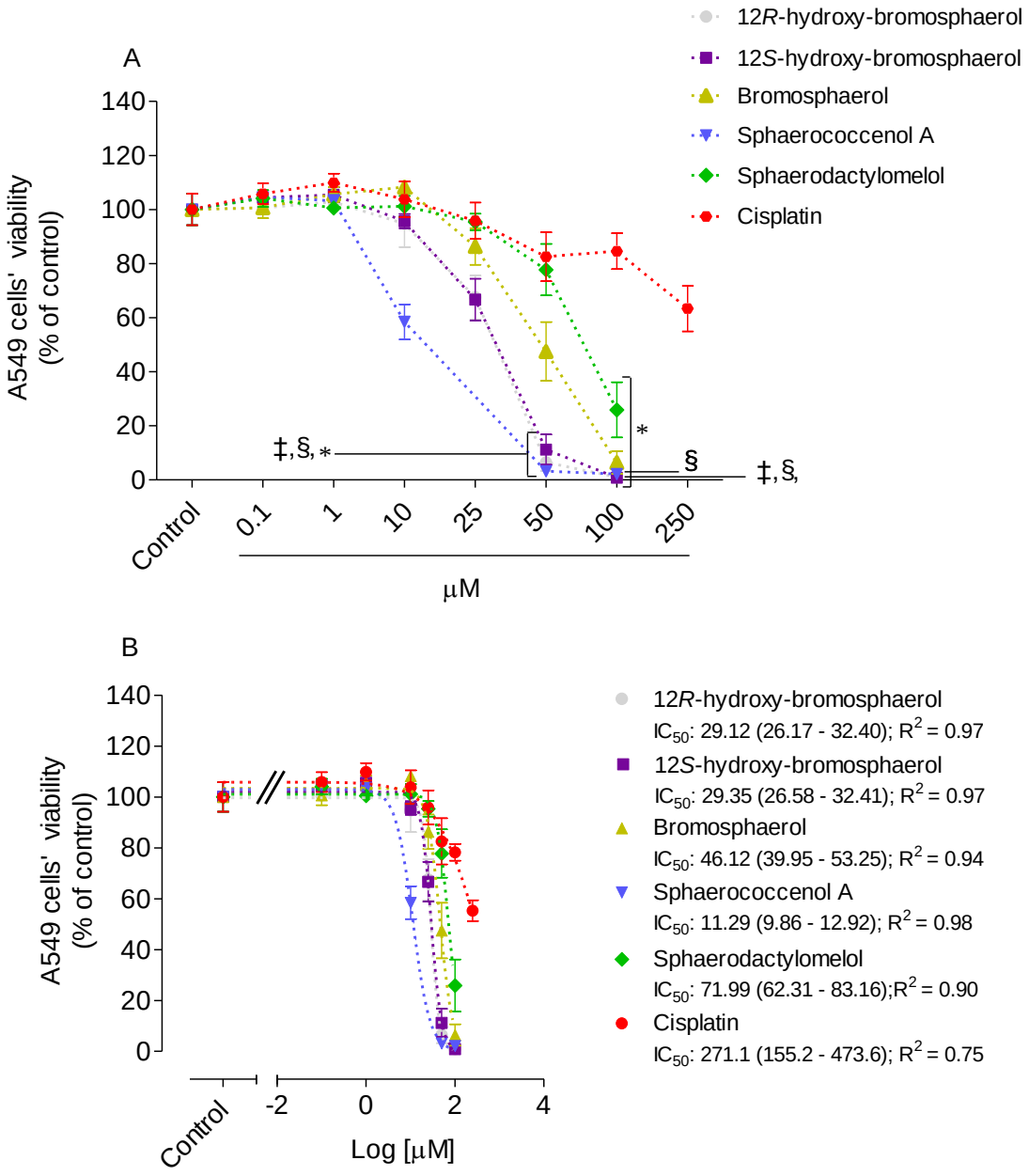


Figure 4.8. Cytotoxic effects of *Sphaerococcus coronopifolius* isolated compounds (0.1 - 100 µM) and cisplatin (0.1 - 250 µM) on A549 cells' viability (% of control) after treatment for 24 hours (A). Dose-response curves of *S. coronopifolius* compounds (0.1 - 100 µM; 24h) and cisplatin (0.1 - 250 µM; 24h) for IC_{50} determination (B). Symbols represent statistically significant differences (Two-way ANOVA, Bonferroni test; *p* - value < 0.05) when compared to: ‡ bromosphaerol; § sphaerodactylomelol; * cisplatin. MTT assay was used to evaluate the compounds effects and the values represent mean ± SEM of at least three independent experiments carried out in triplicate.

- The effects on NCI-H226 cells' viability induced by *S. coronopifolius* isolated compounds and by cisplatin are presented in Figure 4.9.

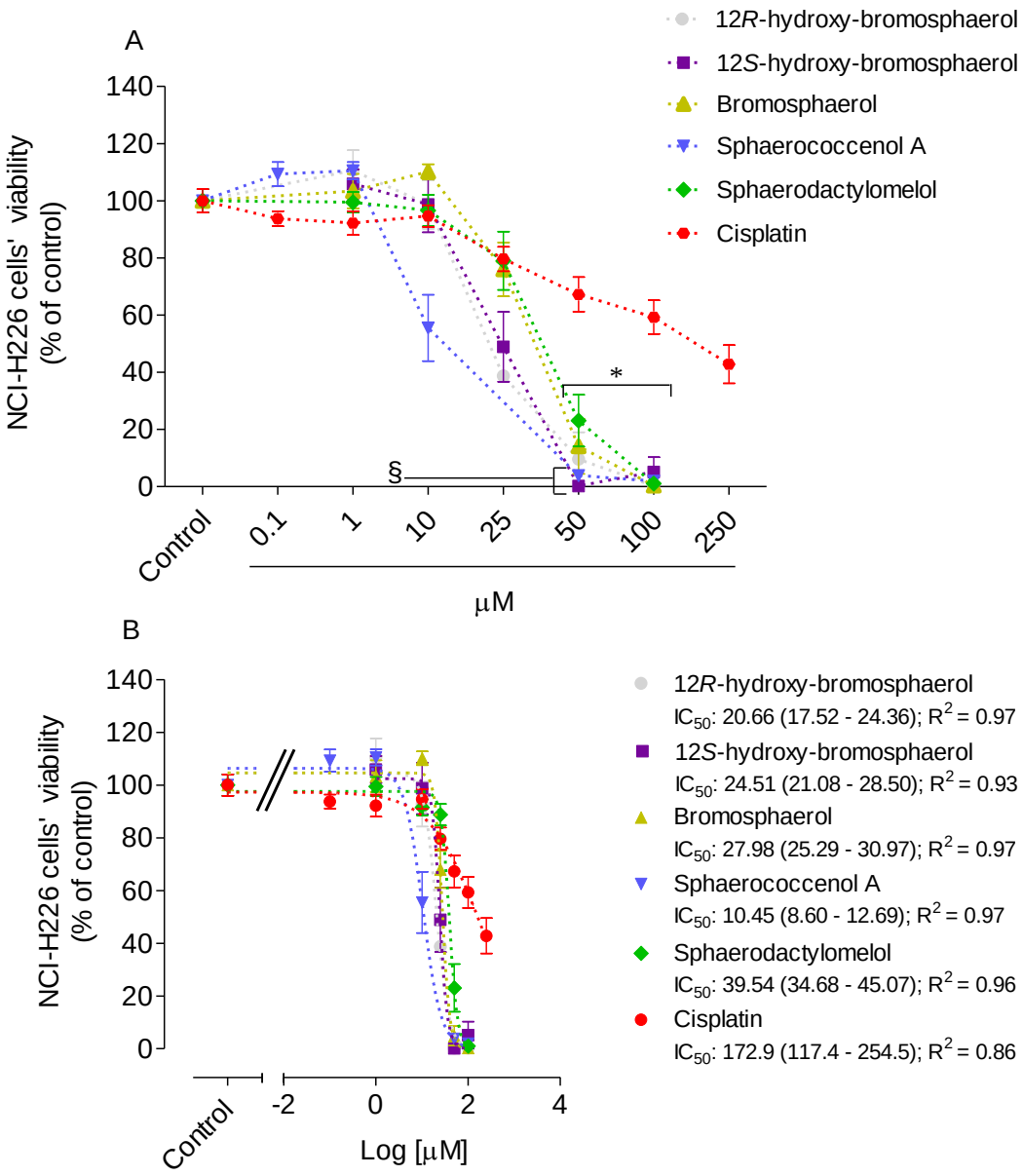


Figure 4.9. Cytotoxic effects of *Sphaerococcus coronopifolius* isolated compounds (0.1 - 100 µM) and cisplatin (0.1 - 250 µM) on NCI-H226 cells' viability (% of control) after treatment for 24 hours (A). Dose-response curves of *S. coronopifolius* compounds (0.1 - 100 µM; 24h) and cisplatin (0.1 - 250 µM; 24h) for IC_{50} determination (B). Symbols represent statistically significant differences (Two-way ANOVA, Bonferroni test; *p* - value < 0.05) when compared to: § sphaerodactylomelol; * cisplatin. MTT assay was used to evaluate the compounds effects and the values represent mean ± SEM of at least three independent experiments carried out in triplicate.

The results obtained revealed that the isolated compounds and cisplatin effects on NCI-H226 cells' viability were concentration dependent (Figure 4.9). At 50 µM, both sphaerococcenol A and 12*S*-hydroxy-bromosphaerol significantly decreased cells' viability, when compared to sphaerodactylomelol (Two-way ANOVA, Bonferroni test; *p* - value < 0.05) (Figure 4.9A). In addition, all compounds (50 and 100 µM) were more efficient at decreasing the cells' viability when compared to cisplatin (Two-way ANOVA, Bonferroni test; *p* - value < 0.05). The analysis of dose-response curves (Figure 4.9B), demonstrated that sphaerococcenol A (10.45 µM) had the highest efficacy followed by 12*R*-hydroxy-bromosphaerol (20.66 µM), 12*S*-hydroxy-bromosphaerol (24.51 µM), bromosphaerol (27.98 µM), and sphaerodactylomelol (39.54 µM). Cisplatin exhibited the biggest IC_{50} (172.9 µM).

- The effects on SK-MEL-28 cells' viability induced by *S. coronopifolius* isolated compounds and by cisplatin are illustrated in Figure 4.10.

According to the results, the decreased SK-MEL-28 cells' viability induced by all compounds tested was concentration dependent (Figure 4.10). Accordingly, 10 µM sphaerococcenol A exhibited a strong cytotoxic activity as compared with the remaining compounds including cisplatin (Figure 4.10A). In turn, 12*R*-hydroxy-bromosphaerol tested at a concentration of 25 µM was far more efficient at reducing the cells' viability than bromosphaerol, sphaerodactylomelol and cisplatin (Two-way ANOVA, Bonferroni test; *p* - value < 0.05). In addition, similar results were verified for sphaerococcenol A and 12*R*-hydroxy-bromosphaerol, when tested at 50 µM. On the other hand, all compounds, with the exception of sphaerococcenol A, were more efficient as cytotoxic agents than cisplatin at a concentration of 100 µM (Two-way ANOVA, Bonferroni test; *p* - value < 0.05). Dose-response curves (Figure 4.10B) revealed that sphaerococcenol A (4.47 µM) exhibited the smallest IC_{50} followed by 12*S*-hydroxy-bromosphaerol (24.47 µM), 12*R*-hydroxy-bromosphaerol (25.11 µM), sphaerodactylomelol (40.51 µM), and bromosphaerol (43.86 µM). Cisplatin exhibited an IC_{50} of 51.52 µM.

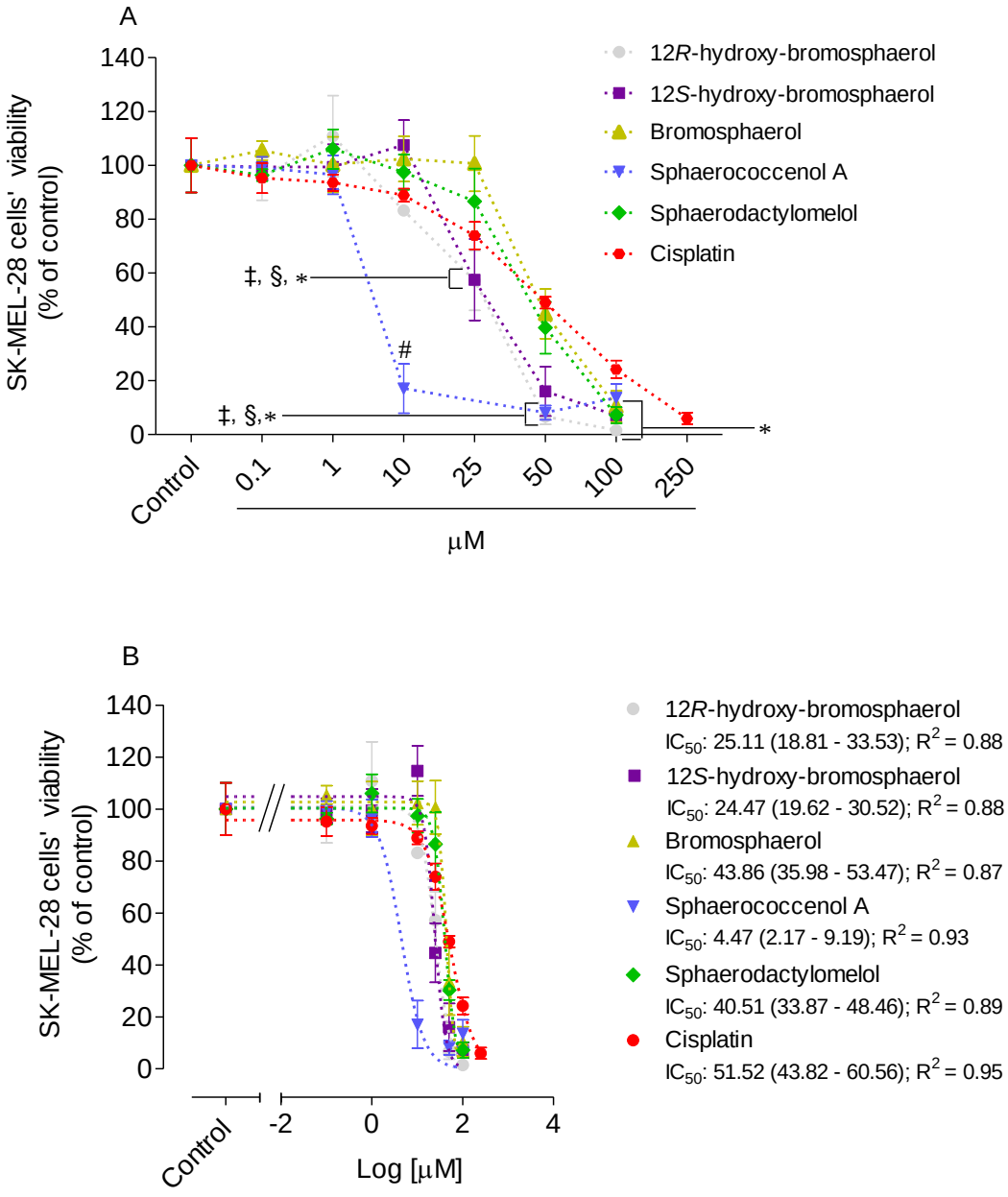


Figure 4.10. Cytotoxic effects of *Sphaerococcus coronopifolius* isolated compounds (0.1 - 100 µM) and cisplatin (0.1 - 250 µM) on SK-MEL-28 cells' viability (% of control) after treatment for 24 hours (A). Dose-response curves of *S. coronopifolius* compounds (0.1 - 100 µM; 24h) and cisplatin (0.1 - 250 µM; 24h) for IC_{50} determination (B). Symbols represent statistically significant differences (Two-way ANOVA, Bonferroni test; *p* - value < 0.05) when compared to: # all compounds; ‡ bromosphaerol; § sphaerodactylomelol; ∗ cisplatin. MTT assay was used to evaluate the compounds effects and the values represent mean ± SEM of at least three independent experiments carried out in triplicate.

- The effects on PC-3 cells' viability induced by *S. coronopifolius* isolated compounds and by cisplatin are presented in Figure 4.11.

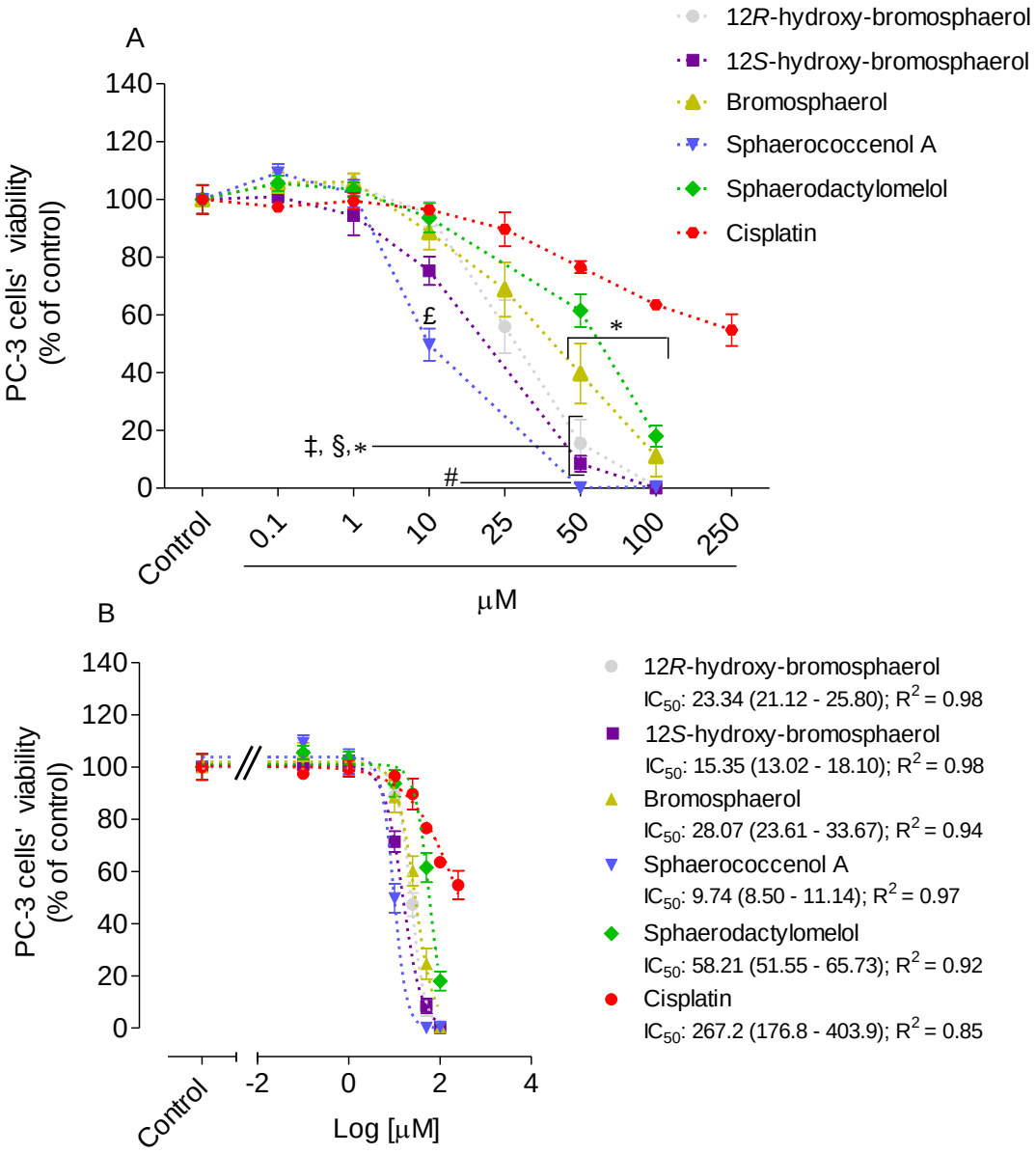


Figure 4.11. Cytotoxic effects of *Sphaerococcus coronopifolius* isolated compounds (0.1 - 100 µM) and cisplatin (0.1 - 250 µM) on PC-3 cells' viability (% of control) after treatment for 24 hours (A). Dose-response curves of *S. coronopifolius* compounds (0.1 - 100 µM; 24h) and cisplatin (0.1 - 250 µM; 24h) for IC_{50} determination (B). Symbols represent statistically significant differences (Two-way ANOVA, Bonferroni test; *p* - value < 0.05) when compared to: # All compounds; £ 12*R*-hydroxy-bromosphaerol; ‡ bromosphaerol; § sphaerodactylomelol; * cisplatin. MTT assay was used to evaluate the compounds effects and the values represent mean ± SEM of at least three independent experiments carried out in triplicate.

From the results presented it was possible to observe that the effects induced on PC-3 cells by *S. coronopifolius* isolated compounds and cisplatin were concentration dependent (Figure 4.11). Ten µM sphaerococcenol A was significantly more effective at decreasing the cells' viability than 12*R*-hydroxy-bromosphaerol. When at a concentration of 50 µM, it displayed the highest cytotoxic activity, comparing with all the other compounds (Two-way ANOVA, Bonferroni test; *p* - value < 0.05) (Figure 4.11A). In the treatment of PC-3 cells with 50 µM of 12*R*-hydroxy-bromosphaerol and 12*S*-hydroxy-bromosphaerol it is possible to observe that those compounds revealed to be more cytotoxic than bromosphaerol, sphaerodactylomelol, and cisplatin. Furthermore, with the exception of sphaerodactylomelol (50 µM), all compounds were statistically more efficient than either 50 or 100 µM cisplatin (Two-way ANOVA, Bonferroni test; *p* - value < 0.05). Regarding the analysis of dose–response curves (Figure 4.11B) sphaerococcenol A was the most efficient exhibiting an IC_{50} of 9.74 µM, followed by 12*S*-hydroxy-bromosphaerol (15.35 µM), 12*R*-hydroxy-bromosphaerol (23.34 µM), bromosphaerol (28.07 µM), and sphaerodactylomelol (58.21 µM). Cisplatin showed the largest IC_{50} with a value of 267.2 µM.

- The effects on 3T3 cells' viability induced by *S. coronopifolius* isolated compounds and by cisplatin, tamoxifen and 5-FU are presented in Figure 4.12.

The results demonstrated that the compounds isolated from *S. coronopifolius* reduced 3T3 cells' viability in a concentration-dependent manner (Figure 4.12). Ten µM sphaeracoccenol A reduced the viability of the 3T3 cells significantly as compared to all the other compounds tested. (Two-way ANOVA, Bonferroni test; *p* - value < 0.05) (Figure 4.12A). Both 10 µM 12*R*-hydroxy-bromosphaerol and 5-FU were more cytotoxic than all the other compounds but sphaerococcenol A. At 25 µM the highest cytotoxic activity was induced by 12*R*-hydroxy-bromosphaerol and tamoxifen. In addition, when tested at 50 and 100 µM, all compounds isolated from *S. coronopifolius* and tamoxifen were significantly more efficient at reducing the cells' viability than cisplatin and 5-FU, with the exception of bromosphaerol (50 µM) (Two-way ANOVA, Bonferroni test; *p* - value < 0.05). It is also possible to observe that 250 µM cisplatin was more cytotoxic than 5-FU when tested at 250 µM. Regarding dose-response studies (Figure 4.12B), sphaerococcenol A displayed the smallest IC_{50} (7.04 µM), followed by 12*R*-hydroxy-bromosphaerol (12.12 µM), 12*S*-hydroxy-bromosphaerol (30.54 µM), sphaerodactylomelol (29.14 µM), and bromosphaerol (51.94 µM). On the other side, the higher values of IC_{50} were verified in the treatments accomplished with the standard drugs cisplatin (137.0 µM) and 5-FU (344.7 µM).

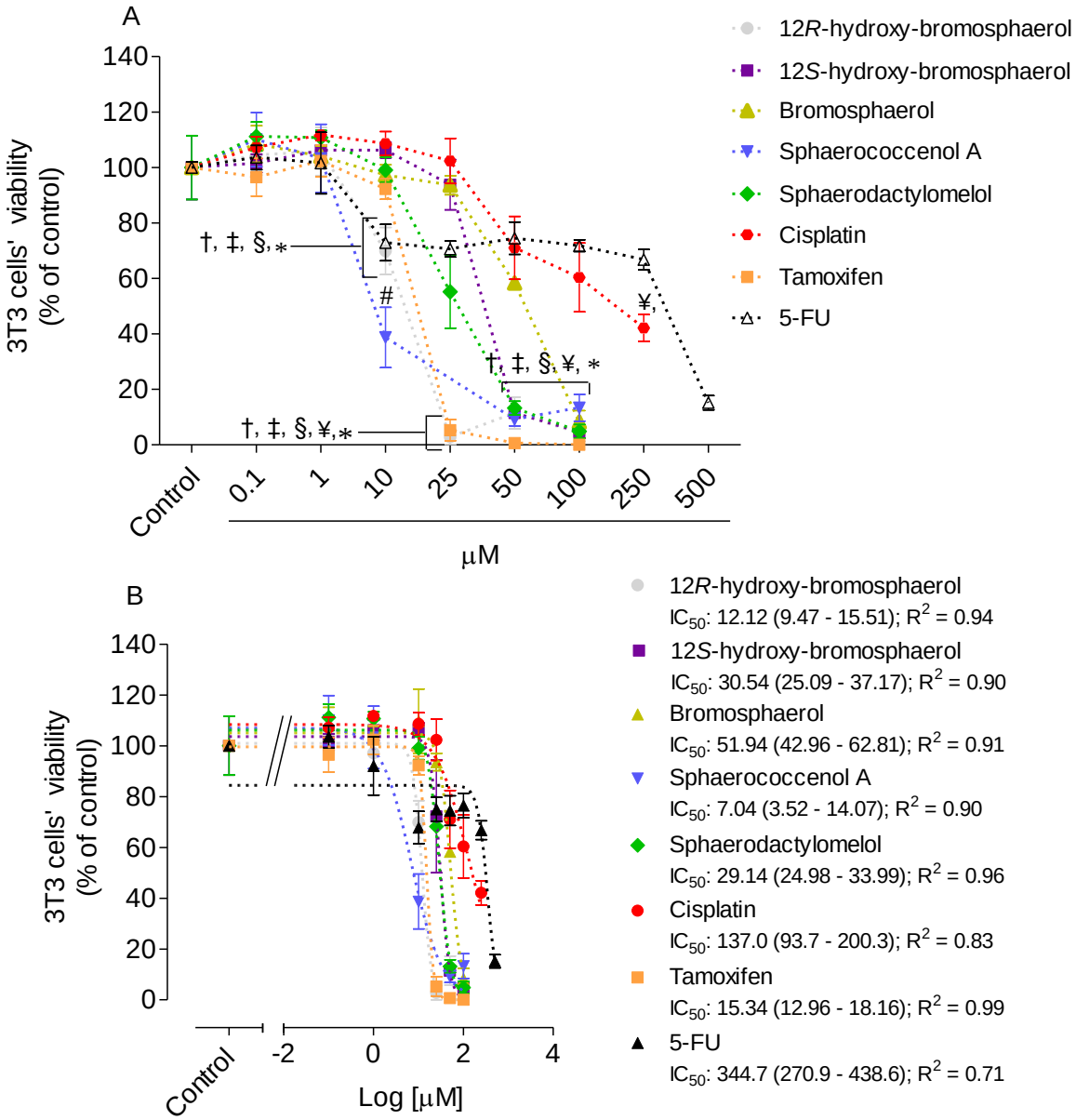


Figure 4.12. Cytotoxic effects of *Sphaerococcus coronopifolius* isolated compounds (0.1 - 100 µM) and cisplatin, tamoxifen and 5-FU (0.1 - 500 µM) on 3T3 cells' viability (% of control) after treatment for 24 hours (A). Dose-response curves of *S. coronopifolius* compounds (0.1 - 100 µM; 24h) and standard drugs cisplatin, tamoxifen and 5-FU (0.1 - 500 µM; 24h) for IC_{50} determination (B). Symbols represent statistically significant differences (Two-way ANOVA, Bonferroni test; *p* - value < 0.05) when compared to: # All compounds; † 12*S*-hydroxy-bromosphaerol; ‡ bromosphaerol; § sphaerodactylomelol; ∗ cisplatin; ¥ 5-FU. MTT assay was used to evaluate the compounds effects and the values represent mean ± SEM of at least three independent experiments carried out in triplicate.

The IC_{50} values of *Sphaerococcus coronopifolius* isolated compounds and standard anticancer drugs on the studied cell lines are summarized in Table 4.1.

Table 4.1. Values of IC_{50} (μM) determined for *Sphaerococcus coronopifolius* compounds (0.1-100 μM) and standard drugs (0.1-500 μM) on cell line models after treatment for 24 hours. The values in parentheses represent the confidence intervals for 95%.

		A549	CACO-2	HCT-15	MCF-7	NCI-H226	PC-3	SH-SY5Y	SK-MEL-28	3T3
Compounds	12*R*-hydroxy-bromosphaerol	29.12 (26.17-32.40)	33.78 (27.98-40.77)	25.08 (22.78-27.62)	18.28 (14.84-22.51)	20.66 (17.52-24.36)	23.34 (21.12-25.80)	21.20 (17.03-26.40)	25.11 (18.81-33.53)	12.12 (9.47 – 15.51)
	12*S*-hydroxy-bromosphaerol	29.35 (26.58-32.41)	53.34 (42.17-67.71)	27.54 (24.61-30.82)	19.06 (14.83-24.51)	24.51 (21.08-28.50)	15.35 (13.02-18.10)	30.97 (24.24-39.57)	24.47 (19.62-30.52)	30.54 (25.09-37.17)
	Bromosphaerol	46.12 (39.95-53.25)	50.37 (36.12-70.26)	24.55 (17.56-34.32)	27.76 (19.40-39.72)	27.98 (25.29-30.97)	28.07 (23.61-33.67)	31.15 (27.06-35.85)	43.86 (35.98-53.47)	51.94 (42.96 – 62.81)
	Sphaerococcenol A	11.29 (9.86-12.92)	16.59 (10.06-27.34)	7.11 (4.67-10.84)	9.40 (5.24-16.87)	10.45 (8.60-12.69)	9.74 (8.50-11.14)	4.63 (1.08-19.77)	4.47 (2.17-9.19)	7.04 (3.52 – 14.07)
	Sphaerodactylomelol	71.99 (62.31-83.16)	89.41 (54.85-145.7)	46.25 (40.37-52.98)	47.19 (36.95-60.26)	39.54 (34.68-45.07)	58.21 (51.55-65.73)	33.04 (29.58-36.90)	40.51 (33.87-48.46)	29.14 (24.98 – 33.99)
Drugs	Cisplatin	271.1 (155.2-473.6)	-	-	-	172.9 (117.4-254.5)	267.2 (176.8-403.9)	13.92 (10.91-17.76)	51.52 (43.82-60.56)	137.0 (93.7-200.3)
	Tamoxifen	-	-	-	27.19 (22.62-32.68)	-	-	-	-	15.34 (12.96-18.16)
	5-Fluorouracil	-	382.7 (247.2-592.3)	155.5 (103.1-234.5)	-	-	-	-	-	344.7 (270.9-438.6)

In Table 4.1 is possible to observe that *S. coronopifolius* isolated compounds exhibited a range of IC_{50} values between 4.47 to 89.41 μM. Amongst all compounds, sphaerococcenol A demonstrated to be the compound with highest cytotoxicity exhibiting IC_{50} values between 4.47 to 16.59 μM. 12*R*-hydroxy-bromosphaerol, 12*S*-hydroxy-bromosphaerol, and bromosphaerol were found to be moderately cytotoxic, displaying an IC_{50} range between 12.12 μM and 53.34 μM. On the other hand, the new compound sphaerodactylomelol demonstrated the lowest cytotoxicity with IC_{50} values between 29.14 and 89.41 μM. Concerning the cytotoxic effects induced by the common antineoplastic

drugs, it is possible to verify that they were less effective than *S. coronopifolius* compounds under similar experimental conditions. The exceptions were the effects observed on MCF-7, SH-SY5Y, and SK-MEL-28 cells, which were similar to the effects induced by *S. coronopifolius* isolated compounds. Tamoxifen exhibited an IC_{50} of 27.19 µM on MCF-7 cells and 5-FU an IC_{50} of 382.7 and 155.5 on CACO-2 and HCT-15 cells, respectively. In turn, cisplatin displayed an IC_{50} range between 13.92 and 271.1 µM. Overall, the largest IC_{50} values were observed on CACO-2 cells, the cell line with higher resistance to all the tested compounds. In addition, it was possible to observe that *S. coronopifolius* isolated compounds do not selectively target cancer cells.

Amongst cancers, tumors related to breast tissues are one of those with highest number of estimated new cases and deaths (Malvezzi *et al.*, 2017; 2018). Therefore, according to the results attained, in order to proceed with the studies linked with intracellular signaling pathways, the MCF-7 cell line, derived from breast tumor tissue, was selected to the subsquent *in vitro* studies.

4.3.2. Time-course effects of compounds on MCF-7 cells' viability

In order to evaluate the time- and concentration-dependent effects of *S. coronopifolius* isolated compounds on MCF-7 cells' viability, time-course experiments were performed exposing those cells to a range of the compounds' concentrations (0.1-100 µM) for 24, 48, and 72 hours. Results are presented in Figure 4.13 to 4.17.

- The time-course effects of 12*R*-hydroxy-bromosphaerol (0.1 - 100 µM) on MCF-7 cells' viability after treatment for 24, 48, and 72 hours are presented in Figure 4.13.

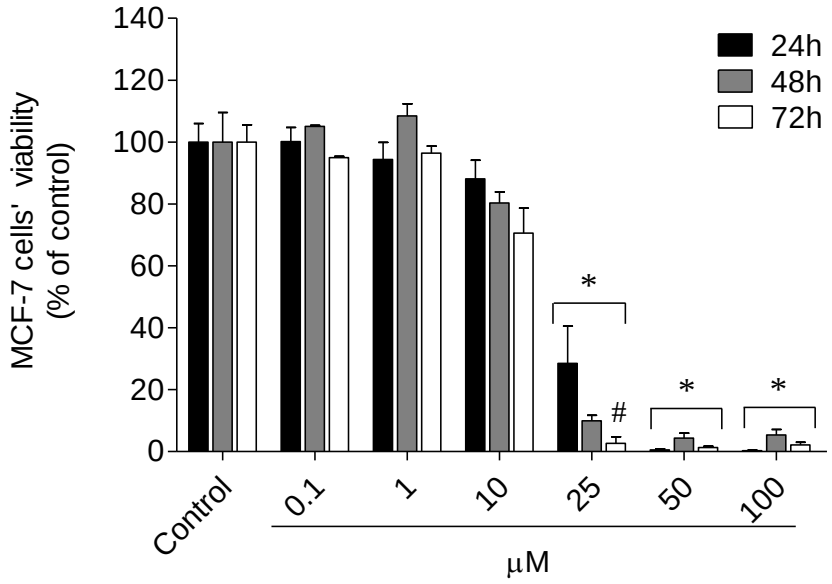


Figure 4.13. Time course-effects of 12*R*-hydroxy-bromosphaerol on MCF-7 cells' viability (0.1 - 100 μM) after treatment for 24, 48 and 72 hours. MTT assay was used to evaluate the effect of the compound and the values represent mean ± SEM of at least three independent experiments carried out in triplicate. Symbols represent statistically significant differences (ANOVA, Tukey test; *p* - value < 0.05) when compared to: *control of respective time; # 24 hours of respective concentration.

The results revealed a concentration dependent decrease on MCF-7 cells' viability ($R^2_{24h} = 0.93$; $R^2_{48h} = 0.99$; $R^2_{72h} = 0.99$), following exposure to 12*R*-hydroxy-bromosphaerol (Figure 4.13). When tested at 25 μM it is possible to observe that after 72 hours, 12*R*-hydroxy-bromosphaerol potentiated its effects above MCF-7 cells as compared to 24 hours treatment, leading to a decrease of their viability. The effects of this compound were not time-dependent.

- The time-course effects of 12*S*-hydroxy-bromosphaerol (0.1 - 100 μM) on MCF-7 cells' viability along 72 hours exposure are illustrated in Figure 4.14

It is possible to observe that 12*S*-hydroxy-bromosphaerol exhibited a concentration-dependent effect on MCF-7 cells' viability, after treatment for 24h ($R^2_{24h} = 0.92$), 48h ($R^2_{48h} = 0.98$), and 72h ($R^2_{72h} = 0.97$) (Figure 4.14). The effects of this compound were not time-dependent.

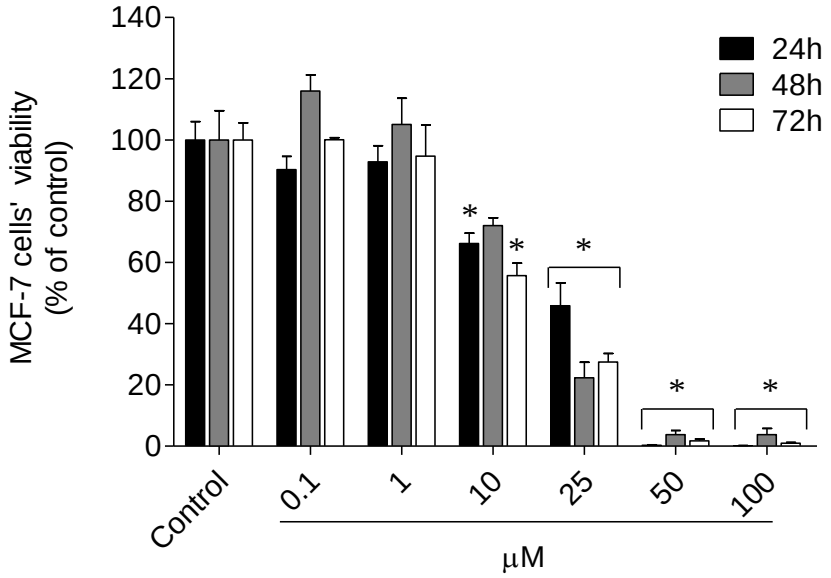


Figure 4.14. Time course-effects of 12*S*-hydroxy-bromosphaerol on MCF-7 cells' viability (0.1 - 100 μM) after treatment for 24, 48 and 72 hours. MTT assay was used to evaluate the effect of the compound and the values represent mean ± SEM of at least three independent experiments carried out in triplicate. Symbols represent statistically significant differences (ANOVA, Tukey test; *p* - value < 0.05) when compared to: *control of respective time.

- The time-course effects of bromosphaerol (0.1 - 100 μM) on MCF-7 cells' viability after 24, 48, and 72 hours of incubation are showed in Figure 4.15.

Through the obtained results (Figure 4.15), it was possible to verify that bromosphaerol displayed a concentration-dependent manner effect ($R^2_{24h} = 0.91$; $R^2_{48h} = 0.94$; $R^2_{72h} = 0.97$) on MCF-7 cells' viability for all times studied. However, when tested at a concentration of 25 μM, bromosphaerol showed to lose activity, after 48 and 72 hours of treatment. On the other side, the effects mediated by this compound did not show time-dependence.

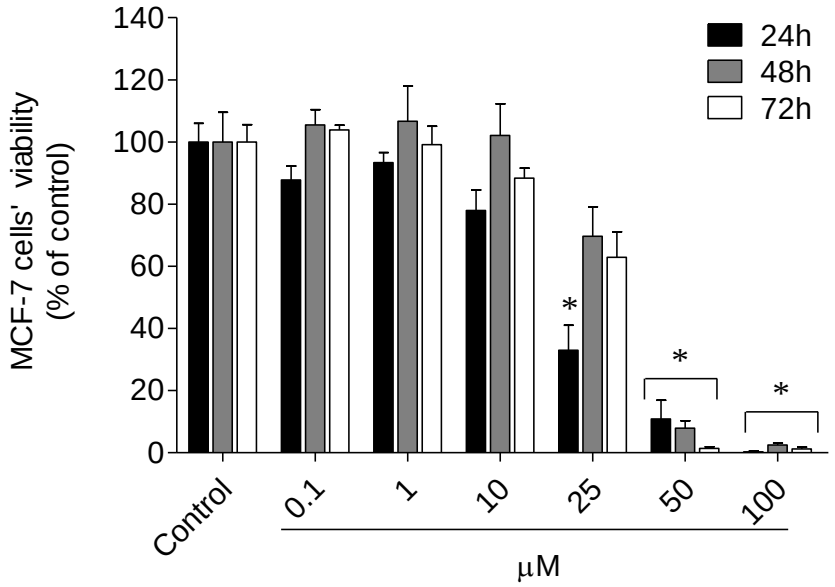


Figure 4.15. Time course-effects of bromosphaerol on MCF-7 cells' viability (0.1 - 100 μM) after treatment for 24, 48 and 72 hours. MTT assay was used to evaluate the effect of the compound and the values represent mean ± SEM of at least three independent experiments carried out in triplicate. Symbols represent statistically significant differences (ANOVA, Tukey test; *p* - value < 0.05) when compared to: *control of respective time.

- The time-course effects induced by sphaerococcenol A (0.1 - 100 μM) on MCF-7 cells' viability after 24, 48, and 72 hours of incubation are displayed in Figure 4.16.

Concerning the achieved results (Figure 4.16), it was possible to verify that sphaeroccocenol A reduces MCF-7 cells' viability in a concentration-dependent manner after incubation for 24h ($R^2_{24h} = 0.94$), 48h ($R^2_{48h} = 0.99$), and 72h ($R^2_{72h} = 0.99$). When tested at a concentration of 1 μM, only the treatment for 24 hours showed a significant reduction of MCF-7 cells' viability (ANOVA, Tukey test; *p* - value < 0.05). On the other side, when tested at 10 μM it is possible to observe that after 48 and 72 hours, sphaerococcenol A potentiated its effects above MCF-7 cells leading to a decrease of their viability. This compound did not show time-dependence.

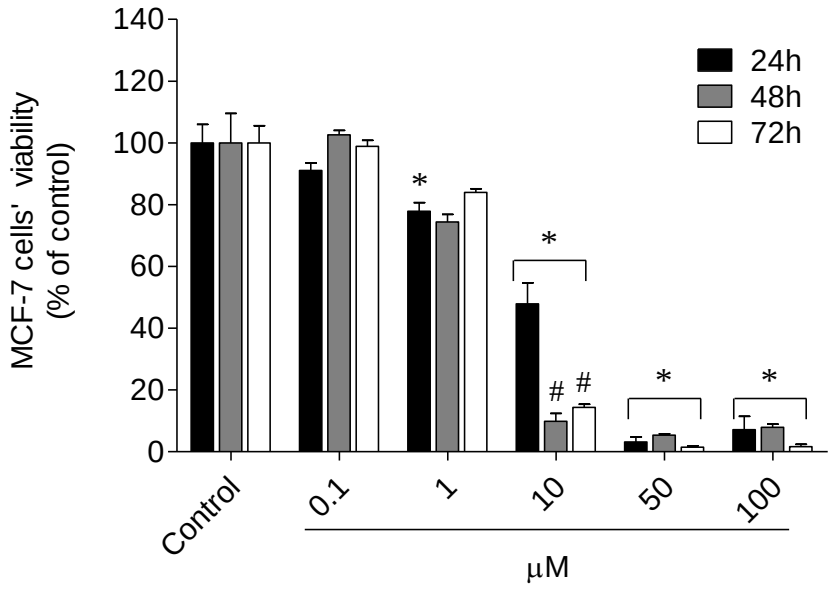


Figure 4.16. Time course-effects of sphaerococcenol A on MCF-7 cells' viability (0.1 - 100 μM) after treatment for 24, 48 and 72 hours. MTT assay was used to evaluate the effect of the compound and the values represent mean ± SEM of at least three independent experiments carried out in triplicate. Symbols represent statistically significant differences (ANOVA, Tukey test; *p* - value < 0.05) when compared to: *control of respective time; # 24 hours of respective concentration.

- The time-course effects mediated by sphaerodactylomelol (0.1 - 100 μM) on MCF-7 cells' viability after 24, 48, and 72 hours of incubation are displayed in Figure 4.17.

Concerning the obtained results (Figure 4.17), it is possible to verify a concentration-dependent effect ($R^2_{24h} = 0.75$; $R^2_{48h} = 0.96$; $R^2_{72h} = 0.96$) mediated by sphaerodactylomelol on MCF-7 cells' viability. On the other hand, the effects induced by this compound on MCF-7 cells' viability did not revealed time-dependence.

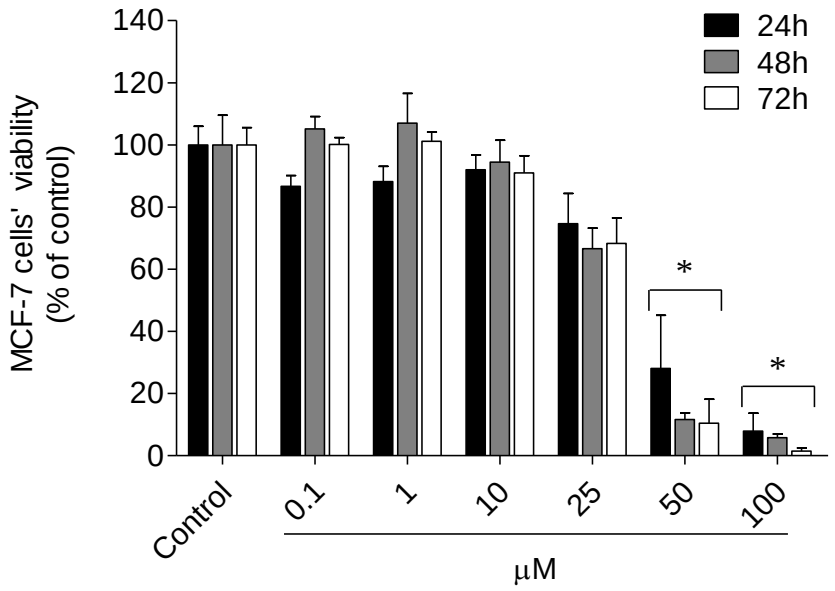


Figure 4.17. Time course-effects of sphaerodactylomelol on MCF-7 cells' viability (0.1 - 100 μM) after treatment for 24, 48 and 72 hours. MTT assay was used to evaluate the effect of the compound and the values represent mean ± SEM of at least three independent experiments carried out in triplicate. Symbols represent statistically significant differences (ANOVA, Tukey test; *p* - value < 0.05) when compared to: *control of respective time.

4.3.3. Cytotoxicity and mitochondrial function

To understand if the effects mediated by the *S. coronopifolius* compounds were linked to the mitochondrial activity, the lactate dehydrogenase (LDH) release was used as an indicator of cell survival and mitochondrial function was assessed by the MTT assay. MCF-7 cells were exposed to the above mentioned compounds (0.1 - 100 μM) for 24 hours. Their effects evaluated by the LDH assay were compared to the effects previously obtained by the MTT assay (0.1 - 100 μM; 24h). Results are presented in Figure 4.18.

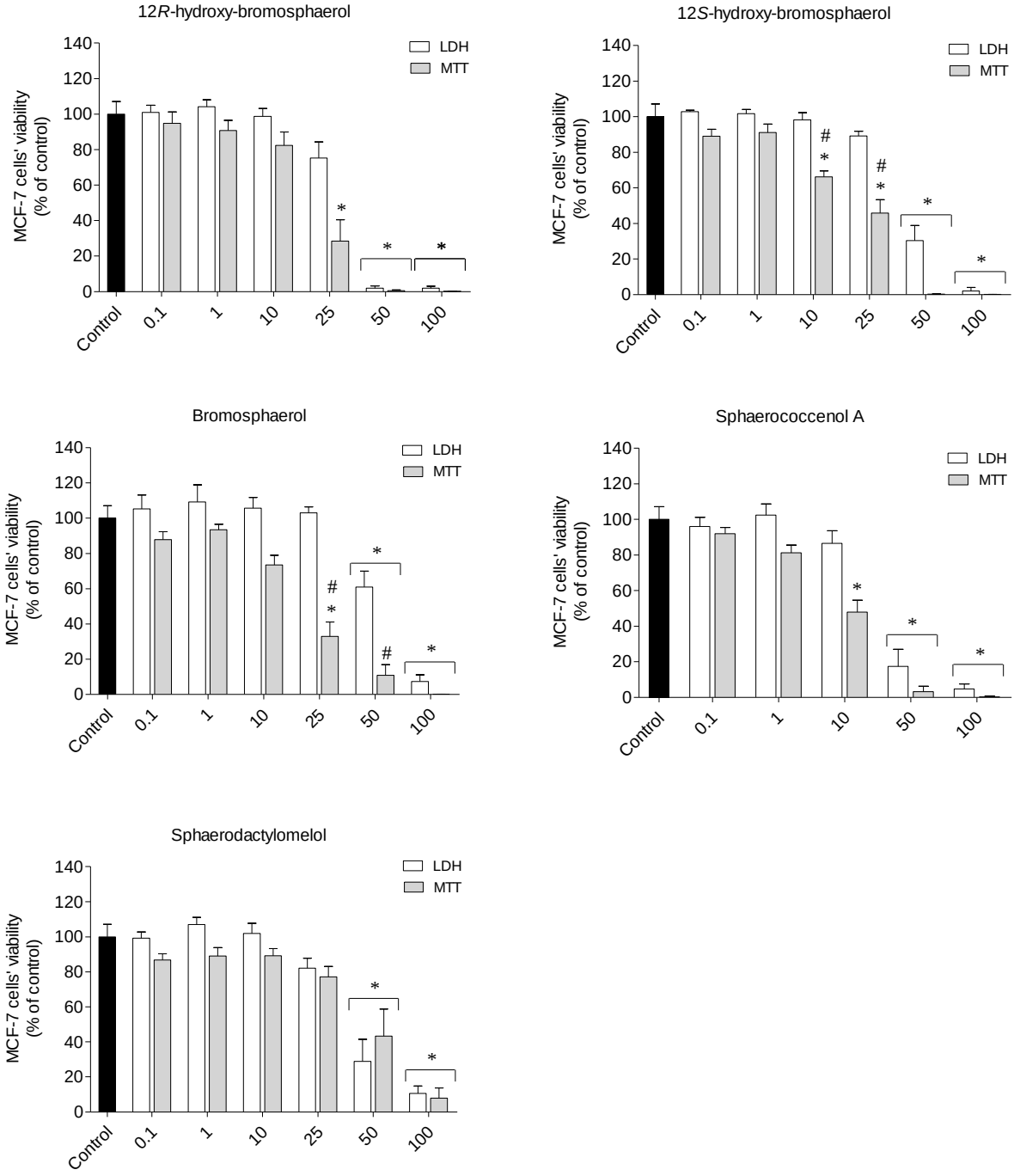


Figure 4.18. Effects of compounds (0.1 - 100 µM) on MCF-7 cells' viability after 24 hours of treatment. MTT and LDH assays were used to evaluate the compounds' effects and the values represent mean ± SEM of at least three independent experiments carried out in triplicate. Symbols represent statistically significant differences (ANOVA, Tukey test; *p* - value < 0.05) when compared to: *control or $^{\#}$ LDH assay.

Through the results expressed in Figure 4.18 is possible to observe that the treatment accomplished with sphaerodactylomelol displayed similar profile when studied by both assays. On the other side, MCF-7 cells, when treated with 12*R*-hydroxy-bromosphaerol and sphaerococcenol A, also exhibited a similar profile by the MTT and LDH assays. However, when tested at 25 and 10 µM, respectively, the effects obtained by the MTT assay showed significant differences comparing with control that were not verified by the LDH assay (ANOVA, Tukey test; p - value < 0.05). Similarly, 12*S*-hydroxy-bromosphaerol and bromosphaerol (25 and 50 µM) also demonstrated to reduce significantly MCF-7 cells' viability, which was not observed by LDH assay. In addition, there are statistical differences between MTT and LDH assays for the treatment with 12*S*-hydroxy-bromosphaerol (10 and 25 µM) and bromosphaerol (25 and 50 µM) (ANOVA, Tukey test; p - value < 0.05).

4.3.4. Hallmarks of oxidative stress – Real-time H_2O_2 production

To understand if the cytotoxic activities mediated by *S. coronopifolius* compounds on MCF-7 cells were associated with oxidative stress, the H_2O_2 levels were quantified. MCF-7 cells were treated with aforementioned compounds (IC_{50}) during 1, 3, and 6 hours, and the H_2O_2 production was determined in real-time. Results are presented in Figure 4.19 and 4.20.

The exposure of MCF-7 cells to compounds isolated from the red alga *S. coronopifolius* led to a significant increase on H_2O_2 levels (ANOVA, Dunnett's test; p - value < 0.05), as compared with control, after 1 and 3 hours (Figure 4.19 and 4.20). Bromosphaerol was the only exception, since it did not induce H_2O_2 generation at all times accomplished. On the other hand, after 6 hours of treatment only sphaerodactylomelol induced H_2O_2 production. The highest H_2O_2 production was induced by sphaerodactylomelol (1h) that duplicated the H_2O_2 levels relative to the control (316.3 ± 31.8% of control). MCF-7 cells exposed to H_2O_2 were used as a positive control.

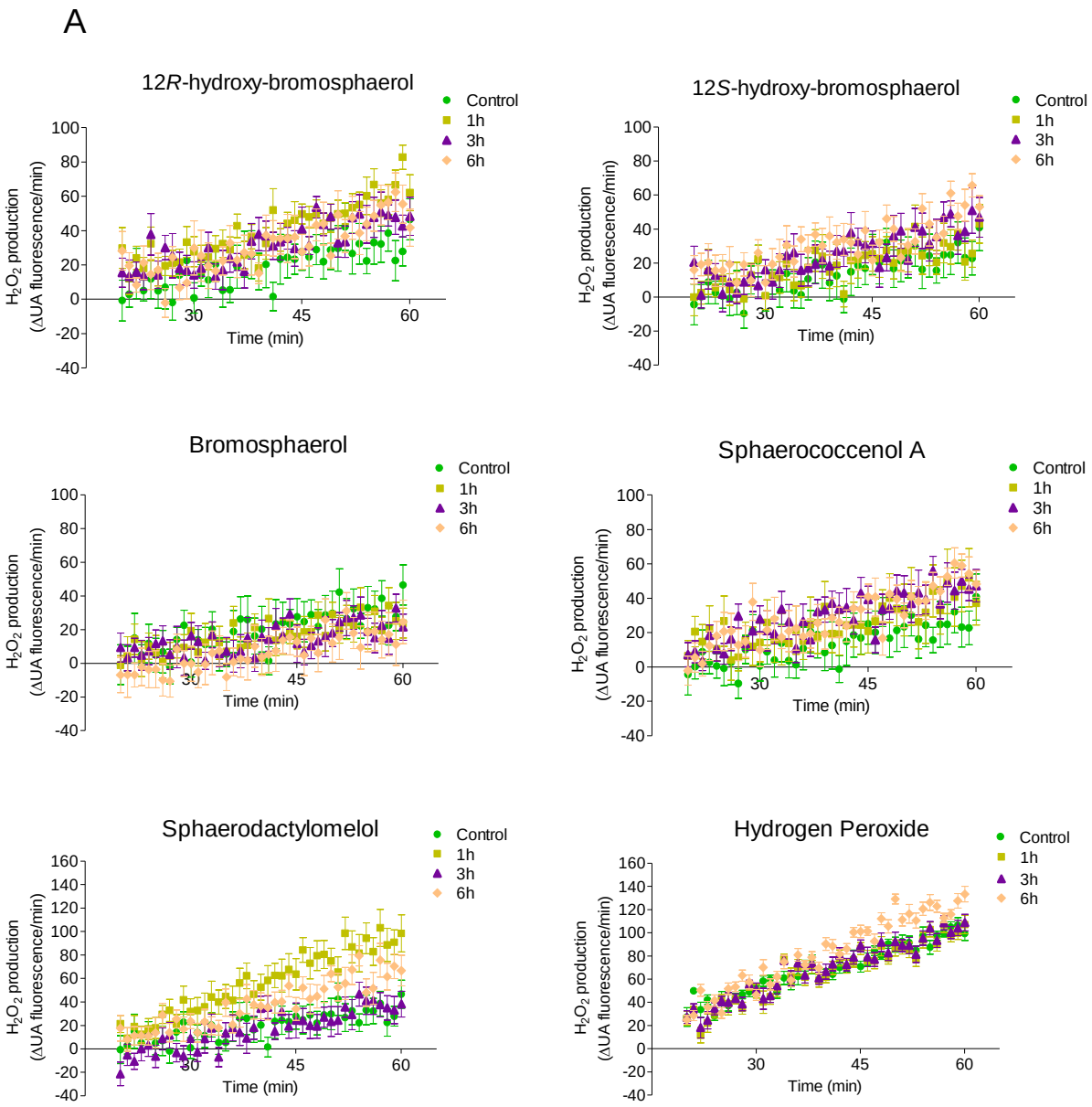


Figure 4.19. Kinetics of hydrogen peroxide (H_2O_2) produced on MCF-7 cells after treatment with *Sphaerococcus coronopifolius* isolated compounds (IC_{50}) and hydrogen peroxide (200 µM) during 1, 3, and 6 hours. The values represent mean ± SEM of at least three independent experiments carried out in triplicate.

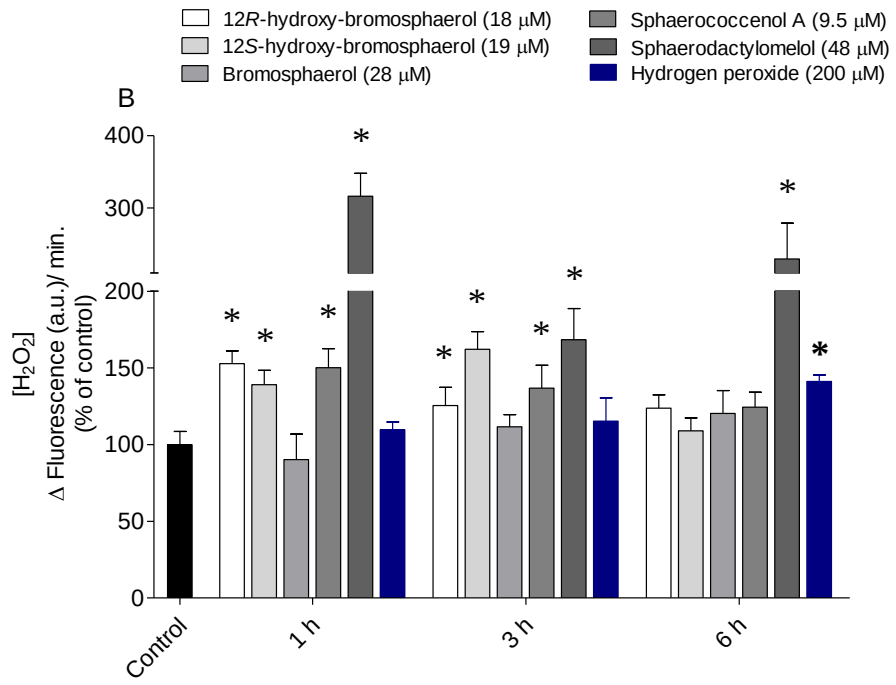


Figure 4.20. Levels of hydrogen peroxide (H_2O_2) produced by MCF-7 cells following 1, 3, and 6 hours of treatment with *Sphaerococcus coronopifolius* compounds (IC_{50}) and H_2O_2 (200 μM). H_2O_2 was quantified fluorimetrically using the "™ Amplex Red Hydrogen Peroxide Assay" kit. H_2O_2 levels were calculated by the slope of the linear phase of fluorescence curve and results were expressed in percentage of control. The values represent mean ± SEM of at least three independent experiments carried out in triplicate. Symbols represent statistically significant differences (ANOVA, Dunnett's test; *p* - value < 0.05) when compared to: *control.

4.3.5. Hallmarks of apoptosis

Several hallmarks of apoptosis, including alterations in mitochondrial membrane potential, translocation of membrane phosphatidylserine, Caspase-9 activity, chromatin condensation, and DNA fragmentation were studied. These studies were performed in order to understand if the effects mediated by the compounds on MCF-7 cells' viability were related with this process. The results are presented in the following sections (4.3.5.1 – 4.3.5.4).

4.3.5.1. Mitochondrial membrane potential (MMP)

Many of the apoptosis key events occur in mitochondria, which plays a central role in this biological process. Amongst them, changes in the inner mitochondrial membrane

permeability leads to the activation of different signals that initiate downstream effects including loss of the MMP (Green and Reed, 1998; Indran *et al.*, 2011). Therefore, the MMP was evaluated following exposure of MCF-7 cells, for 15, 30, and 60 minutes, to the IC_{50} values of the *S. coronopifolius* isolated compounds. Results are expressed as percentage of control (untreated cells) and presented in Figure 4.21.

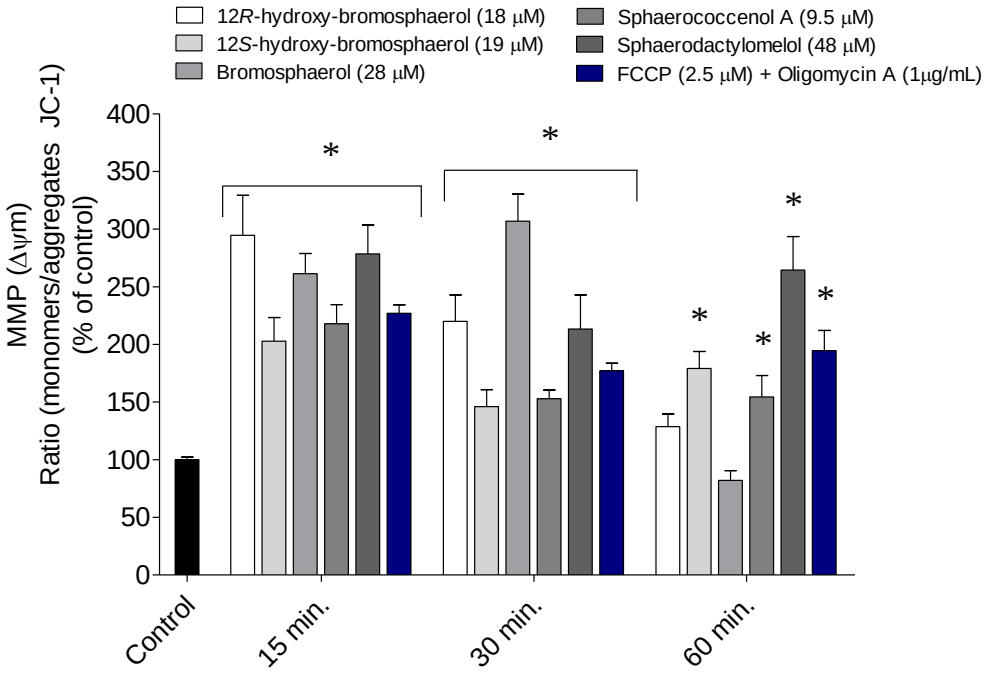


Figure 4.21. Effects on MCF-7 cells mitochondrial membrane potential (MMP) following treatement with *Sphaerococcoccus coronopifolius* compounds (IC_{50}) and FCCP (2.5 µM) plus oligomycin A (1 µg/mL) solution for 15, 30 and 60 minutes. The results were expressed as the ratio between the monomers/ aggregates of JC-1. The values of each column represent the mean ± SEM of 3 or 4 independent experiments. Symbols represent statistically significant differences (ANOVA, Dunnett's test; *p* - value < 0.05) when compared to: * control.

The treatment of MCF-7 cells with isolated compounds induced changes on MMP after 15 and 30 minutes exposure (Figure 4.21). However, after treatment for 60 minutes, only 12*S*-hydroxy-bromosphaerol (179.23 ± 14.6%), sphaerococcenol A (154.44 ± 18.5%), and sphaerodactylomelol (264.55 ± 29.1%) exhibited a significant depolarization of MMP compared to the control (ANOVA, Dunnett's test; *p* - value < 0.05). In addition, 12*R*-hydroxy-bromosphaerol (294.61 ± 37.7%), bromosphaerol (306.9 ± 23.5%), and sphaerodactylomelol (264.55 ± 29.1) mediated the highest depolarization after MCF-7 cells

exposition to the compounds for 15, 30, and 60 minutes, respectively. Furthermore, the FCCP plus oligomycin solution treatment revealed to induce depolarization of MMP on all times tested.

4.3.5.2. Annexin V and propidium iodide (PI) staining

Translocation of membrane phosphatidylserine (PS) from the inner side of the plasma membrane to the surface is one of the earlier events of apoptosis. Cells stained with Annexin V/ PI conjugate allow to distinguish apoptosis from necrosis, since Annexin V has high affinity to PS, and PI has only capacity to enter cells when the integrity of their membranes is compromised (Crowley *et al.*, 2016). Hence, MCF-7 cells were stained with Annexin V/ PI conjugate after exposition to the aforementioned compounds for 24 hours. Results are shown as percentage of events (viable cells, apoptosis, late apoptosis and necrosis) and are presented in Figure 4.22.

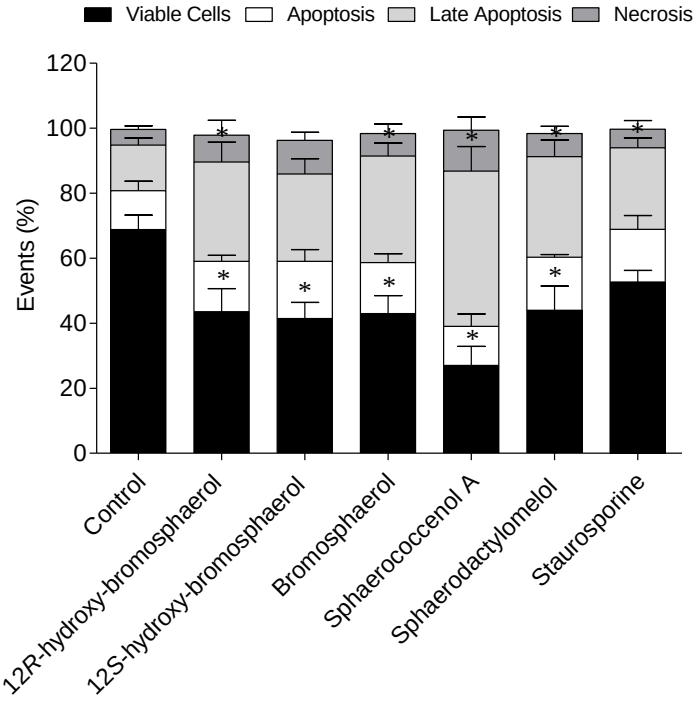


Figure 4.22. MCF-7 cells treated with *Sphaerococcus coronopifolius* compounds (12*R*-hydroxy-bromosphaerol - 18.0 µM; 12*S*-hydroxy-bromosphaerol – 19.0 µM; bromosphaerol – 28.0 µM ; sphaerococcenol A – 9.5 µM; sphaerodactylomelol – 48.0 µM ; staurosporine – 1 µg/mL) during 24h. Cells were then labeled with Annexin V/ PI conjugate and analyzed by flow cytometry. The results are presented as mean ± SEM of four independent experiments (10,000 events). Symbols represent statistically significant differences (Student's *t* test; *p* - value < 0.05) when compared to respective event on the: * control.

It is possible to observe that under control conditions MCF-7 cells were mainly viable or in late apoptosis stage (Figure 4.22). Treatment with sphaerococcenol A demonstrated the highest number of cells in late apoptosis stage exhibiting a value around 48%. In addition, this compound also displayed the smaller number of viable cells (± 27%). As to the other compounds, 12*R*-hydroxy-bromosphaerol, bromosphaerol and sphaerodactylomelol presented significant differences in the percentage of viable cells and cells in late apoptosis stage, when compared to the control (Student's *t* test; *p* - value < 0.05). 12*S*-hydroxy-bromosphaerol only displayed significant differences to control situation in the percentage of viable cells (Student's *t* test; *p* - value < 0.05). As to apoptosis and necrosis levels, there were no differences between treated cells and control (untreated cells). In addition, staurosporine showed significant differences in late apoptosis stage, when compared to control (Student's *t* test; *p* - value < 0.05).

4.3.5.3. Caspase -9 activity

Caspase-9 is involved in the activation of downstream caspases (e.g. Caspase-3, -6 and -7) initiating the caspase cascade, which plays a central role in apoptotic response (Kuida, 2000). Thus, MCF-7 Caspase-9 activity was evaluated following exposure of the cells to the compounds (IC_{50}) for 3, 6, and 9 hours. Results are expressed in percentage of control (untreated cells) and are presented in Figure 4.23.

Analyzing the results (Figure 4.23) it was possible to verify that all compounds induced MCF-7 Caspase-9 activity as compared to control. The highest increase of Caspase-9 activity was mediated by the sphaeroccocenol A after 3 hours (333.97 ± 37.86%) and 6 hours (483.67 ± 52.51%), as well as by sphaerodactylomelol after 6 hours (266.73± 33.26%) and 12*R*-hydroxy-bromosphaerol after 9 hours (372.66 ± 16.8%). On the other hand, bromosphaerol only induced activity of Caspase-9 after the treatment for 6 hours. Staurosporine, used as apoptosis positive control, induced Caspase-9 activity after incubation for 6 hours. The time exposure of MCF-7 cells to sphaerococcenol A, bromosphaerol and 12*R*-hydroxy-bromosphaerol influenced Caspase-9 activity being possible to observe a significant increase between the two times tested.

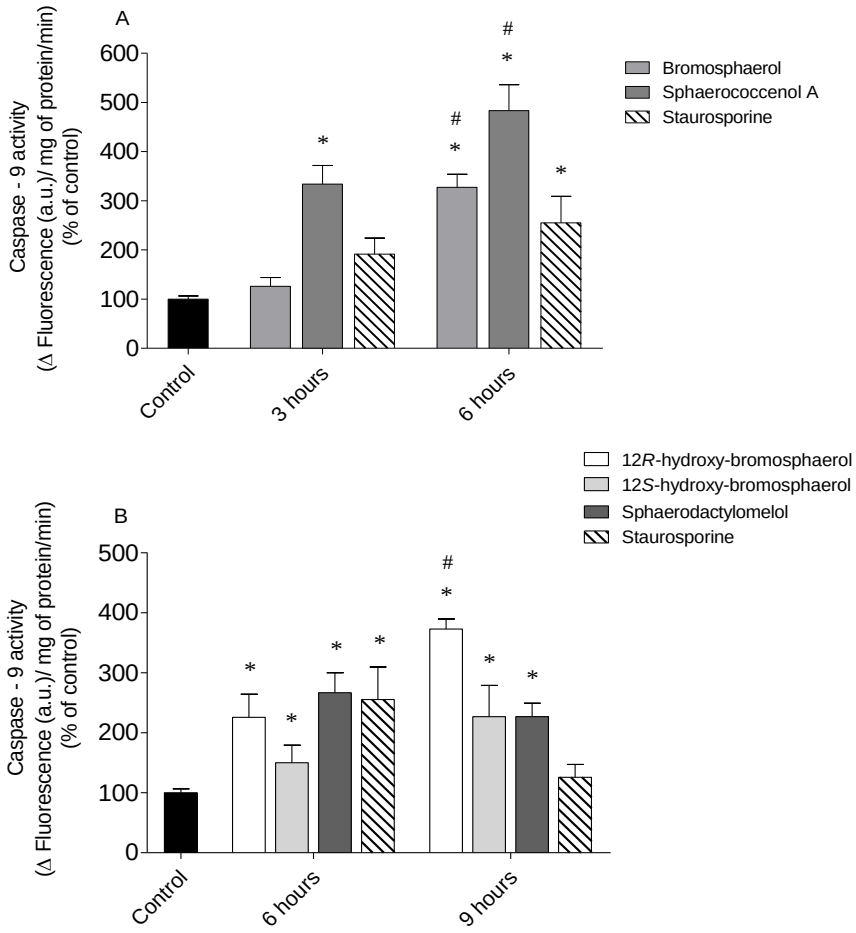


Figure 4.23. MCF-7 Caspase-9 activity after *Sphaerococcus coronopifolius* compounds (IC_{50}) and staurosporine (1 µg/mL) treatment for 3, 6 and 9 hours. The activity was quantified by the slope of the linear phase accumulation of 7-amino-4-(trifluoromethyl) coumarin (between 20 and 80 min). The results are presented in percentage of control (Δ of fluorescence arbitrary units per mg protein per min). The values in each column represent the mean ± SEM of four independent experiments. Symbols represent statistically significant differences (ANOVA, Tukey test; *p* - value < 0.05), when compared to: * control; # different times of each compound.

4.3.5.4. Nuclear condensation and/ or DNA fragmentation by DAPI staining

Nuclear morphological changes such as chromatin condensation and/ or DNA fragmentation are characteristics of the apoptotic process (Tounekti *et al.*, 1995). Thus, following treatment of MCF-7 cells with the IC_{50} values of the isolated compounds for 18h, 24h, 36h, 48h, and 72h, the cells' DNA was stained with DAPI. Results are presented in Figures 4.24 and 4.25.

- The changes mediated by the 12*R*-hydroxy-bromosphaerol, 12*S*-hydroxy-bromosphaerol, bromosphaerol, and sphaerodactylomelol treatment on MCF-7 cells' DNA after 24, 48 and 72 hours, are illustrated in Figure 4.24.

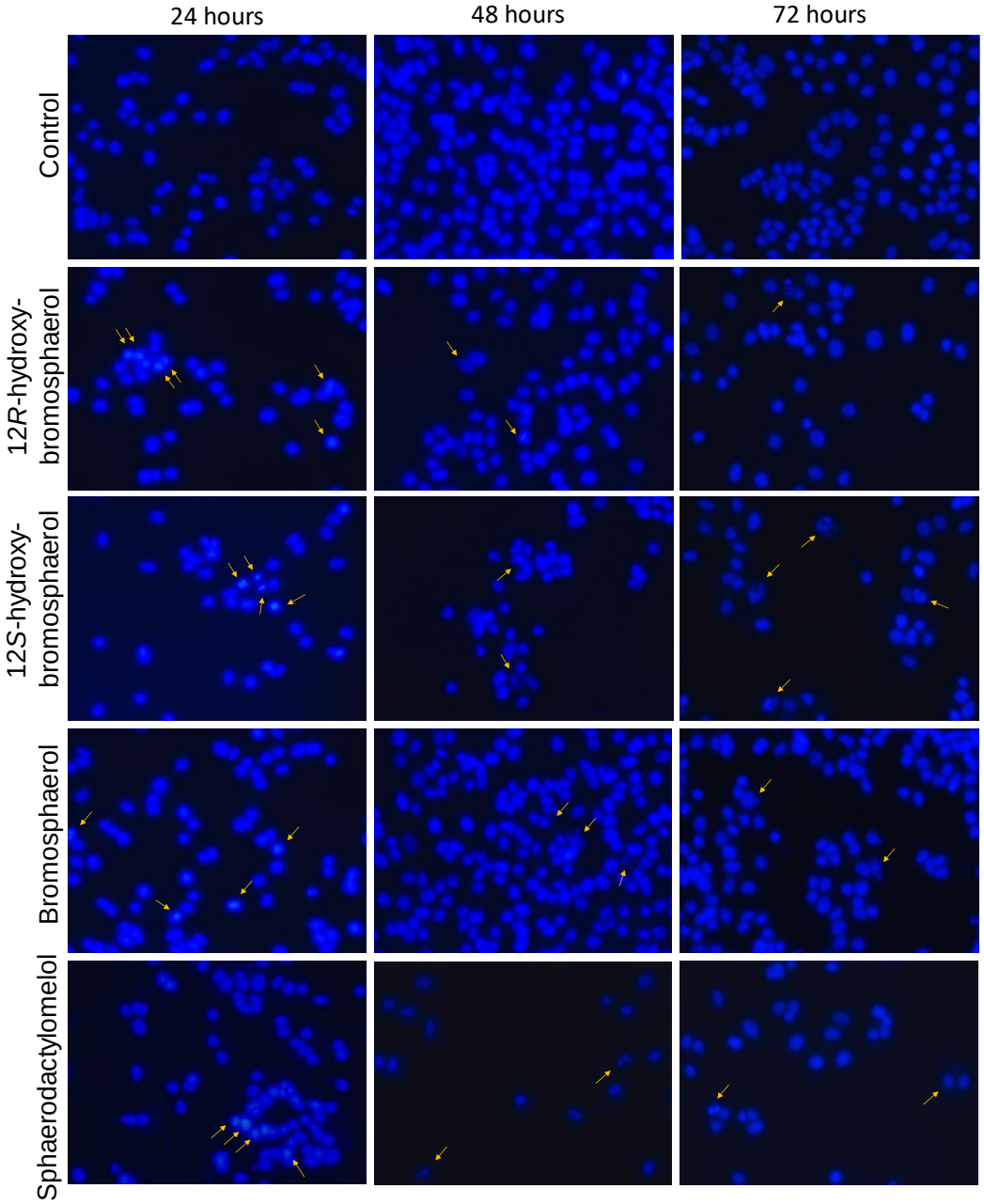


Figure 4.24. MCF-7 cells' DNA changes when treated with 12*R*-hydroxy-bromosphaerol (18.0 µM), 12*S*-hydroxy-bromosphaerol (19.0 µM), bromosphaerol (28.0 µM) and sphaerodactylomelol (48.0 µM) for 24, 48 and 72 hours. Images of DAPI stained cells were obtained using inverted fluorescence microscope at ×400. Arrows indicate alterations in DNA as comparing with control. The images are representative of one well of each situation tested.

Observing the results (Figure 4.24) it is possible to conclude that exposure to the isolated compounds induced changes on MCF-7 cells' DNA. Exposures of 24h and 48h to 12*R*-hydroxy-bromosphaerol seems to induce DNA condensation, but the effects were not clear. However, prolonging the exposure to 72 hours overtly induced DNA fragmentation. The treatment of MCF-7 cells with 12*S*-hydroxy-bromosphaerol induced clearly DNA condensation at 24 hours exposures, and extensive DNA fragmentation for prolongued exposures i.e., 48 and 72 hours. Thus, the effects of 12*S*-hydroxy-bromosphaerol are clearly time dependent. Similarly, bromosphaerol demonstrated to induce DNA condensation after 24 hours, and DNA fragmentation after 48 and 72 hours. Sphaerodactylomelol induced DNA condensation after 24 hours and, after 72 hours promoted DNA fragmentation.

- The changes mediated by the sphaerococcenol A and staurosporine treatment on MCF-7 cells' DNA, after 18, 24 and 36 hours, are illustrated in Figure 4.25.

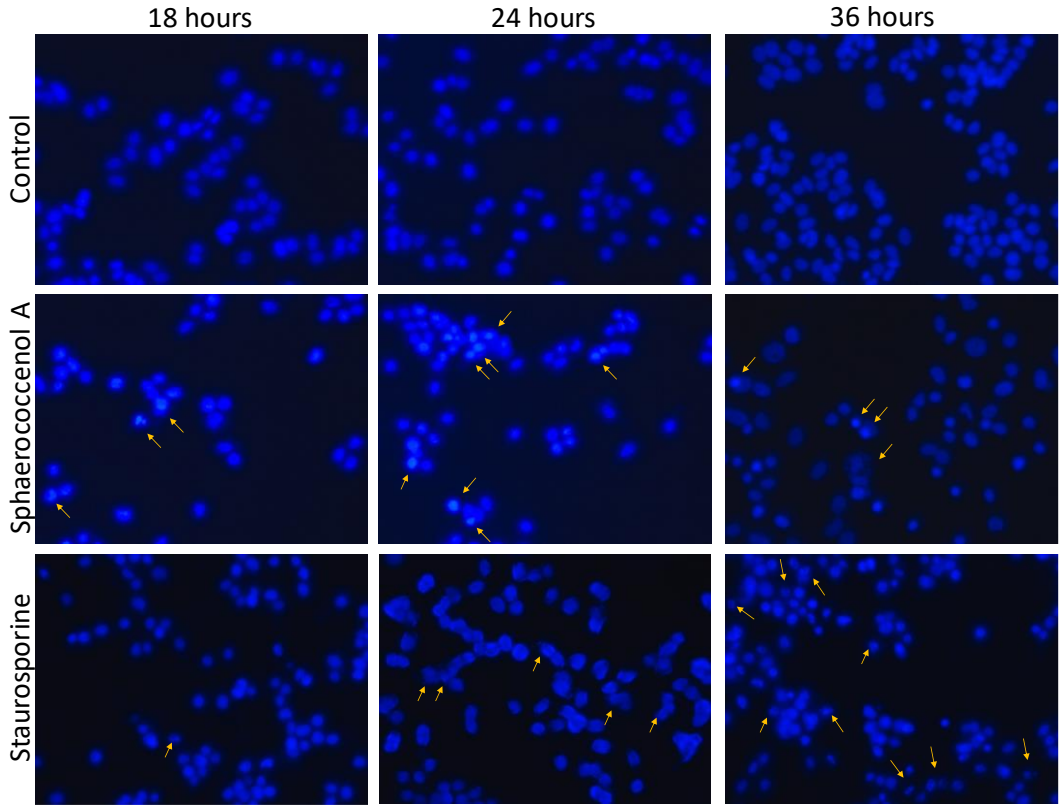


Figure 4.25. MCF-7 cells' DNA changes when treated with sphaerococcenol A (9.5 µM) and staurosporine (1 µg/mL) for 18, 24 and 36 hours. Images of DAPI stained cells were obtained using inverted fluorescence microscope at ×400. Arrows indicate alterations in DNA as comparing with control. The images are representative of one well of each situation tested.

MCF-7 cells exposure to sphaerococcenol A induced DNA condensation after 18, 24 and 36 hours (Figure 4.25). In addition, after 36 hours it was also possible to observe DNA fragmentation. The treatment with staurosporine, a positive control for apoptosis, after 18 hours did not exhibit differences compared to control. However, after 24 hours, there were evident changes on MCF-7 cells' DNA and after 36 hours, most of them displaying fragmented DNA.

4.3.6. Genotoxic activities of the isolated compounds on L929 fibroblasts

One of the essential assessments to protect human health is the study of genotoxic potential of compounds to ensure their safety for therapeutic purposes (Corvi and Madia, 2017). Therefore, the potential of *S. coronopifolius* compounds to promote DNA damage was assessed on L929 mouse fibroblasts, after treatment for 3 hours (25 and/ or 50 µg/mL). The results obtained by the Comet assay, are illustrated in Figure 4.26.

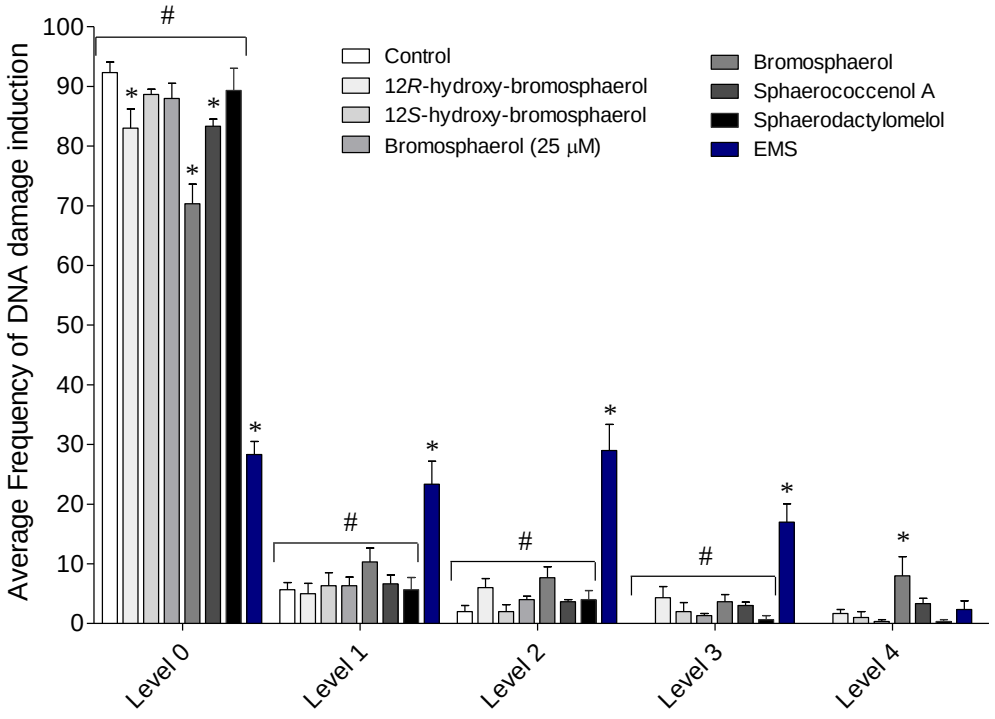


Figure 4.26. Frequency of L929 fibroblasts DNA changes following exposure to treatment with compounds (25 and/ or 50 µM) for 3 hours. Ethyl methanesulfonate (EMS) was used as positive control. The values in each column represent the mean ± SEM of three independent experiments. Damage index: Σ (comet class: 1, 2, 3, 4). 0, nucleus without DNA damage. Symbols represent statistically significant differences (Two-way ANOVA, Bonferroni test; *p* - value < 0.05) when compared to: * control; # EMS.

The results showed (Figure 4.26) that 12*R*-hydroxy-bromosphaerol, bromosphaerol, sphaerococcenol A and EMS exhibited statistical significant differences when compared to control at level 0 (Two-way ANOVA, Bonferroni test; p - value < 0.05). At levels 1, 2 and 3, only EMS displayed significant differences compared to control (Two-way ANOVA, Bonferroni test; p - value < 0.05). On the other hand, it is possible to observe that all compounds tested, and control revealed statistical significant differences compared to EMS at the levels 0, 1, 2, and 3 (Two-way ANOVA, Bonferroni test; p - value < 0.05). At the level 4 of DNA damage index, only bromosphaerol at 50 µM induced effects on L929 cells. However, when tested at 25 µM, this compound exhibited a similar behaviour to all the other tested compounds.

4.4. Discussion and main remarks

The present chapter was designed to evaluate the antitumor potential of compounds derived from the red alga *S. coronopifolius* on several *in vitro* human cancer models. The data gathered here indicates that compounds studied do not demonstrate selective activity on the studied cells suggesting that their cytotoxic activities may be mediated by similar mechanisms of action. Additionally, the results attained in the experimental activities associated to oxidative stress and apoptosis hallmarks lead us to suppose that these two processes may be involved in their activities. Concerning cytotoxic activities, compounds displayed a range of IC_{50} between 4.5 and 90 µM. Sphaerococcenol A was the metabolite with the highest cytotoxicity in the different studied cellular models, exhibiting a range of IC_{50} between 4.5 and 16.6 µM. In fact, the cytotoxic activity mediated by this compound had already been observed in the work described in Chapter 2, exhibiting the highest antiproliferative activity on HepG2 cells (Rodrigues *et al.*, 2015). Moreover, amongst sixteen compounds isolated from *S. coronopifolius* by Smyrniotopoulos and co-workers (2010), sphaerococcenol A also displayed the highest cytotoxicity on several human cancer cells lines, namely U373, A549, NSCLC, SK-MEL-28, PC-3, and LoVo. In addition, the IC_{50} values observed for A549 (3.7 µM), SK-MEL-28 (5.2 µM) and PC-3 (3.7 µM) cancer cells were not so different from the values attained in this study (11.3, 4.5 and 9.7 µM, respectively). Also, 12*R*-hydroxy-bromosphaerol, 12*S*-hydroxy-bromosphaerol and bromosphaerol, demonstrated moderate cytotoxicity exhibiting an IC_{50} of 15 to 46 µM for A549, PC-3 and SK-MEL-28 cells. The IC_{50} values here obtained were also similar to the values (9 – 35 µM) described by Smyrniotopoulos and co-workers (2010) for these compounds in the same cancer cell lines. Moreover, in the study accomplished by Smyrniotopoulos and collaborators (2010), these compounds also did not induce selective activity on the cancer cells studied, exhibiting similar effects on the different cancer cell models. Looking to the cytotoxic activities mediated by *S. coronopifolius* molecules it is interesting to observe that the effects were similar or more potent than the effects induced by standard drugs when tested in the same experimental conditions. This fact is evident in the treatment of CACO-2 and HCT-15 cells with 5-FU, where the effects promoted by the compounds were substantially more potent. Since these two cell lines are described to have intermediate and high resistance, respectively, to 5-FU, this may explain the results observed (Bracht *et al.*, 2010). Despite the largely employement of cisplatin for the last 40 years in cancer therapy, its application has been limited due to the undesired side effects or by occurrence of drug resistance (Ratzon *et al.*, 2016; Cai *et al.*, 2017). In the present

study, A549, NCI-H226, and PC-3 cells also revealed resistance to the treatment with cisplatin comparing to the *S. coronopifolius* compounds for exposures up to 24 hours. Previous studies also observed resistance of these cells to cisplatin treatment. (Zhang *et al.*, 2003; Whiteside *et al.*, 2006; Gumulec *et al.*, 2014). However, the efficiency of cisplatin was less potent than the effects observed in other studies with the same cell lines (Gumulec *et al.*, 2014; Cai *et al.*, 2017). This fact may be due to very prolongued exposure conditions (72h) used in those studies. For instance, Gumulec and co-workers (2014) demonstrated that the cytotoxic effects of cisplatin on PC-3 cells were time dependent which may explain the achieved results. Thus, it is expected that an increase of cisplatin exposure time lead to more pronounced cytotoxic effects.

Currently, a variety of assays have been employed in the pharmacological area to evaluate cytotoxic activities (Menyhárt *et al.*, 2016). In this study, the effects of compounds on MCF-7 cells' viability were assessed by the MTT and LDH assays. Despite those assays providing important informations on the cytotoxicity of a compound, the cellular targets of each one are distinct. For instance, MTT principle is related to dehydrohgenases activities (both mitochondrial and cytoplasmatic) and LDH with membrane integrity (Menyhárt *et al.*, 2016). The results obtained with both tests revealed that some of the compounds, particularly, 12*S*-hydroxy-bromosphaerol and bromosphaerol, evidenced differences on both tests, being the effects revealed by the MTT assay more marked. These facts suggest that the cytotoxic activities mediated by these two compounds may target mitochondrial activity. Additionally, although the treatment with 12*S*-hydroxy-bromosphaerol and sphaerococcenol A did not demonstrated statistic differences between the two assays, it is also interesting to observe that the effects on MCF-7 cells' viability were more marked by using MTT method than by the LDH assay, at a concentration near of their IC_{50}, 25 and 10 µM, respectively.

Despite *S. coronopifolius* compounds in previous studies having shown interesting cytotoxic activities, the intracellular signaling pathways underlying their activities were not studied until now. Here, the treatments of MCF-7 cells with the compounds led to a stimulation of H_2O_2 production, changes in mitochondrial membrane potential, increase of cells population at late apoptosis stage, induction of Caspase-9 activity and DNA condensation and/or fragmentation, suggesting that their cytotoxic activities may be associated with oxidative stress and apoptosis. In fact, many studies have demonstrated that compounds belonging to terpenes chemical class promote antiproliferative activities, which are generally associated to oxidative stress and apoptosis (Huanjie and Dou, 2010;

Lai *et al.*, 2016; Chen *et al.*, 2018). Moreover, it is widely recognized that some anticancer drugs, such as cisplatin, lead to an increase on ROS levels during therapeutic regimens, which have been proposed as important apoptosis mediators (Ozben, 2007). The controlled production of H_2O_2 on biological systems plays a central role in vital cellular processes (Lennicke *et al.*, 2015; Hopkins, 2017). However, the increase of its levels may promote mitochondrial dysfunction leading to cellular apoptosis (Mao *et al.*, 2006; Singh *et al.*, 2007; Pinteus *et al.*, 2017). Actually, H_2O_2 has ability to mediate the downregulation and upregulation of antiapoptotic (Bcl-2) and proapoptotic proteins (BAX), respectively, cytochrome C release, loss of MMP, and increase on Caspase-3/-7/-9 activity (Stridh *et al.*, 1998; Mao *et al.*, 2006; Cui *et al.*, 2015; Kaushik *et al.*, 2015). These facts can corroborate the hypothesis that the cytotoxic activities mediated by the *S. coronopifolius* compounds, excepting bromosphaerol, may be associated to an increase on H_2O_2 levels leading to apoptosis. In fact, *S. coronopifolius* compounds lead to a decrease on cells' viability, which was accompanied by a simultaneos increase on H_2O_2 levels (Figure 4.19 and 4.20), depolarization of MMP (Figure 4.21), stimulation of Caspase-9 activity(Figure 4.23) and induction of DNA condensation and/ or fragmentation (Figure 4.24 and Figure 4.25). Among all compounds tested, sphaerodactylomelol is a strong candidate to support this theory, since it displayed the highest H_2O_2 generation, a clearly depolarization of MMP, and induction of Caspase-9 activity. Caspase-9 has been implicated in apoptosis being involved in the formation of the multiprotein complex, apoptosome, which activates then Caspase-3, and -7 initiating the caspase cascade (Kuida, 2000; Wu and Bratton, 2013). Moreover, the treatment of MCF-7 cells with *S. coronopifolius* compounds led to changes on MMP and Caspase-9 activity, suggesting that their cytotoxic activities may be mediated by activation of the intrinsic apoptotic pathway. Also, it was also possible to verify that *S. coronopifolius* compounds promoted DNA fragmentation. By apoptosis, this event is usually initiated by Caspase-3 (Wolf *et al.*, 1999). However, MCF-7 breast carcinoma cells do not express this enzyme suggesting that other player may be mediating DNA fragmentation. This theory is sustained by the study of MC Gee and co-workers (2002), in which PBOX-6 compound induced apoptosis on MCF-7 promoting DNA fragmentation. However, this event was not induced by Caspase-3 but by Caspase-7, suggesting that Caspase-3 is not necessarily essential for DNA fragmentation associated to apoptosis.

One of the earlier events of programmed cell death is the translocation of membrane phosphatidylserine (Rysavy *et al.*, 2014; Segawa and Nagata, 2015). The flow cytometry analysis using AnnexinV/PI conjugate demonstrated an accumulation of cells at the late apoptosis stage. Due to the fact that cells, at this stage, are double labeled, these features

can be interpreted as corresponding to primary necrotic cells and not to apoptotic cells (Wlodkowic *et al.*, 2011). However, those unexpected results may be explained by the long time (24 hours) that MCF-7 cells were exposed to the *S*. *coronopifolius* compounds, not being possible to observe earlier apoptosis. This point of view is sustained by the results attained with the apoptosis inducer, staurosporine, which also revealed an increase on the number of cells in the late apoptosis stage as compared to control and did not show apoptotic cells. In addition, at later stages of apoptosis, the membrane may become more permeable, and thus PI can enter cells driving to double positive population (Henry *et al.*, 2013). Therefore, to check the apoptosis stages, time-courses analyses and additional assays such as caspase activities, which is one of the major biochemical events that occur in apoptosis (Henry *et al.*, 2013; Brauchle *et al.*, 2014), were carried out. Furthermore, the idea that cytotoxic activities mediated by these compounds may be associated with apoptosis is also supported by recent study performed with the red alga *S. coronopifolius* by Salhi *et al.* (2018), where dichloromethane extract (such as the extract from where the compounds were isolated) increased the number of SKBR-3 apoptotic cells, which are derived from breast cancer tissues (Salhi *et al.*, 2018).

Similar results were observed with other marine compounds, such as heteronemin, tuberatolide B, DDSD and peroxy sesquiterpenoids, which promoted ROS generation and triggered apoptosis (Miyazato et al., 2016; Velatooru et al., 2016; Choi et al., 2017; Lee et al., 2018). For instance, spatane diterpenoid previously isolated from the brown alga *Stoechospermum marginatum* induced morphological alterations, nuclear condensation, and DNA fragmentation on B16F10 melanoma cells as well as promoted ROS production. The increase of these species led to a change in Bax/Bcl-2 ratio and triggered the loss of mitochondrial transmembrane potential, the release of cytochrome c, and activated caspase-mediated apoptotic pathway (Velatooru *et al.*, 2016). In the same point of view, Miyazato and co-workers (2016) verified that peroxy sesquiterpenoids derived from soft coral *Sinularia sp*. induced cell death on HCT116 human colon cancer cells by apoptosis-induction via caspase activation. Moreover, the treatment with the bromoterpenes drove to an accumulation of intracellular H_2O_2, which seems to trigger apoptosis. Those studies corroborate the hypothesis that *S. coronopifolius* bromoterpenes mediated similar effects on MCF-7 cells.

Opposite to the other compounds, bromosphaerol effects do not seem to be associated with H_2O_2 production, since the MCF-7 cells treatment with this compound did not influenced the concentration of H_2O_2. Many chemotherapeutic drugs mediate their

cytotoxic activities through the induction of DNA damage (Karran, 2001). This strategy overloads the capacity of cellular DNA repair systems leading to the activation of cell death mechanisms, including apoptosis intrinsic pathway (Nowsheen and Yang, 2012; Cheung-Ong *et al.*, 2013). In the genotoxicity assay accomplished on L929 cells, bromosphaerol was the only compound that induced DNA damage, suggesting that this compound targets directly the DNA. However, additional studies, including on MCF-7 cells, are required to confirm the direct DNA damage as the main mechanism underlying bromosphaerol effects. As to the remaining compounds they do not seem to induce genotoxicity effects.

In summary, the cytotoxic activities mediated by *Sphaerococcus coronopifolius* bromoterpenes are not cell specific, and sphaerococcenol A displayed the highest cytotoxicity. Moreover, the possible mechanisms of action induced by those compounds on MCF-7 cells seem to be associated with an increase in H_2O_2 levels, loss of MMP, and consequently the activation of Caspase-9 and DNA condensation and/ or fragmentation. Conversely, bromosphaerol did not induce ROS generation even though it caused DNA, damage, MMP depolarization, increased Caspase 9 levels, and ultimately DNA fragmentation (Figure 4.27). To the best of our knowledge, this is the first study accomplished to understand the intracellular signaling pathways associated with the antitumor activities mediated by those compounds.

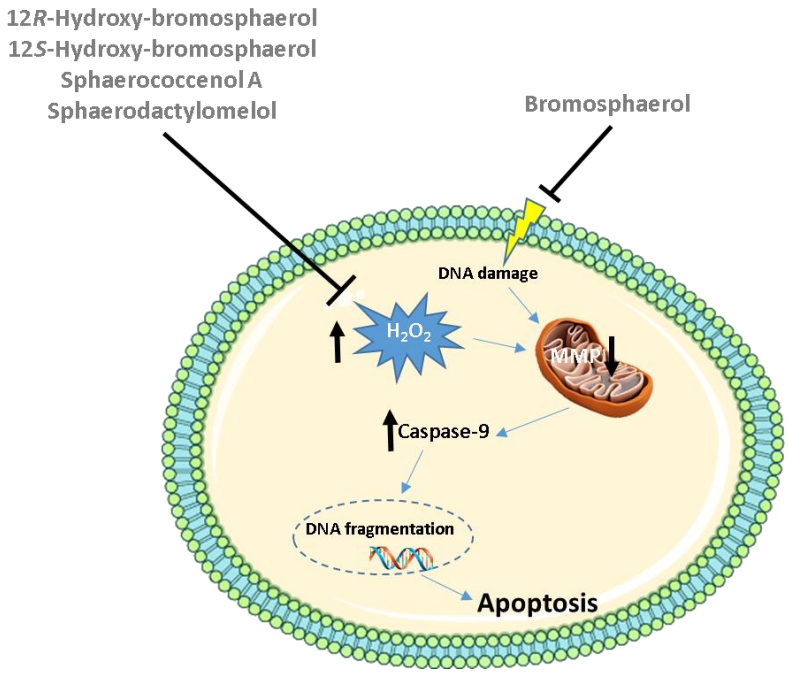


Figure 4.27. Possible mechanism of action underlying the effects of the bromoterpenes isolated from the red alga *Sphaerococcus coronopifolius* on MCF-7 cells.

4.5. References

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Chapter 5

Sphaerococcus coronopifolius compounds as cancer stem cell-targeting agents

5.1. Introduction

Despite the advances concerning cancer biology and cancer therapeutic strategies achieved in the last years, this illness remains a huge threat for our society and one of the most deadly in the world. Due to widespread smoking habits and increasing accumulation of atmosphere pollutants, lung cancer continues to be one of the main causes of cancer-related deaths worldwide having been reported 1.8 million of new cases and 1.6 million of deaths in 2012 (Jacques *et al.*, 2015; Siegel *et al.*, 2018). In 2018, lung cancer remains as one of the lowest 5-year relative survival rate (18%) (Siegel *et al.*, 2018). Cancer therapeutic resistance is one of the factors that more contributes for this reality since it is related with the ability of cancers to develop resistant mechanisms against therapeutics regimens which can be intrinsic or acquired (Ayob and Ramasamy, 2018). Usually, this capacity is linked with distinct mechanisms including specific genetic and epigenetic changes on cancer cells and/or on their microenvironment (Housman *et al.*, 2014; Knoechel *et al.*, 2014). Tumor resistance to chemotherapeutic agents is often related with therapeutic failure and, consequently, cancer relapse, being one of the greatest challenges in the treatment of oncologic patients (Godwin *et al.*, 2013; Prieto-Vila *et al.*, 2017). Several mechanisms are associated with drug resistance, including drug efflux, detoxification or inactivation, changes of drug targets, improvement of the DNA repair mechanisms, apoptosis blockage, epithelial-to-mesenchymal transition, tumor microenvironment and tumor heterogeneity (Luqmani, 2005; Mansoori *et al.*, 2017; Senthebane *et al.*, 2017).

Advances in cancer research identified, inside the tumor mass, a small subpopulation of cells designated as cancer stem cells (CSC´s) which seems to play a crucial role in tumorigenesis, tumor maintenance, metastasis and resistance to conventional cancer drugs (Zhao, 2016; Aponte and Caicedo, 2017; Lau *et al.*, 2017) (Figure 5.1). Usually, chemotherapy and radiation therapy target rapidly dividing cancer cells that comprise the majority of tumor. However, quiescent CSC´s may keep viable after the treatment by activating intrinsic survival mechanisms (Nguyen *et al.*, 2015). CSC´s reveal similar features with the normal stem cells exhibiting the ability to self-renew, originate multi-lineage and possess potential for long-term tumor repopulation (Rich, 2016; Ayob and Ramasamy, 2018). The CSC's theory stating that this cells are responsible for tumors' heterogenicity, progression and relapse following therapy as gained considerable strenght in the last years (Pietras, 2011; Ayob and Ramasamy, 2018; Lleonart *et al.*, 2018). These cells have the capability to manipulate intrinsic and extrinsic mechanisms of adaptation as

well as to interact with the microenvironment. In fact, CSC´s through the secretion of signals "manipulate" the surrounding cells (e.g. stromal and immune cells) influencing their recruitment, transformation and functions. This capacity creates the necessary conditions for their survival, expansion and resistance (Zhao, 2016; Ayob and Ramasamy, 2018). Moreover, bioinformatics-based studies demonstrated that the presence of CSC´s biomarkers are related with poor patient prognosis evidencing the crucial role of those cells in cancer resistance (Horst *et al.*, 2008; Eppert *et al.*, 2011; Cheng *et al.*, 2016). Additionally, CSC´s threat becomes more complex with recent evidences, which demonstrated the ability of differentiated non-CSC´s to dedifferentiate to CSC´s, through a reprogramming mechanism (Chaffer *et al.*, 2011; Gupta *et al.*, 2011; Chaffer *et al.*, 2013; Kiyoung *et al.*, 2017; Rodrigues *et al.*, 2018). Moreover, several studies reported the presence of different CSC's populations into tumor masses with distinct molecular signatures (Schober and Fuchs, 2011; Emlet *et al.*, 2014; Zheng *et al.*, 2018). Thus, future directions of CSC's target therapy should not address only the CSC's existing within the tumors' but all the others cellular populations that may generate, by dedifferentiation, new CSC's with different molecular signatures (Kiyoung *et al.*, 2017). Regarding lung cancer, due to its genotypic and histological varieties it is considered one of the cancer type with higher complexity (Zakaria *et al.*, 2017). The resistance to chemotherapy according to ongoing experimental and clinical observations suggest that CSC's are involved in its malignant phenotype development (Hardavella *et al.*, 2016). Despite the high advances in CSC´s biology, the development of these cells remains unclear. Thus, advances in this area to a better understanding of their role in cancer development and metastasis could be one of the keys to identify potential therapeutic targets and design multimodal therapies. These multimodal therapies could abolish not only bulk tumor cells, but also CSC´s and tumor microenvironment signaling (Rich, 2016; Lau *et al.*, 2017) (Figure 5.1).

Due to the success of NPs and their derivatives in cancer treatments, as well as their ability to mediate several signaling pathways and cause fewer side effects, there is a growing interest to understand their potential to be applied on CSC's therapeutics (Taylor and Jabbarzadeh, 2017). Several studies have demonstrated that NPs mediate interesting activities on CSC´s. For instance, the natural product gigantol extracted from *Dendrobium draconis* plant exhibited ability to suppress CSC phenotypes and CSC markers CD133 and ALDH1A1 on non-small cell lung cancer. This effect seems to be linked with the inhibition of intracellular signaling pathway AKT, and consequently decreases of cellular levels of pluripotency and self-renewal factors Oct4 and Nanog (Bhummaphan and Chanvorachote, 2015; Chanvorachote *et al.*, 2016).

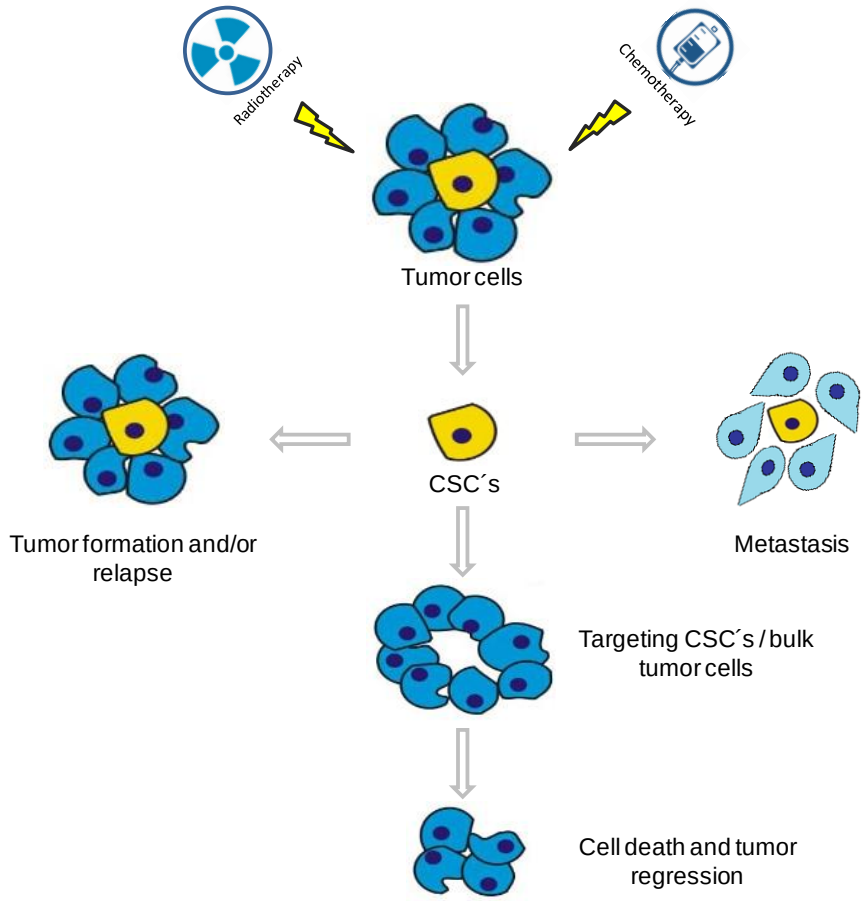

Figure 5.1. Overview of cancer stem cells role in cancer resistance development, and their potential to be target for therapeutics.

In the same way, crude extract and 4-vinylphenol obtained from the medicinal herb *Hedyotis diffusa* also inhibited the development of colorectal and breast cancer cells with stem-like phenotype mediating several intracellular signaling pathways (Sun *et al.*, 2016; Leung *et al.*, 2018). In this area there are also reports regarding MNP potential. Marine extract of *Crambe crambe* sponge inhibited the resistance to apoptosis, self-renewal ability, and proliferation of pancreatic cancer cells with CSC´s phenotype. Moreover, its co-administration with gemcitabine induced a total tumor abolishment on an *in vivo* model (Sabine *et al.*, 2012). Algae compounds have also demonstrated interesting potential to be applied on CSC´s therapy. For instance, polyhalogenated monoterpenes extracted from the red alga *Plocamium cornutum* showed selective activity to inhibit the development of MCF-7 mammosphere structures, without cytotoxicity on either adherent MCF-7 cells or MCF-12A non-transformed cells (De la Mare *et al.*, 2013). Extracts obtained from brown alga, *Saccharina japonica*, suppressed sphere-forming ability of glioblastoma stem cells (Kim *et al.*, 2018). Also, the compound derived from fucoxanthin, fucoxanthinol, demonstrated

ability to promote apoptosis, suppressed epithelial-to-mesenchymal transition, sphere formation and *in vivo* tumorigenesis of human colorectal cancer stem cells (Terasaki *et al.*, 2017; Terasaki *et al.*, 2018). Recently, Rodrigues and co-workers (2018) used hexavalent chromium [Cr(VI)] as a model of cancer carcinogenesis exposing human bronchial epithelial cell line BEAS-2B (Bronchial Epithelial Airway System-2B) to Cr(VI). This treatment led to the transformation of BEAS-2B cells into RenG2 cells (malignant human bronchial epithelial cells), which possess malign characteristics. After that, RenG2 cells were injected in nude mice and from the resulting tumor two additional cell lines were isolated (DRenG2 and DDRenG2). Inside of the tumor mass it was also possible to verify the presence of cancer cells with stem cell properties, named as SC-DRenG2 cells (cancer stem cells isolated from RenG2 cells derived tumors), due to their potential to grow without anchorage and capacity to form sphere structures (Rodrigues *et al.*, 2018) (Figure 5.2).

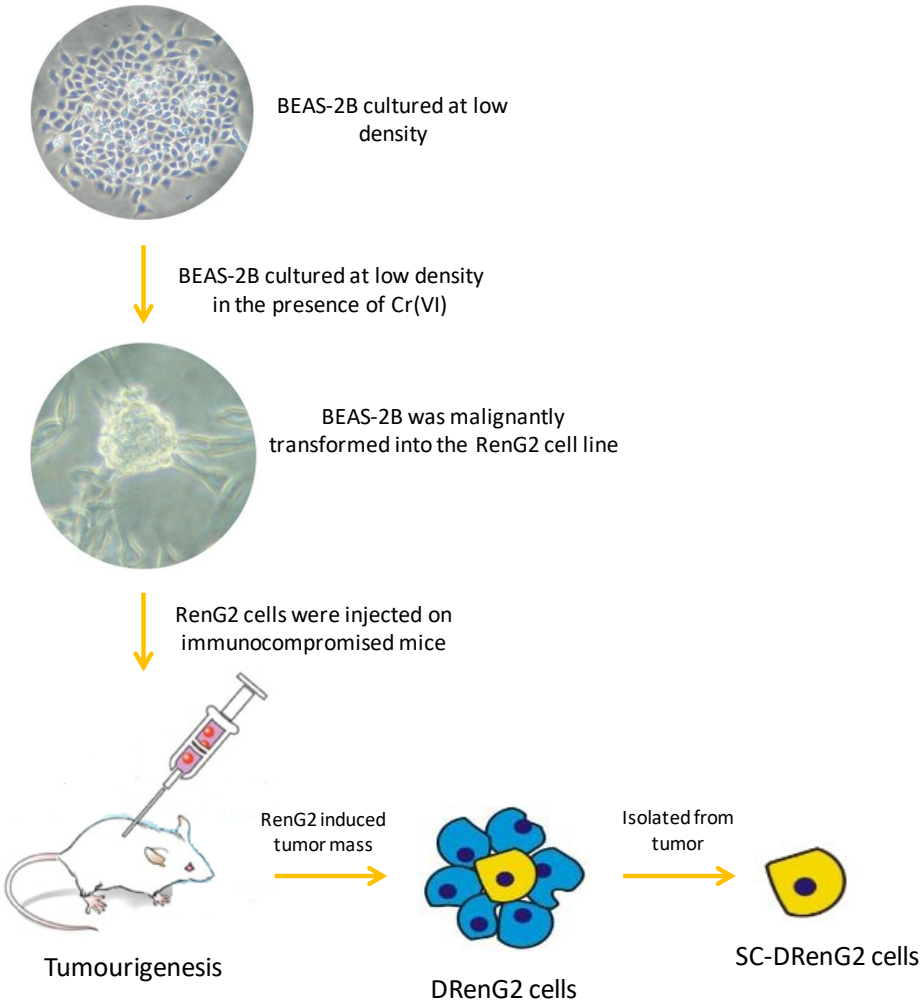

Figure 5.2. Design of the proceeds accomplished to transform BEAS-2B cells into RenG2 cells and origin of the SC-DRenG2 cells (adapted from Rodrigues *et al*., 2018).

These results led to the development of a co-culture system composed by normal human bronchial fibroblasts (HBF) and RenG2 cells. Their cultivation in co-culture system promotes the arising of tumor cells with stem cells characteristics mimetizing the events that happen on *in vivo* model. Therefore, the main aim of this chapter is to understand the potential of *Sphaerococcus coronopifolius* compounds to suppress the development of CSC´s on an *in vitro* human lung cancer co-culture system. According with our best knowledge, it is the first study accomplished to access the antitumor potential of these compounds to target CSC´s.

5.2. Materials and methods

The procedures accomplished to assess the potential of *S. coronopifolius* compounds to target CSC´s are described in the following sections (5.2.1 – 5.2.4).

5.2.1. Compounds isolated from the red algae *Sphaerococcus coronopifolius*

Compounds tested in co-culture system were extracted and isolated according to the procedures described in Chapter 3. Chemical information and their structures are presented in Figure 4.1 (Chapter 4). As previously referenced, compounds were dissolved in DMSO whose concentration in all the experiments performed was lower than 0.2% to avoid its toxicity. Controls were always treated with highest concentration of DMSO as vehicle.

5.2.2. Evaluation of compounds cytotoxic activities on monoculture system

The cytotoxic activities of compounds were evaluated on *in vitro* human cellular models derived from normal and carcinogenic lung tissues. Preliminary studies were carried out in monoculture system to define the concentrations to be used in co-culture system. BEAS-2B cells (Bronchial Epithelial Airway System-2B), RenG2 cells (malignant human bronchial epithelial cells), SC-DRenG2 cells (Cancer stem cells derived from RenG2 cells) and HBF cells (Human Bronchial Fibroblasts) were used in the experiments.

5.2.2.1. Maintenance of cell cultures

BEAS-2B cells were acquired from the European Collection of Cell Cultures (ECCAC, Salisbury, UK; ECCAC no. 95102433). Malignant human bronchial epithelial cells (RenG2) and cancer stem cells derived from RenG2 cells (SC-RenG2 cells) were attained according to Rodrigues and co-workers (2018), following the procedures displayed in Figure 5.2. Cells were cultured in either CloneticsTM BEGM medium (Lonza, Basel, Switzerland) supplemented with bovine insulin, bovine pituitary extract, epinephrine, human epidermal growth factor, hydrocortisone, retinoic acid, triiodothyronine, transferrin and the antibiotics amphotericin B sulphate and gentamicin (Lonza Basel, Switzerland) or LHC-9 medium (Gibco, USA). HBF were previously obtained from non-malignant human lung tissue from a patient at the Centro Hospitalar e Universitário de Coimbra (CHUC), through appropriate informed consents and according to the ethical procedures approved by the Ethical Committee of the Faculty of Medicine of the University of Coimbra. HBF cells were maintained in DMEM medium supplement with 10% FBS (Biochrom, Germany), 20 U/mL penicillin, 20 µg/mL streptomycin and 50 ng/mL amphotericin B (Biochrom, Germay). Cells were kept at 37ºC in a 95% air/ 5% CO_2 incubator. Culture flasks were coated with gelatin solution 2 hours before use and, excepting otherwise stated, cells were seeded at a recommended initial density of 4×10^3 cells/cm^2. Subculture was performed using a 0.25% trypsin-1 mM EDTA solution (Biochrom, Cambridge, UK) whenever cultures reached 80% confluence.

5.2.2.2. Cytotoxic activities of compounds on cellular models

In order to determine the most interesting compounds to be tested on co-culture system, as well as the concentrations to be used, preliminary assays were performed on BEAS-2B (4×10^3 cells/ well), HBF (4×10^3 cells/ well), RenG2 (4×10^3 cells/ well), and SC-DRenG2 (4×10^3 cells/ well) cells using monoculture system. BEAS-2B, HBF, RenG2, and SC-DRenG2 cells were seeded in 96-well plates and incubated overnight. Cells were then treated with the compounds at 10, 50 and 500 µM for 72 hours. After the treatment, the medium was removed and cells were washed with PBS buffer and incubated at 37ºC during 1 hour with a MTT solution (1.2 mM) previously dissolved in PBS buffer. After washing off excess MTT, cells were disaggregated with DMSO and the absorbance of the crystals of formazan was measured at 570 nm using a spectrophotometer plate reader (Bio-Tek

Synergy plate reader, Bedfordshire, UK). At least three independent experiments were performed each in triplicate and results expressed in percentage of control.

5.2.3. Experimental assays on co-culture system

Co-culture system was established with RenG2 cells and HBF according to the scheme illustrated in Figure 5.3. The co-culture of RenG2 and HBF cells mimics the conditions that RenG2 cells faced in lumbar subcutaneous compartment of mice, where dedifferentiation of terminally differentiated cancer cells originate cells with stem-like properties. This system was created as presented in Figure 5.3.

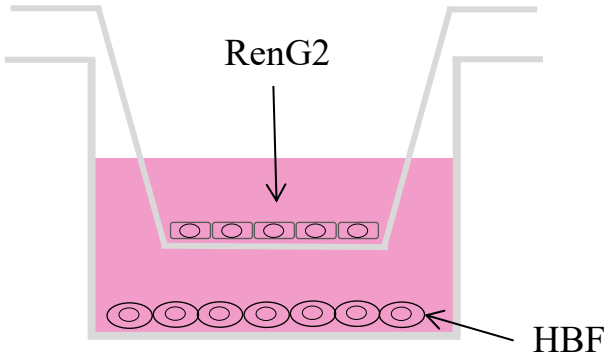

Figure 5.3. Co-culture experimental design. Using a 6-well plated adapted transwell system, RenG2 and HBF cells were co-cultured for 2 months. HBF cells were cultured in the lower compartment, while RenG2 cells were housed in the upper compartment.

5.2.3.1. Co-culture of RenG2 and HBF: the attainment of the RenG2 system and treatment with compounds

HBF cells were seeded as feeder layers in 6-well plates equipped with 4.5 cm^2 Transwell® insert (SPL Life Sciences, Korea) at a density of 1.4×10^4 cells/ well. After 3 days, RenG2 cells were seeded in Transwell® insert at a density of 8×10^3 cells/ well (Figure 5.3). Co-cultures were kept in the incubator at 37ºC in a 95% air/ 5% CO_2 for two months and mediums were changed every 15 days. After two months, both cell lines were treated with *S. coronopifolius* isolated compounds for 72 hours, with the concentrations previously defined, to evaluate their ability to avoid the development and if possible the differentiation of the existing CSC's. The presence of cells with stem properties was evaluated performing

the sphere-forming assay (5.2.3.2.). At least three independent experiments were carried out in triplicate and results expressed in percentage of control.

5.2.3.2. Sphere-forming assay for assessment of CSC´s formation

Sphere-forming assay is an *in vitro* technique widely used in cancer stem cells research to isolate cancer cells with stem-like properties. This assay is based on the intrinsic properties of CSC´s, such as intrinsic self-renewal and differentiation, which lead to formation of tridimensional spheres when cultured under low-adherence conditions with appropriate mediums and supplements (Mimura *et al.*, 2010; Pastrana *et al.*, 2011; Shaheen *et al.*, 2016). The steps accomplished during sphere-forming assay are described in next sections (5.2.3.2.1 – 5.2.3.2.3).

5.2.3.2.1. Preparation of CSC´s isolation medium

CSC´s isolation medium was prepared according to Rodrigues (2014). Briefly, 250 mL of DMEM:F12 (1:1) cell culture medium (Gibco, USA) was supplemented with 5 mL of penicillin (5000 U/mL) - streptomycin (5000 µg/mL) (Gibco, USA), 10 µL of progesterone solution (1mM) (Sigma, China) and 5 mL of the commercialized insulin, transferrin, selenium (ITS) sodium pyruvate solution (Gibco, USA). Hence, 0.6 g of sodium bicarbonate ($NaHCO_3$) (Sigma, USA) was dissolved in the liquid phase, along with 0.08 g of putrescine (Sigma, Switzerland). The final volume of 500 mL was reached by the addition of a solution containing 2% methylcellulose (Sigma, USA).

5.2.3.2.2. Preparation of low adherence 6-well plates for CSC´s isolation

Low adherence conditions are mandatory for CSC´s isolation. Thus, low adherence 6-well plates were prepared according to Rodrigues (2014). The 6-well plates were coated with a 2% poli-(2-hydroxyethyl methacrylate) (poli-HEMA) (Sigma, USA) solution. To prepare 200 mL of a 2 mg/mL coating solution, 4 g of poli-HEMA were added to 200 mL of 95% ethanol in a glass flask, and allowed to dissolve over a stirring plate with constant agitation for 8 hours at room temperature. After complete dissolution, 6-well plates were coated with solution at 0.4 mL/ well and allowed to dry at room temperature, in a sterile atmosphere over a stable bench. Afterwards, plates were further sterilized by exposing to

UV light for 20 min. Whenever plates were not immediately necessary, they were sealed with Parafilm® and stored at 4ºC.

5.2.3.2.3. Procedure for CSC´s isolation

The proceedings to isolate CSC´s were accomplished according to Rodrigues (2014)**.** After the treatment with compounds for 72 hours, cells housed in the upper compartment were washed with PBS buffer, bring up with 0.25 % trypsin-1 mM EDTA solution (Biochrom, Cambridge, UK) and collected by centrifugation at 380 *g* for 5 min at room temperature. Cells were then resuspended in the isolation medium at a concentration of $3x10^4$ cells/ mL, and 2 mL of this suspension were added to each well of the 6-well plate previously prepared. The isolation medium was supplemented with 10 ng/mL of both human EGF (E9644, Sigma-Aldrich) and bFGF (100-18B, PeproTech, London) and cells were maintained at 37ºC in a 95% air/ 5% CO_2. Supplements' concentration was replaced every two days. Whenever sphere formation was verified, and spheres reached a satisfactory volume (which normally happens around 15 days after platting), they were collected, and analyzed using optic microscopy (Axio observer z1 Carl Zeiss; Camara AxioCam HR R3; Fiji ImageJ software, Wayne Rasband, National Institutes of Health, USA). The spheres size was registered and photos were taken (Fiji ImageJ software, Wayne Rasband, National Institutes of Health, USA). At least three independent experiments were carried out in triplicate.

5.2.4. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) with Dunnett's multiple comparison of group means to determine significant differences relatively to control treatment. In addition, for the remaining multiple comparisons the Tukey's test was applied. For all analyzes, all data were checked for normality and homoscedasticity. Results are presented as mean ± standard error of the mean (SEM). Differences were considered statistically significant at level of 0.05 (p - value < 0.05). The determination of IC_{50} was performed by the analysis of non-linear regression by means of the equation:

$$y = \frac{100}{[1+10(X-log(IC_{50}))]}$$

Calculations were performed using IBM SPSS Statistics 24 (IBM Corporation, Armonk, NY, USA) and GraphPad v5.1 (GraphPad Software, La Jolla, CA, USA) softwares.

5.3. Results

The results achieved to assess the potential of *S. coronopifolius* compounds to target CSC´s are reported in the following sections (5.3.1 and 5.3.2).

5.3.1. Cytotoxic activities of compounds on monoculture system

Cytotoxicity of *Sphaerococcus coronopifolius* isolated compounds was assessed on BEAS-2B, HBF, RenG2, and SC-DRenG2 cell lines following 72 hours exposure. The effects were assessed by the MTT assay. The results were expressed in percentage of control, and are displayed in Figures 5.4 to 5.10.

- The cytotoxic activities induced by the compounds (10, 50 and 500 µM) on HBF cells following 72 hours exposure are presented in Figure 5.4.

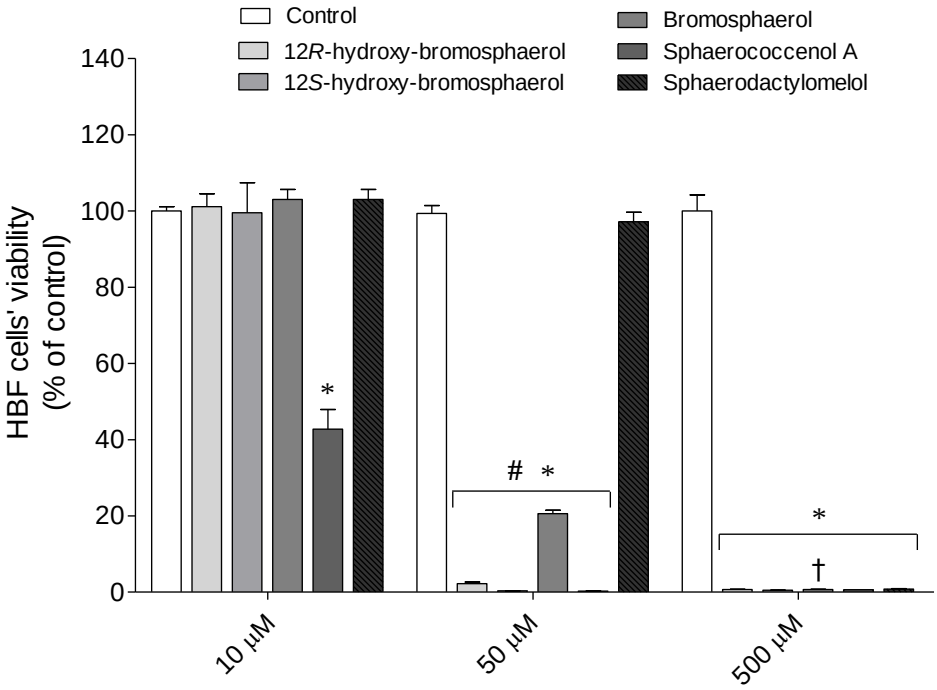

Figure 5.4. HBF cells' viability following 72 hours of exposure to the *Sphaerococcus coronopifolius* isolated compounds (10, 50 and 500 µM) expressed as % of the control. MTT was used as previously described and the values correspond to mean ± SEM at least three independent experiments carried out in triplicate. Symbols represent statistically significant differences (One-way ANOVA, Tukey test; *p* - value < 0.05) when compared to: * control of respective concentration; # 10 µM treatment; † 10 and 50 µM treatment.

The results revealed that only 10 µM sphaerococcenol A reduced significantly (55%) the HBF cells' viability (One-way ANOVA, Tukey test; *p* - value < 0.05) (Figure 5.4). When tested at 50 and 500 µM all compounds decrease the cells' viability in more than of 75%. Additionally, it was also possible to verify that a higher concentration resulted in more marked effects on HBF cells' viability. In this point of view, bromosphaerol exhibited similar effects between all concentrations tested. On the other hand, the remaining compounds displayed a marked effect between 10 and 50 µM and maintained the cytotoxicity observed at 50 µM, when tested at 500 µM.

- The cytotoxic activities induced by the *S. coronopifolius* isolated compounds (10, 50 and 500 µM) on RenG2 cells following 72 hours exposure are presented in Figure 5.5.

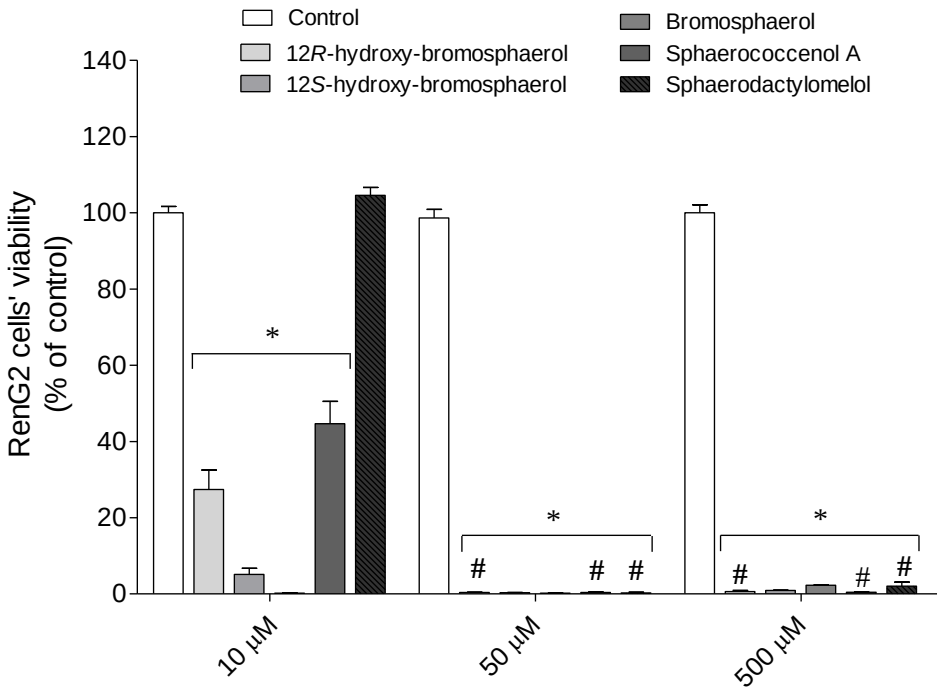

Figure 5.5. RenG2 cells' viability following 72 hours of exposure to the *Sphaerococcus coronopifolius* isolated compounds (10, 50 and 500 µM) expressed as % of the control. MTT assay was used as previously described and values correspond to mean ± SEM at least three independent experiments carried out in triplicate. Symbols represent statistically significant differences (One-way ANOVA, Tukey test; *p* - value < 0.05) when compared to: * control of respective concentration; # 10 µM treatment.

The increase of compounds concentration was translated in RenG2 cells' viability decrease, excepting for bromosphaerol and 12*S*-hydroxy-bromosphaerol (Figure 5.5). The effects were observed when RenG2 cells were exposed to concentrations of 10 and 50 µM of the aforementioned compounds. After exposition to 500 µM the effects observed did not revealed significant differences to the ones obtained with 50 µM concentration (One-way ANOVA, Tukey test; *p* - value < 0.05). In addition, it was also possible to observe that the highest decrease on RenG2 cells' viability was mediated by 12*S*-hydroxy-bromopshaerol and bromosphaerol when tested at a concentration of 10 µM. The treatment with 50 and 500 µM concentrations decreased the cells' viability in more than of 95%.

According to preliminary results attained with RenG2 and HBF cells, the cells exposition to 50 and 500 µM induced a non selective marked effect on the viability of both cell lines. Therefore, it was decided to select the concentration of 10 µM to be tested on BEAS-2B and SC-RenG2 cells.

- The cytotoxic activities induced by the *S. coronopifolius* isolated compounds (10 µM) on SC-DRenG2 cells following 72 hours exposure are displayed in Figure 5.6.

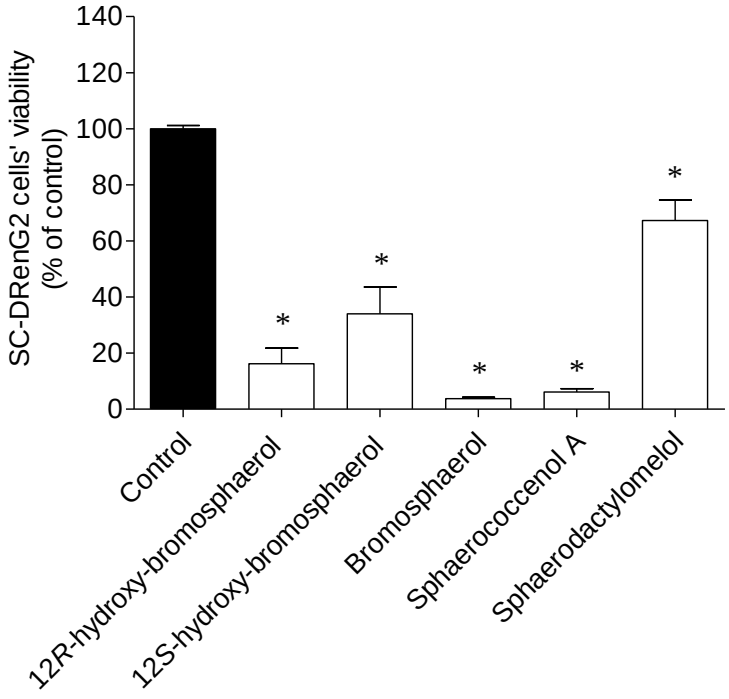

Figure 5.6. SC-RenG2 cells' viability following 72 hours of exposure to the *Sphaerococcus coronopifolius* isolated compounds (10 µM) expressed as % of the control. MTT assay was used as previously described and values correspond to mean ± SEM at least three independent experiments carried out in triplicate. Symbols represent statistically significant differences (One-way ANOVA, Dunnett's test; *p* - value < 0.05) when compared to: * control.

The results showed (Figure 5.6) that all compounds (10 µM; 72 hours) decreased SC-DRenG2 cells' viability exhibiting significant differences compared to control (One-way ANOVA, Dunnett's test; *p* - value < 0.05). Amongst them, the highest reduction of cell viability was mediated by bromosphaerol (3.75 ± 0.54% of viable cells) and sphaerococcenol A (6.16 ± 1.15 of viable cells). On the other hand, sphaerodactylomelol (67.25 ± 7.34% of viable cells) exhibited the lowest effect on cells' viability

In order to assess whether the *S. coronopifolius* isolated compounds were selective towards malignant cells, these compounds were tested on BEAS-2B cells, since those cells are derived from human normal bronchial epithelial cells.

- The cytotoxic activities induced by the *S. coronopifolius* isolated compounds (10 µM) on BEAS-2B cells after 72 hours of treatment are illustrated in Figure 5.7.

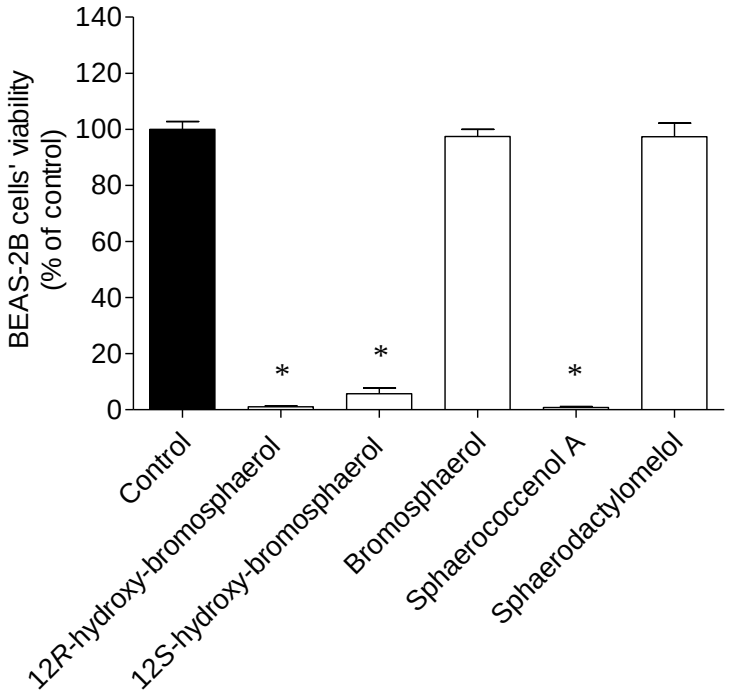

Figure 5.7. BEAS-2B cells' viability following 72 hours of exposure to the *Sphaerococcus coronopifolius* isolated compounds (10 µM) expressed as % of the control. MTT assay was used as previously described and values correspond to mean ± SEM at least three independent experiments carried out in triplicate. Symbols represent statistically significant differences (One-way ANOVA, Dunnett's test; *p* - value < 0.05) when compared to: * control.

Through the results obtained (Figure 5.7) it was possible to understand that bromosphaerol and sphaerodactylomelol did not induced cytotoxicity on BEAS-2B cells as compared to control, when tested at 10 µM. In contrast, 12*R*-hydroxy-bromosphaerol (1.01 ± 0.28% of viable cells), 12*S*-hydroxy-bromosphaerol (5.70 ± 2.07% of viable cells) and sphaerococcenol A (0.82 ± 0.29% of viable cells) decreased the cells' viability in more than of 90%.

Ten µM 12*R*-hydroxy-bromosphaerol and 12*S*-hydroxy-bromosphaerol reduced the viability of RenG2 cells but were non-cytotoxic towards HBF (Figure 5.5 and Figure 5.4, respectively). However, these compounds revealed toxicity when tested on BEAS-2B cells (Figure 5.7). Therefore, it was decided to perform dose-response assays for these two compounds. BEAS-2B cells were treated with different concentrations (2 - 10 µM) of 12*R*-hydroxy-bromosphaerol and 12*S*-hydroxy-bromosphaerol for 72 hours. MTT assay was used to access the induced effects. Results were expressed in percentage of control and are presented in Figures 5.8 and 5.9.

- BEAS-2B IC_{50} evaluation for 72 hours exposure to 12*R*-hydroxy-bromosphaerol (2 – 10 µM) displayed in Figure 5.8.

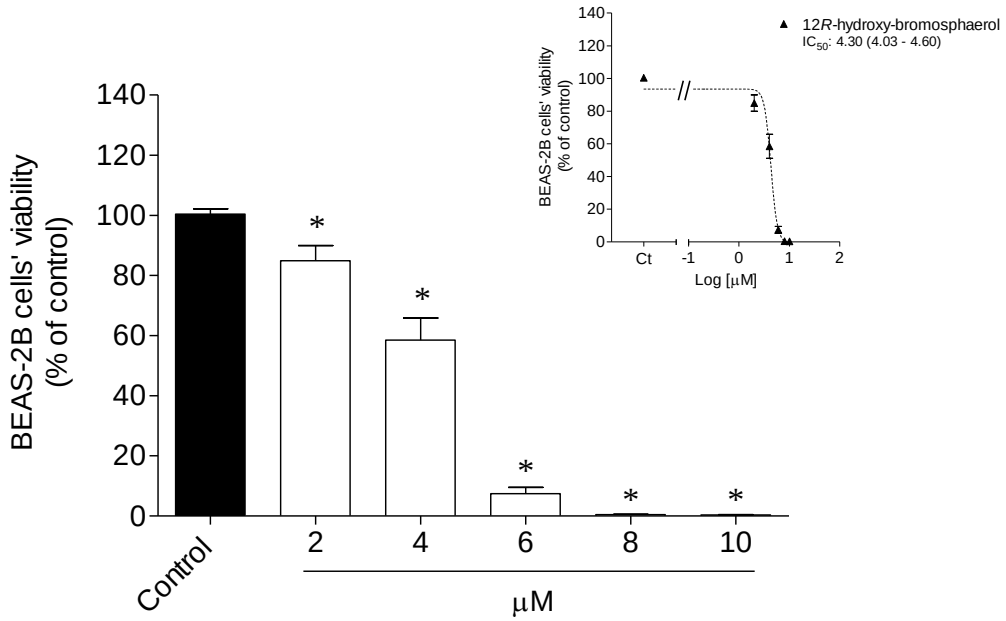

Figure 5.8. BEAS-2B dose-response curve following 72 hours exposure to12*R*-hydroxy-bromosphaerol (2 – 10 µM). The results were assessed by the MTT assay. Values correspond to mean ± SEM at least three independent experiments carried out in triplicate. Symbols represent statistically significant differences (One-way ANOVA, Dunnett's test; *p* - value < 0.05) when compared to: * control.

The results (Figure 5.8) revealed that the effects induced by 12*R*-hydroxy-bromosphaerol on BEAS-2B cells' viability were concentration-dependent ($R^2 = 0.93$). Moreover, all concentrations displayed significant differences when compared to control (One-way ANOVA, Dunnett's test; *p* - value < 0.05). Regarding dose-response assay, 12*R*-hydroxy-bromosphaerol exhibited an IC_{50} of 4.30 µM.

- BEAS-2B IC_{50} evaluation for 72 hours exposure to 12*S*-hydroxy-bromosphaerol (2 – 10 µM) presented in Figure 5.9.

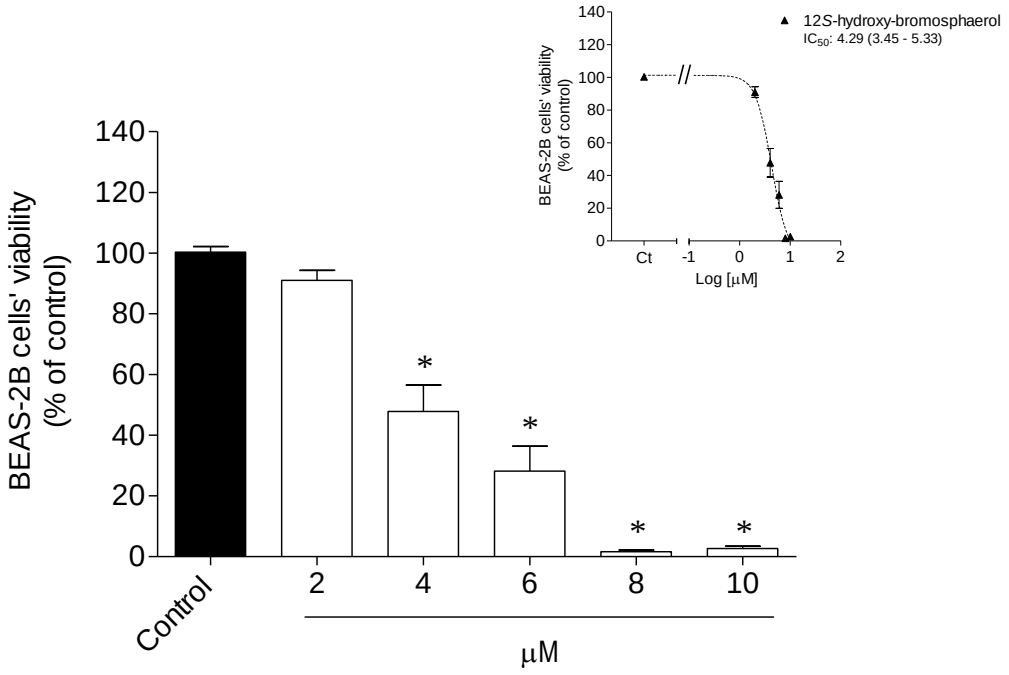

Figure 5.9. BEAS-2B dose-response curve following 72 hours exposure to 12*S*-hydroxy-bromosphaerol (2 – 10 µM). The results were assessed by the MTT assay. Values correspond to mean ± SEM at least three independent experiments carried out in triplicate. Symbols represent statistically significant differences (One-way ANOVA, Dunnett's test; *p* - value < 0.05) when compared to: * control.

The treatment of BEAS-2B cells with different concentrations of 12*S*-hydroxy-bromosphaerol revealed that the effects were concentration dependent ($R^2 = 0.87$). All concentrations tested, between 4 and 10 µM, exhibited statistically significant differences as compared to control (One-way ANOVA, Dunnett's test; *p* - value < 0.05). Concerning dose-response assay, 12*S*-hydroxy-bromosphaerol exhibited an IC_{50} of 4.29 µM.

Therefore, following the determination of IC_{50} for these two compounds, it was decided to test 12*R*-hydroxy-bromosphaerol, 12*S*-hydroxy-bromosphaerol and bromosphaerol at 4 µM during 72 hours on SC-DRenG2 cells' viability. The effects were revealed by the MTT assay. Results were expressed in percentage of control and are presented in Figure 5.10.

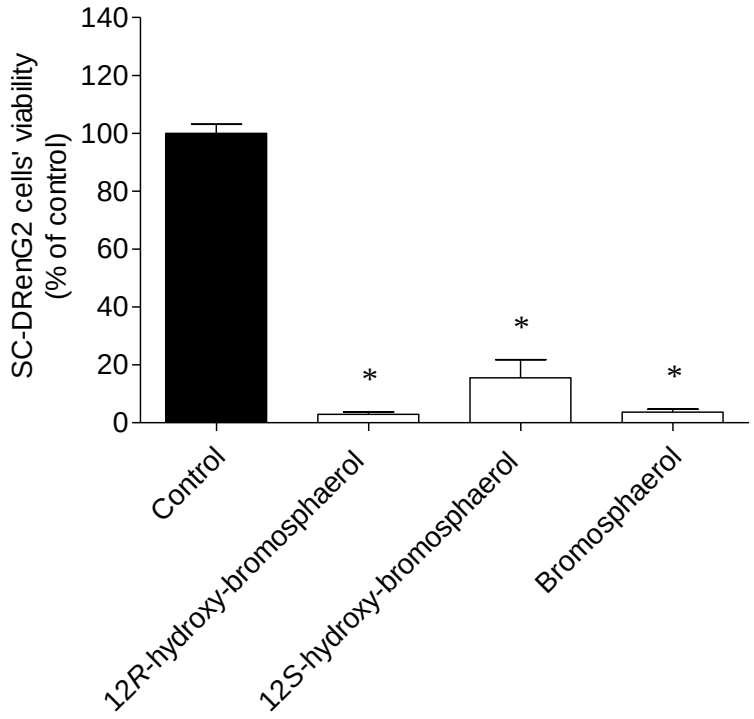

Figure 5.10. SC-DRenG2 cells' viability following 72 hours of exposure to 4 µM 12*R*-hydroxy-bromosphaerol, 12*S*-hydroxy-bromosphaerol and bromosphaerol, expressed as % of the control. MTT assay was used as previously described and the values correspond to mean ± SEM at least three independent experiments carried out in triplicate. Symbols represent statistically significant differences (One-way ANOVA, Dunnett's test; *p* - value < 0.05) when compared to: * control.

The results showed (Figure 5.10) that the treatment with 12*R*-hydroxy-bromosphaerol, 12*S*-hydroxy-bromosphaerol and bromosphaerol, at 4 µM, on SC-DRenG2 cells led to a decrease of SC-DRenG2 cells' viability in more than of 80%. All compounds revealed statistical significant differences as compared to control (One-way ANOVA, Dunnett's test; *p* - value < 0.05). Additionally, 12*R*-hydroxy-bromosphaerol and bromosphaerol were selected for the co-culture assay since they induced the highest reduction on SC-DRenG2 cells' viability (> 90%).

5.3.2. Effects of compounds on CSC´s formation on co-culture system

According to the results obtained in previous section (5.3.1) for the monocultures, 12*R*-hydroxy-bromosphaerol and bromosphaerol were selected to be tested on the co-culture system composed by RenG2 and HBF cells. Cells on co-culture system were treated with the aformentioned compounds (4 µM) for 72 hours and the presence of CSC´s was screened using the sphere-forming assay. The results were expressed as perimeter (µm) and are presented in Figure 5.11.

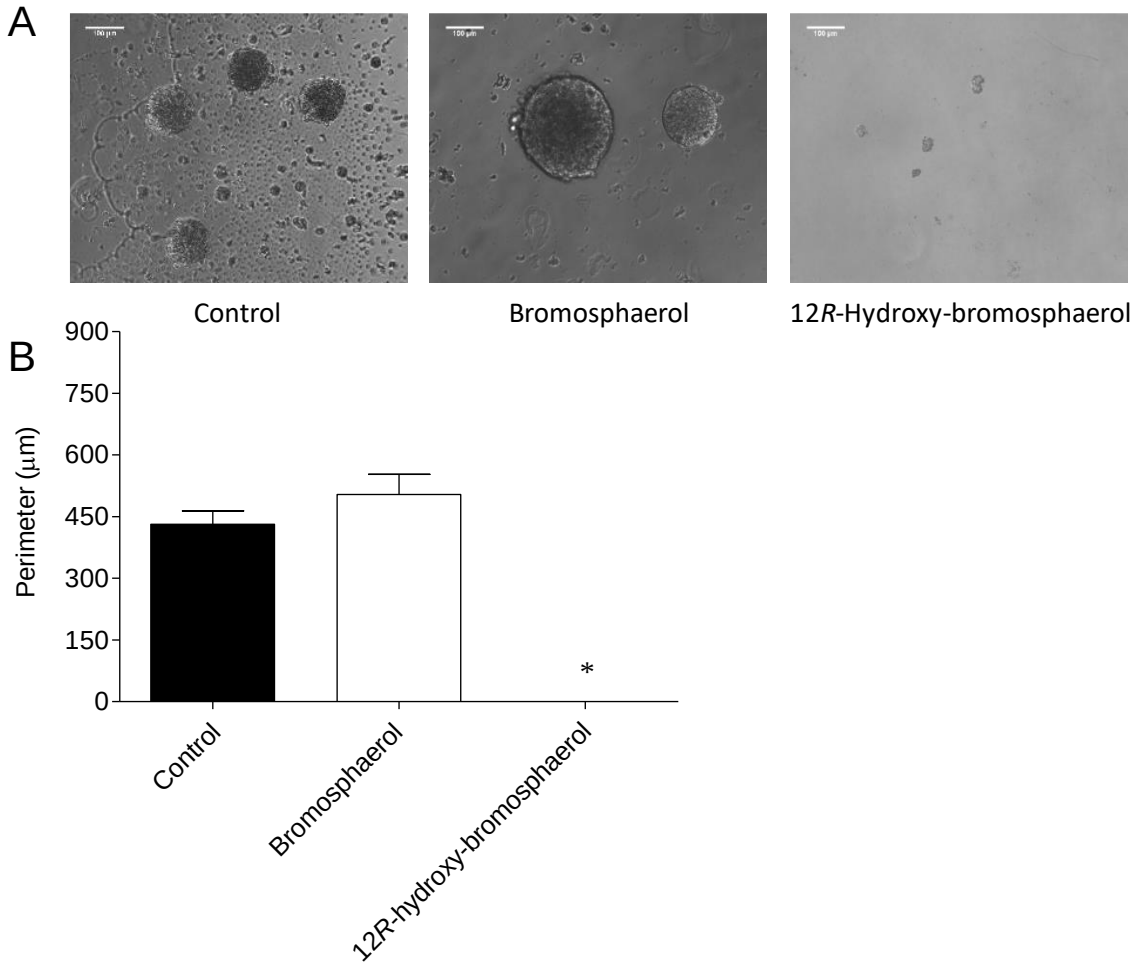

Figure 5.11. Spheres attained after co-culture system treated (4 µM) with 12*R*-hydroxy-bromosphaerol and bromosphaerol for 72 hours. The images are representative of each treatment accomplished (A). Perimeter (µm) analysis of the attained spheres after 2 weeks in culture under low-adherence conditions (B). Twenty spheres were measured per treatment. The results were revealed by the sphere-forming assay. Values correspond to mean ± SEM at least three independent experiments carried out in triplicate. Symbols represent statistically significant differences (One-way ANOVA, Dunnett's test; *p* - value < 0.05) when compared to: * control.

The results showed (Figure 5.10) that the treatment with 12*R*-hydroxy-bromosphaerol induced a totally abolishment of cancer cells development with stem-like phenotype. Additionally, bromosphaerol did not exhibit significant differences compared to control (One-way ANOVA, Dunnett's test; p - value < 0.05).

5.4. Discussion and main remarks

One of the major challenges in cancer diseases is associated with the drug resistance to traditional therapeutics being of utmost importance to improve the currently strategies and to develop new approaches to fight this burden (Housman *et al.*, 2014). Advances in cancer biology have demonstrated that this disease is much more complex than the simple continuous uncontrolled proliferation of cancer cells, which is sustained by several factors that contribute for cancer resistance and relapse (Zahreddine and Borden, 2013). Amongst them, tumor heterogeneity, which is associated with the presence of CSC´s, and tumor surrounding environment have revealed to be key players in cancer development (Albini *et al.*, 2015; Sun and Yu, 2015). Thus, the present chapter was designed to study the potential of *Sphaerococcus coronopifolius* compounds to suppress CSC´s on an *in vitro* human lung cancer co-culture system. The data gathered here indicates that 12*R*-hydroxy-bromosphaerol, 12*S*-hydroxy-bromosphaerol, and bromosphaerol have the ability to target cancer cells with more aggressive phenotype (SC-DRenG2) in monoculture system. However, in co-culture system, the 12*R*-hydroxy-bromosphaerol has revealed to hamper the development of spheres suggesting their potential to target CSC´s.

Concerning the experiments accomplished in monoculture system with RenG2 and HBF cells, 12*R*-hydroxy-bromosphaerol, 12*S*-hydroxy-bromosphaerol and bromosphaerol displayed selectivity towards cancer cells. On the other hand, sphaerodactylomelol did not exhibited cytotoxic activity, and sphaerococcenol A was not selective as it targets both RenG2 and HBF cell lines. In addition, the treatment performed on cells with a more aggresive phenotype, SC-DRenG2 cells, all compounds exhibited ability to reduce their viability being the highest reduction mediated by sphaerococcenol A and bromosphaerol. However, when tested on cells derived from human bronchial epithelial tissue (BEAS-2B), only bromosphaerol and sphaerodactylomelol did not induce cytotoxicity. Therefore, due to the lack activity of sphaerodactylomelol as well as the absence of selectivity evidenced by sphaerococcenol A, these two compounds were excluded from the co-culture assays. As the, 12*R*-hydroxy-bromosphaerol and 12*S*-hydroxy-bromosphaerol did not displayed cytotoxicity on HBF cells but exhibited cytotoxic activities on BEAS-2B, it was decided to performed dose-response assays in these cells to define their IC_{50}, which revealed an IC_{50} near to 4 µM. Since an ideal antitumor drug should be cytotoxic and specifically target cancer cells (Blagosklonny, 2004), 12*R*-hydroxy-bromosphaerol and bromosphaerol were

selected for the co culture assays as they selectively target SC-DRenG2 and do not target HBFs. Regarding cytotoxic activities of *S. coronopifolius* isolated compounds, few studies were driven to assess their potential on *in vitro* human lung cancer models. In previous report, sphaerococcenol A and bromosphaerol revealed to be inactive on tumor cell lines derived from lung cancer tissues, namely A549 and NSCLC-N6-L16 cells (Smyrniotopoulos *et al.*, 2008). These facts are not in agreement with results attained for RenG2 cells, as well as with the results observed in Chapter 4, in which those compounds exhibited cytotoxicity on *in vitro* human lung cancer models (A549, NCI-H226 and RenG2). On the other hand, our findings are according to the results achieved by Smyrniotopoulos and collaborators (2010), where sphaerococcenol A and bromosphaerol displayed cytotoxicity on A549 cells exhibiting an IC_{50} of 3.7 and 35 µM, respectively.

As referenced before, Rodrigues and co-workers (2018) developed a co-culture system using HBF and RenG2 cells demonstrating that microenvironment generated inside of the system led to the dedifferentiation of RenG2 cells into SC-DRenG2 cells, which exhibited more aggressive phenotype. In addition, the authors also verified that interleukin 6 (IL-6) and Activin-A cytokines play a central role in the stimulation of RenG2 cells dedifferentiation, since their neutralization impaired CSC´s formation. Concerning the experiments accomplished, 12*R*-hydroxy-bromosphaerol was the only compound that exhibited ability to prevent the development of spheres on low adherent conditions. On the other hand, bromosphaerol, that in the experiments driven in monoculture system seemed to be the most promising compound, did not exhibited activity. A possible explanation for the discrepant bromosphaerol behaviour observed in mono and co-culture systems may relay on the fact that IL-6 is a multifunctional cytokine found in the microenvironment of many tumors at high levels. It is involved in tumorigenesis through the regulation of cancer hallmarks and multiple signaling pathways being also associated to cancer therapeutic resistance (Culig and Puhr, 2012; Kumari *et al.*, 2016). Hence, due to the role of IL-6 on CSC´s formation and according to our findings we hypothesize that 12*R*-hydroxy-bromosphaerol may target this cytokine production, release and/ or signaling avoiding the dedifferentiation of RenG2 cells into CSC´s, possibly, in contrast to bromosphaerol. The fact that the expression of IL-6 seems to be stimulated by the microenvironment created inside of the co-culture system may explain why bromosphaerol, when tested in monoculture system with SC-DRenG2 cells, decrease their viability but in co-culture did not showed ability to inhibit the dedifferentiation of RenG2 cells. These results can suggest that this compound may not influence the levels of IL-6. For instance, diterpene glycosides named pseudopterosins, previously isolated from soft coral *Antillogorgia elisabethae*

demonstrated to block the key inflammatory signaling pathway NF-κB triggering the pro-inflammatory cytokines IL-6, TNFα, and monocyte chemotactic protein 1 on triple-negative breast cancer and monocytic leukemia cells (Sperlich *et al.*, 2017). Recent reports have also demonstrated that inteurleukin-8 (IL-8) produced by tumor tissues promotes loss of their epithelial characteristics and the acquisition of mesenchymal characteristics through epithelial-to-mesenchymal transition (EMT) process. These adaptations endorse the increase of metastatic dissemination, stemness, and intrinsic resistance (Waugh and Wilson, 2008; David *et al.*, 2016). In addition, for instance, IL-8 signaling pathway is also associated with the resistance of lung carcinoma cells to anticancer drug erlotinib (Fernando *et al.*, 2016). According to this point of view it is interesting to observe that dichloromethane extract derived from *S. coronopifolius* in LPS-stimulated endothelial cells displayed to be the most active extract promoting the highest decrease of IL-8 secretion (Salhi *et al.*, 2018). Therefore, since 12*R*-hydroxy-bromosphaerol was isolated from dichloromethane extract there is the possibility that results attained in the co-culture system may be related with the decrease of interleukins levels, such as IL-6, strengthen our hypothesis.

Despite advances on CSC´s biology have been only achieved in the last two decades, there are several reports attesting the therapeutically potential of marine natural products, including metabolites from terpenes chemical class (Reya *et al.*, 2001; Al-Hajj *et al.*, 2003; Ottinger *et al.*, 2012; De la Mare *et al.*, 2013; Terasaki *et al.*, 2017; Kim *et al.*, 2018). For instance, two polyhalogenated monoterpene stereoisomers (RU017 and RU018) and one sesquiterpene (smenospongine) isolated from the red alga *Plocamium cornutum* and sponge *Spongia pertusa Esper*, respectively, prevent the tumor sphere formation on *in vitro* breast cancer models. In the case of smenospongine, it promoted cell cycle arrest and intrinsic apoptosis as well as mediated the downregulation of specific stem cell markers, namely Nanog, Sox2, and Bmi1. This metabolite also inhibited the development of tumors *in vivo* (De la Mare *et al.*, 2013; Tang *et al.*, 2018). Additionally, the potential of algae to target CSC´s is also reinforced by the studies driven with the extracts derived from the brown alga *Saccharina japonica*, and the metabolite derived from fucoxanthin, fucoxanthionol, which inhibited tumor sphere formation on glioma and colorectal cancer stem cells, respectively (Terasaki *et al.*, 2017; Kim *et al.*, 2018).

Summarizing, in preliminary results attained on the monoculture system 12*R*-hydroxy-bromosphaerol and bromosphaerol displayed to be the most interesting compounds to be tested on co-culture system composed by HBF and RenG2 cells. Furthermore, in the experiments accomplished on the co-culture system, suggesting

instead 12*R*-hydroxy-bromosphaerol as the compound that completely abrogated CSC´s sphere formation. According to the best of our knowledge, this was the first approach that study the potential of *S. coronopifolius* compounds to target CSC´s on human lung cancer *in vitro* model.

5.5. References

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Chapter 6

General discussion and concluding remarks

6.1. General discussion and concluding remarks

Worldwide, cancer incidence rate has increased, posing a huge health challenge to the society, especially for low-income and middle-income countries, where is expected an increase of 60% of this burden by 2030. The growing magnitude of this phenomenon is directly related with population ageing and growth, but also with societal, economic and lifestyle changes (Fidler *et al.*, 2018; You and Henneberg, 2018; Znaor *et al.*, 2018). Thus, to fight this burden, a concerted effort by the research and healthcare communities is needed in order to provide society with new solutions for prevention, diagnosis and treatment of cancer diseases (Rahib *et al.*, 2014). NPs have played a crucial role in cancer therapy being estimated that more than 60% of currently anticancer drugs are natural biomimetic inspired (Cragg and Newman, 2009). From marine origin, six of nine drugs in the market are used in cancer therapy and twenty-four compounds undergoing clinical trials have indication for oncologic area attesting the potential of MNP as a source of anticancer drugs (Alves *et al.*, 2018). Amongst marine organisms, algae-derived compounds have displayed interesting antitumor activities mediating specific inhibitory activities on a number of key cellular processes linked to cancer development (Alves *et al.*, 2018).

Nowadays, it is widely recognized that the biological and physical conditions of surrounding environment strongly influence the production of secondary metabolites by the marine organisms (Hay, 1996; Ianora *et al.*, 2006). Our study driven with a specie (*Sphaerococcus coronopifolius*) collected from the Atlantic coast in Berlenga Nature Reserve (Peniche, Portugal) allowed to isolate seven compounds, two of them described for the first time as marine natural products, which were named as sphaerodactylomelol and 6-acetyl-sphaeroeudesmanol. Sphaerodactylomelol is a rare example of dactylomelane family that seems to be restricted to Atlantic specimens. 6-acetyl-sphaeroeudesmanol presents the same relative sterochemistry than a synthetic derivative of the austradiol acetate (Brennan and Erickson, 1982); however, this was the first report of its isolation from a natural resource. In fact, along the last four decades, *S. coronopifolius* has revealed to be a rich source of terpenes and more than forty compounds were already identified on this alga (Rodrigues *et al.*, 2015; Alves *et al.*, 2018). Most of those studies were carried out in the Mediterranean sea existing just a few studies performed in the Atlantic coast. These facts, as well as our results, demonstrated undoubtedly that this alga is a huge source of terpene natural compounds. In addition, the results attained suggest that the distinct biological and physical conditions of Mediterranean sea and Atlantic Ocean

can promote the synthesis of distinct compounds. One of the major challenges in marine biodiscovery is associated with the sustainable production of the compounds of interest to "supply" sufficient quantity for preclinical, clinical assays and further commercialization (Martins *et al.*, 2014; Jaspars *et al.*, 2016). The vast majority of the compounds here isolated displayed interesting yields, namely sphaerococcenol A, guaranteeing enough supply for our laboratory assays. Unfortunately, this limitation was observed for the new compound, 6-acetyl-sphaeroeudesmanol, making impossible to evaluate its antitumor potential due to the low yield obtained. However, since there are no studies about its pharmacological potential, it will be relevant to find strategies to obtain considerable amounts.

Sphaerococcus coronopifolius has been the focus of numerous studies to evaluate its pharmacological potential, e.g antimicrobial, anti-inflammatory, antimalarial, antiviral and cytotoxic activities (Etahiri *et al.*, 2001; Smyrniotopoulos *et al.*, 2008; Smyrniotopoulos *et al.*, 2010a; Smyrniotopoulos *et al.*, 2010b; Pinteus *et al.*, 2015; Rodrigues *et al.*, 2015; Alves *et al.*, 2016; Alves *et al.*, 2018; Salhi *et al.*, 2018). The biological activities observed are not uncommon, since most of compounds isolated and characterized from this alga belong to terpenes class. Terpenes have displayed a great range of biological activities, including the activities exhibited by *S. coronopifolius* extracts and compounds. Artemisinin and paclitaxel (Taxol®) are two of the most renowned terpene-based drugs applied in antimalarial and anticancer therapeutics, respectively (Wang *et al.*, 2005). Moreover, marine terpenes have aroused great interest due to their ability to induce potent cytotoxic activities (Wang *et al.*, 2005; El Gamal, 2010; Li *et al.*, 2015; Zhang and Liu, 2015). The results attained in the present study are in agreement with those facts, since the diterpenes studied mediated cytotoxic activities on different *in vitro* tumor cells models. Among all compounds tested, sphaerococcenol A displayed the strongest cytotoxicity, exhibiting a range of IC_{50} between 4.5 and 16.6 µM. Despite the interesting results, it is also true that the potency of the compounds effects were much smaller when compared to Taxol® anticancer drug. This drug when tested on *in vitro* breast cancer cell lines for 72 hours exhibited an IC_{50} range between 5 to 9 nM (George *et al.*, 2015). However, the effects of Taxol® on human lung cancer cells revealed to be most effective when the exposition time is prolonged (Liebmann *et al.*, 1993). For instance, Taxol® treatment accomplished on twenty-eight cell lines derived from non-small and small cell lung cancer for 3, 24, and 120 hours, exhibited schedule-dependent effect on cytotoxicity (Georgiadis *et al.*, 1997). Looking to time course effects displayed by sphaerococcenol A at 10 µM (~IC_{50} on MCF-7 cells) and 12*R*-hydroxy-bromosphaerol at 25 µM (~IC_{50} on MCF-7 cells) it was possible to observe that their effects were more intense for prolongued exposures i.e., 48 and 72 hours. These results, as well as the performance

of Taxol®, suggest that the effects of sphaerococcenol A and 12*R*-hydroxy-bromosphaerol may be potentiated.

It is also interesting to note that two of the isolated compounds, 12*R*-hydroxy-bromosphaerol and 12*S*-hydroxy-bromosphaerol, are diastereoisomers, which means that the compounds have the same molecular formula and constitutions around the carbon atoms but the spatial arrangement of the groups around those atoms differ only in the configuration around the chiral center. Sometimes, these spatial differences are enough to mediate distinct pharmacological activities as observed with thalidomide. This drug, released in the late 1950's, revealed to be a disaster, due to be a chiral compound with two enantiomeric states, *R* and *S*, facts that were unknow at the time. The (*R*)-enantiomer was responsible for the sedative effects, the therapeutic properties of interest, while (*S*)-isomer induced teratogenic activities provoking a range of severe birth defects (Vargesson, 2015). However, despite 12*R*-hydroxy-bromosphaerol and 12*S*-hydroxy-bromosphaerol displayed different spatial arrangement, this fact seems not influence the cytotoxic activies observed, since those compounds exhibited similar activities on the different *in vitro* human cancer cell lines studied.

Due to the challenges imposed by cancer diseases, every therapeutic strategy should be studied for the purpose to improve the performance of currently chemotherapeutic drugs. Some of the compounds that exhibited pharmacological activities less potent than currently anticancer drugs, a strategy to be explored can be their application in co-administration regime. In fact, MNP have demonstrated great potential to be used as co-adjuvants leading to increased therapeutic efficiency of standard drugs, reducing their undesirable side-effects, as well as to overcome drug resistance of some cancers (Williams and Jacobs, 1993; Dyshlovoy *et al.*, 2015; Chen *et al.*, 2017). For instance, algae-derived compounds, such as carotenoids and polysaccharides, when co-administrated with cisplatin and 5-FU, improved their antitumor activity and mitigated the immunocompetence damage induced by 5-FU (Zhou *et al.*, 2006; Lins *et al.*, 2009; Liu *et al.*, 2013; Wang and Zhang, 2014). In the same point of view, combined treatment of docetaxel with limonene, that belongs to terpenes class, enhanced its antitumor activity without inducing cytotoxicity on normal prostate epithelial cells (Rabi and Bishayee, 2009). Hence, since the cytotoxic activities mediated by *S. coronopifolius* metabolites were less powerful than most of anticancer drugs, such as Taxol®, in upcoming experiences, their use in co-administration should be evaluated.

In the studies here accomplished, the isolated compounds seem to have no selective activity for cancer cells, since as they target either 3T3 fibroblasts or malignant cells with the same proficiency. One of the strategies to avoid this problem can be to adjust the range of concentrations to sub-toxic concentrations. For instance, in Chapter 5, all compounds, excepting sphaerococcenol A, when tested at 10 μM, did not induce cytotoxicity on human bronchial fibroblasts (HBF) but reduced RenG2 cells' viability around 95%. However, when tested on BEAS-2B epithelial cells, only bromosphaerol did not induced cytotoxicity. Actually, the flawless antitumor drug should target cancer cells without inducing toxicity on normal cells (Blagosklonny, 2004). However, the classical chemotherapy that induces considerable side effects, due to poor selectivity, such as acute and chronic toxicity, continues to be a corner stone in cancer therapeutics because, it promotes a clear effect on cancer cells eradication (Bendale *et al.*, 2017; Gerhards and Rottenberg, 2018). To overcome this problem, several approaches have been suggested, including the development and improvement of drug delivery systems that recognize specific markers in the surface of cancer cells leading to an increase in the bioavailability, as well as decrease on the side-effects associated to anticancer drugs (Maranhão *et al.*, 2017; Senapati *et al.*, 2018). Regarding cytotoxic activities observed in Chapter 3, and previously discussed, the most part of compounds inhibited the HepG2 cells' proliferation at sub-toxic concentrations. In addition, the IC_{50} values observed for HepG2 cells were substantially higher than the values verified in Chapter 4, suggesting low hepatotoxicity. These facts are particularly interesting since the administration of anticancer drugs is usually associated to liver injuries, being necessary to assess the liver function of the patients undergoing chemotherapy, prior and during the treatment (King and Perry, 2001; Vincenzi *et al.*, 2016).

Although most of the *S. coronopifolius* compounds here studied had been isolated and identified some decades ago, few studies were accomplished to evaluate their cytotoxic activities (Smyrniotopoulos *et al.*, 2008; Smyrniotopoulos *et al.*, 2010a; Rodrigues *et al.*, 2015). Moreover, the intracellular signaling pathways mediated by those compounds underlying the observed activities are unknown. However, a grossly approach using computer-assisted phase-contrast microscopy (quantitative videomicroscopy) was performed for sphaerococcenol A and 12*S*-hydroxy-bromosphaerol, two of the most active compounds in the study accomplished by Smyrniotopoulos and co-workers (2010a) on apoptosis-resistant U373 glioblastoma cell line. The attained results indicated that activities mediated by those compounds were related with cytostatic and not with cytotoxic effects, since the U373 GBM cells treated with sphaerococcenol A exhibited a marked decrease in

mitosis entry, whereas 12*S*-hydroxy-bromosphaerol displayed a marked increase in mitosis length. According to this point of view, the possible involvement of those compounds in cell cycle regulation may explain and corroborate the results obtained in Chapter 3, where the treatment with sphaerococcenol A and 12*S*-hydroxy-bromosphaerol led to the inhibition of HepG2 cells' proliferation without inducing cytotoxicity. Furthermore, the findings achieved in our study (Chapter 4) suggest that the activities mediated by the compounds on MCF-7 may be associated with ROS generation, apoptosis and/ or DNA damage. Actually, the treatment accomplished with *S. coronopifolius* metabolites, excepting bromosphaerol, increased H_2O_2 production. Interesting, bromosphaerol was the only compound that induced DNA damage. Concerning hallmarks associated to apoptotic process, all compounds induced MMP depolarization, enhanced Caspase-9 activity and promoted DNA condensation and/or fragmentation.

Despite cell proliferation and apoptosis to be two distinct biological processes, they are strictly linked by different players, such as cell-cycle regulators and apoptotic stimulators that have multiple functions and can influence both processes (Pucci *et al.*, 2000; Alenzi, 2004; Maddika *et al.*, 2007). In fact, there are anticancer drugs that arrest cell cycle during mitosis inducing cytostasis, one condition poorly tolerated by any cell, and consequently, can trigger cell death by apoptosis (Rixe and Fojo, 2007). These facts allow to establish a relationship between the results obtained in our study, as well as the results observed by Smyrniotopoulos and co-workers (2010a) for sphaerococcenol A and 12*S*-hydroxy-bromosphaerol, suggesting that those compounds may have the ability to arrest cell cycle and to induce apoptosis. For instance, the marine anticancer drug ET-743, displayed the capacity to induce cell cycle arrest at S and G2/M phases, and subsequent apoptosis (Gajate *et al.*, 2002). However, to validate this hypothesis it will be essential to study the cell cycle progress on the cells previously treated with the compounds to understand if their effects affect its regulation. One of the key players involved in the regulation of cell cycle and/or induction of apoptosis in humans is the p53 tumor suppressor protein which plays multiple functions (Chen, 2016). The expression of p53 protein can be activated by multiple cellular stress factors, including increase of ROS levels and DNA damage (Roos and Kaina, 2006; Saha *et al.*, 2014). Through the treatments accomplished on MCF-7 cells, all compounds, excepting bromosphaerol, promoted H_2O_2 generation. Additionally, bromosphaerol was the only compound that induced DNA damage in the treatments performed on L929 cells. Due to these evidences, even as the fact that MCF-7 cells express p53 protein (Gartel *et al.*, 2003), it is possible that this protein may be involved in the observed effects.

The programmed cell death is characterized by two main apoptotic pathways, intrinsic and extrinsic pathways (Elmore, 2007). Regarding intrinsic pathway, mitochondria plays a central role, since it is the site where several anti-apoptotic and pro-apoptotic proteins interact but also a source of signals leading to the activation of protease enzymes named as caspases (Wang and Youle, 2009). Moreover, due to mitochondria involvement in cell death, this organell has aroused as chemotherapy potential target (He *et al.*, 2015). The findings gathered in our study suggest that the reduction of MCF-7 cells' viability may be mediated by the apoptotic intrinsic pathway. This hypothesis is supported by the results attained, since two hallmarks essential to trigger this pathway were strongly influenced by the treatment performed with *S. coronopifolius* compounds on MCF-7 cells, namely loss of MMP and Caspase-9 activation. In fact, a decrease in MMP is a hallmark of cells that preceed to apoptosis following mitochondria insults (He *et al.*, 2015) and activation of Caspase-9. This enzyme plays a key role in the intrinsic or mitochondrial pathway, leading to the activation of executioner Caspases -3, -6, and -7, resulting in cell death by apoptosis (Li *et al.*, 2017). In addition, the importance of Caspase-9 is reinforced by previous reports which described that the inhibition of its activity seems to be related with the development of chemotherapeutic resistance in specific types of *in vitro* human cancer models (Wu and Ding, 2002; Kuwahara *et al.*, 2003; Mueller *et al.*, 2003; Oudejans *et al.*, 2005).

Increasing levels of ROS, like H_2O_2, promote cell death processes such as apoptosis (Redza-Dutordoir and Averill-Bates, 2016). H_2O_2 has the ability to induce apoptosis by the intrinsic pathway leading to loss of mitochondrial membrane permeability, cytochrome *c* release and, thereby, activating the caspase cascade through its interaction with Apaf-1 protein. This events trigger the recruitment and activation of Caspase-9 and consequently of the downstream caspases (Singh *et al.*, 2007; Pinteus *et al.*, 2017b). Moreover, previous studies accomplished by our research group have also showed that MCF-7 cells treated with H_2O_2 showed a depolarization of MMP and an increase of Caspase-9 activity (Pinteus *et al.*, 2017a; Pinteus *et al.*, 2017b; Pinteus *et al.*, 2017c). In addition, apoptosis induced by H_2O_2 via the mitochondrial pathway is mediated by p53 as an upstream factor (Kitamura *et al.*, 1999; Singh *et al.*, 2007). In fact, the treatment of MCF-7 cells performed with the compounds, excepting bromosphaerol, led to an increase of H_2O_2 levels. Hence, according to this data, together with results attained in hallmarks associated to apoptosis, we hypothesize that the reduction of MCF-7 cells' viability may be mediated by ROS-activated intrinsic apoptotic pathway. However, additional assays will be needed to attest the involvement of ROS in cell death induced by *S. coronopifolius* compounds. For instance, it will be important to verify the ROS accumulation, as well as the cells' viability in the

presence or absence of antioxidant compounds, such as NAC (N-acetyl-cysteine), which has the ability to neutralize these species (Jiao *et al.*, 2016). Koul and co-workers (Koul *et al.*, 2017) proved that ROS generation mediated by Cladosporol A treatment has a crucial role in the induction of cell death by the apoptotic mitochondrial pathway because, in the presence of NAC, the compound was unable to elevate ROS levels and failed the induction of cell death. Our hypothesis is also supported by the results mediated by spatane diterpene, previously isolated from *Stoechospermum marginatum* brown alga, which increased ROS production and thereby trigger the intrinsic apoptotic pathway as described before (Velatooru *et al.*, 2016).

The mechanism of action of many anticancer drugs is associated with the induction of high levels of DNA damage, that trigger cell cycle checkpoints, leading to cell cycle arrest and/or cell death (Swift and Golsteyn, 2014). This strategy is based on two fundamental assumptions. The first, one relies on the fact that cancer cells divide more often than normal cells, and second, that normal cells have the mechanisms of DNA damage response and DNA repair mechanism intact, having the ability to arrest proliferation for more efficient DNA repair, and to avoid the damage mediated by the drug (Swift and Golsteyn, 2014; Liu *et al.*, 2015). NPs have the ability to induce DNA damage leading to cell cycle arrest and to apoptosis by mitochondrial pathway (Srivastava *et al.*, 2016; Choi *et al.*, 2017; Chung *et al.*, 2017). Thereby, according to the findings observed following bromosphaerol treatment, it is possible to suppose that its effects may be associated with induction of DNA damage leading to activation of cell death by the apoptotic intrinsic pathway. Amongst all compounds, bromosphaerol was the only that induced DNA damage levels on L929 cells. However, it is also true that when tested at similar concentration (25 μM, the IC_{50} for MCF-7 cells, 24h), L929 cells were not affected. Moreover, since the treatment on MCF-7 cells was prolongued, this possibly drove to DNA damage or activate DNA repair mechanisms recovering the effects mediated by bromosphaerol observed on L929 cells after 3 hours. The preliminary results attained for the remaining compounds suggest that their activities may be not related with the induction of DNA damage. Nevertheless, more studies are needed to validate the different hypothesis, including the evaluation of the genotoxic effects along the time on L929 cells, accomplishing those assays on MCF-7 cells, as well as studying the cell cycle.

Since the human genome has already been sequenced (Venter *et al.*, 2001), the use of genomics and proteomics tools can be interesting approaches to deeply dissect the intracellular signaling pathways involved in the effects mediated by the *S. coronopifolius*

bromoterpenes. Actually, the use of those tools will give us a general view about the differential expression of genes and proteins between the clustering genes or proteins involved in specific intracellular signaling pathways (Martinez *et al.*, 2001; Parikh *et al.*, 2010; Mieczkowski *et al.*, 2012).

In order to assess the potential candidates to therapeutic drugs, including anticancer, accomplishment of experiences in different models should be assured (Cagan and Meyer, 2017). For instance, tumor microenvironment plays a central role in cancer development; however, this factor is not taken into account in studies performed in mono-culture systems, since there is no establishment of cell-cell interactions between different cellular players (Miki *et al.*, 2012). Therefore, the antitumor potential of isolated compounds was studied in co-culture system aiming to understand their potential to prevent the development of cancer cells with stem-like phenotype. The obtained findings revealed that the most promising compound was 12*R*-hydroxy-bromosphaerol, which seems to block the differentiation of RenG2 cells, since cancer cells with stem-like phenotype after expansion were not observed. This was the first approach accomplished with those compounds in co-culture system to target CSC´s, opening a new research line to be explored. As discussed previously in Chapter 5, the cytokines IL-6 and Activin-A seem to play a key role in the differentiation of RenG2 cells into CSC´s cells (Rodrigues *et al.*, 2018). Due to the involvement of these cytokines, as well as the fact that a recent report showed that dichloromethane extracts of *S. coronopifolius* possess anti-inflammatory activities (Salhi *et al.*, 2018) lead us to suggest that the effects mediated by 12*R*-hydroxy-bromosphaerol may be linked with those cytokines. IL-6 has been associated with several biological processes essential to cancer development, including progression and metastasis through the regulation of tumor microenvironment and cancer stem cells (Rossi *et al.*, 2015). These facts have increased the interest of research community in IL-6 as target for cancer therapeutics (Hong *et al.*, 2007; Rossi *et al.*, 2015). In this area, MNP have displayed ability to reduce the levels of IL-6 contributing to the antitumor activities promoted by those compounds (Roberto *et al.*, 2014; Chen *et al.*, 2017; Sperlich *et al.*, 2017). For instance, the treatment accomplished with the anticancer drug etoposide on human colorectal cancer HCT116 increases the expression of IL-6 leading to activation of JAK1-STAT3 pathway that mediates anti-senescence and promotes tumor growth. In addition, the increase of IL-6 levels drive the inhibition of p53, TGF-β and apoptosis in cancer cells treated with etoposide. However, etoposide treatment supplemented with oligo-fucoidan, a sulfated polysaccharide derived from algae, prevented the expression of IL-6 and cooperates with p53 suppressing ATM signaling and tumor progression (Chen *et al.*, 2017). Also, triple-negative breast

cancer and monocytic leukemia cells treated with MNP pseudopterosin suppressed cytokine release, promoting the down-regulation of IL-6 and IL-8 (Sperlich *et al.*, 2017). According to this evidence, as well as the facts observed by Rodrigues and co-workers (2018) in this co-culture model, make us to believe that the activities mediated by the 12*R*-hydroxy-bromosphaerol may be associated with the production, release and/ or signaling of these interleukins.

In conclusion, the chemical analysis of *Sphaerococcus coronopifolius* specimens collected from Berlenga Nature Reserve, Peniche, Portugal, allowed to isolate seven compounds, including five already known terpenes, identified as alloaromadendrene (**1**), bromosphaerol (**3**), sphaerococcenol A (**4**), 12*S*-hydroxy-bromosphaerol (**5**), and 12*R*-hydroxy-bromosphaerol (**7**), and two new natural metabolites, named as sphaerodactylomelol (**2**) and 6-acetyl-sphaeroeudesmanol (**6**). Concerning the cytotoxic actitivities, *S. coronopifolius* compounds exhibited an IC_{50} range between 4.47 to 89.41 µM. Sphaerococcenol A revealed the highest cytotoxicity (range of IC_{50} between 4.47 to 16.59 µM) and sphaerodactylomelol exhibited the lowest cytotoxicity (range of IC_{50} between 33.04 and 89.41 µM). In addition, compounds tested did not showed selectivity for any specific tumor cell. Regarding the study of intracellular signaling pathways, the attained results suggested that the cytotoxic activities induced by 12*R*-hydroxy-bromosphaerol (**7**), 12*S*-hydroxy-bromosphaerol (**4**), sphaerococcenol A (**3**) and sphaerodactylomelol (**2**) on MCF-7 cells were mediated by ROS-activated intrinsic apoptotic pathway. However, the cytotoxic activities promoted by bromosphaerol (**3**) seems to be associated with induction of DNA damage, triggering intrinsic apoptotic pathway. Relating to co-culture experiences, the most promising compound was 12*R*-hydroxy-bromosphaerol (**7**), which exhibited the highest ability to abrogate cancer cells with stem-like phenotype.

Altogether, the integrative strategy used in this thesis succeeded in attaining most of the initially established goals as well as to answer the questions raised, namely: 1) it was proved that the distinct conditions of Atlantic Ocean and Mediterranean sea seem to contribute to the production of compounds with different features. Despite the major metabolites isolated in the present work had alread been described for *S. coronopifolius* specimens collected in Mediterranean sea, two new compounds from natural origin were identified; 2) it was showed that the potency of the effects of the studied compounds was medium-high (4.5 to 90 µM); however they did not exhibit selective activity for tumor cells as well as among the human cancer cell´s models used; 3) it was evidenced that *Sphaerococcus coronopifolius* isolated compounds induced cytotoxicity by intracellular

signaling pathways linked to ROS generation, apoptosis and DNA damage; 4) it was defined that amongst the isolated compounds, the 12*R*-hydroxy-bromosphaerol displayed high activity in the prevention of cancer cells development with stem-like phenotype, suggesting potential to overcome the challenge imposed by tumors resistance to chemotherapeutic drugs.

6.2. References

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Chapter 7

Future perspectives

Overall, the scientific work developed into the present thesis allowed to achieve several findings, opening new research opportunities to be explored. Therefore, different approaches can be outlined to reinforce the results here attained.

Regarding the isolation and identification processes, the major metabolites of F1, F2 and F3 fractions derived from *S. coronopifolius* dichloromethane extract were identified. Therefore, due to the great potential of this alga as a source of new compounds, it is of utmost interest to proceed with the chemical characterization of the remaining fractions being expectable to discover new compounds. In addition, it will be relevant to do efforts in order to increase the available mass of the new compound 6-acetyl-sphaeroeudesmanol to evaluate its antitumor potential.

On the other hand, in order to evaluate the antitumor potential of *S. coronopifolius* compounds in all aspects, their application in co-administration with standard anticancer drugs should also be explored. Moreover, to overcome the cytotoxic activities mediated by *S. coronopifolius* compounds on normal cells and potentiate their effects on cancer cells, the use of nanotechnology can be an interesting strategy.

In order to strengthen the evidences related to the apoptosis induction, it will be important to evaluate other key players involved in this signaling pathway, namely the expression of pro-apoptotic (e.g. Bax) and anti-apoptotic (e.g. Bcl-2) proteins, release of cytochrome C, as well as the activation of Caspase -7. In addition, the study of cell cycle as well as the involvement of p53 protein should be studied. In line with these experiences, it will be relevant to evaluate the role of ROS in the cytotoxic activities observed, it will be important in upcoming experiences to perform the studies in the presence or absence of antioxidant compounds, such as NAC (N-acetyl-cysteine), which has the ability to neutralize these species.

Concerning the co-culture assays, it will be interesting to verifiy if the effects mediated by 12*R*-hydroxy-bromosphaerol are associated with the production, release and/ or signaling of interleukin-6. Moreover, due the promising results mediated by 12*R*-hydroxy-bromosphaerol on co-culture system it will be interesting to understand if this compound influences the regulation of specific stem cell markers, such as Nanog, Sox2, and Bmi1, as well as to validate its potential on human tumor xenograft model. Regarding genotoxic assays, in upcoming experinces, the time-course effects on L929 cells as well as on MCF-7 cells should be performed.

The accomplishment of these approaches, together with the main results attained in this thesis will contribute to the better understand of the effective pharmacological potential of the red alga *Sphaerococcus coronopifolius* compounds in cancer therapeutics.

Annexes

Annex I

Chapter 1 article

frontiers in Pharmacology

REVIEW
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From Marine Origin to Therapeutics: The Antitumor Potential of Marine Algae-Derived Compounds

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Marine environment has demonstrated to be an interesting source of compounds with uncommon and unique chemical features on which the molecular modeling and chemical synthesis of new drugs can be based with greater efficacy and specificity for the therapeutics. Cancer is a growing public health threat, and despite the advances in biomedical research and technology, there is an urgent need for the development of new anticancer drugs. In this field, it is estimated that more than 60% of commercially available anticancer drugs are natural biomimetic inspired. Among the marine organisms, algae have revealed to be one of the major sources of new compounds of marine origin, including those exhibiting antitumor and cytotoxic potential. These compounds demonstrated ability to mediate specific inhibitory activities on a number of key cellular processes, including apoptosis pathways, angiogenesis, migration and invasion, in both *in vitro* and *in vivo* models, revealing their potential to be used as anticancer drugs. This review will focus on the bioactive molecules from algae with antitumor potential, from their origin to their potential uses, with special emphasis to the alga *Sphaerococcus coronopifolius* as a producer of cytotoxic compounds.

Keywords: cancer, marine natural products, seaweeds, intracellular signaling pathways, biodiversity, marine chemical ecology, *Sphaerococcus coronopifolius*

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INTRODUCTION

Natural products (NPs) have been used as therapeutic agents for the treatment of a wide spectrum of illnesses for thousands of years, playing an important role in meeting the basic needs of human populations. In 1985, World Health Organization estimated that ∼65% of world population ensured their primary health care using predominately plant-derived traditional medicines, existing a less prevalence in developed countries (Cragg and Newman, 2013). Due their unusual chemical features, NPs have functioned as scaffolds for the development of new products with huge therapeutic and industrial potential. Moreover, these compounds present a greater efficiency and specificity with target sites since were originated in co-evolution with biological systems. These interesting compounds result from the interactions between organisms and their environment,

which promote the production of diverse complex chemical compounds by the organisms to increase their survival and competitiveness (Mishra and Tiwari, 2011).

Comparing with terrestrial organisms, marine organisms do not have a distinguished history of use in traditional medicine. However, in the last 50 years, advances in new technologies and engineering such as scuba diving techniques, manned submersibles and remotely operated vehicles (ROVs) opened up the marine environment to scientific exploration (Cragg and Newman, 2013). The coexistence of several species in these habitats of limited extent increases their competitiveness and complexity. For example, sessile organisms such as algae, corals, sponges, and other invertebrates are in constant competition and many of them have evolved chemical means to defend themselves against predation or overgrowth of competing species or, conversely, to subdue motile prey species for ingestion. These chemical adaptations are generally defined as "secondary metabolites" and involve different classes of chemical compounds, which have evidenced great pharmacological potential (Simmons et al., 2005). Therefore, marine organisms have revealed to be an exceptional reservoir of NPs, some of them with different structural features from those of terrestrial sources. Despite considerable challenges, some marine compounds arrived in the market and are currently used in therapeutics, providing a useful roadmap for future translational efforts (for details, please see topic 4; Arizza, 2013). Among the different illnesses, cancer is a growing threat to public health, particularly in developed countries, and it is expected that their occurrence and associated deaths will increase in the next years (American Cancer Society, 2015). This problem is directly associated with the growth and aging of the population and the adoption of behaviors that contribute to increase cancer risk (American Cancer Society, 2015). Moreover, owing to the tumor cells resistance to drugs, significant toxicity, and undesirable side effects observed with synthetic drugs, there is an urgent need for new antitumor drugs development (Sawadogo et al., 2015; Torre et al., 2015). Given that cancer is a multifactorial and multi-targeting disease that cannot be prevented by mono-targeted therapies, many researchers have focused their efforts toward NPs, especially those from marine environments, to identify novel anticancer compounds. Today, it is estimated that more than 60% of anticancer drugs presently in the market are of natural origin (Cragg and Newman, 2009). In addition, there are several natural compounds originated or derived from marine life presently undergoing clinical trials with oncological indications (AndisInsight, 2018; Calado et al., 2018; EMA, 2018a,b; FDA, 2018a,b; Mayer, 2018).

This review contains six topics condensing the importance of marine resources as source of antitumor compounds, summarizing the most important works accessing the potential of algae as source of marine drugs for cancer therapeutics.

CANCER BIOLOGY—GENERAL OVERVIEW

Cancer is one of the major human health problems worldwide, with high social and economic impacts. There is evidence of this disease in antiquity, dating back to the times of the Pharaohs in ancient Egypt and the classical world (Nobili et al., 2009). Currently, worldwide, cancer is responsible by one in each seven deaths causing more deaths than AIDS, tuberculosis and malaria combined (American Cancer Society, 2015). Only surpassed by cardiovascular diseases, cancer is the second leading cause of death in high-income countries while being the third leading cause of death in low- and middle-income countries following cardiovascular, infectious and parasitic diseases (American Cancer Society, 2015). By 2030, it is estimated that the incidence of this illness grows to over 21.7 million new cases and 13 million deaths (American Cancer Society, 2015). Beyond the social impact, cancer is also associated with high financial costs at the individual and social level for both the person with cancer and for society as a whole. For example, in 2011 in the US the direct medical costs (total of all health care expenditures) associated with cancer was estimated in $88.7 billion, which are related with hospital outpatient or office-based provider visits, patient hospital stays and medical prescriptions (American Cancer Society, 2015).

However, what we simply call "human cancer" comprises, in fact, more than 100 different diseases that result from the continuous uncontrolled proliferation of cancer cells (Urbano et al., 2011), which have the capability to invade organs and normal tissues, as well as metastasizing through the body. These cells do not respond properly to the signals that regulate their normal behavior (Schulz, 2007; Cooper and Hausman, 2013). In line with this view, Hanahan and Weinberg (2000) published a review article that combined information about cancer biology and defined six hallmarks (sustaining proliferative signaling, resisting cell death, inducing angiogenesis, enabling replicative immortality, activating invasion and metastasis and evading growth suppressors) that all cancer cells have and that are responsible for their malignant properties. Subsequently, an upgrade of this list was done adding two new hallmarks, deregulating cellular energetics and evading immune destruction. Nevertheless, the occurrence of these hallmarks is directly associated with the genome instability, which is responsible by genetic diversity that stimulates their acquisition, and inflammation and promotes multiple hallmark functions (Hanahan and Weinberg, 2011). Recently, case–control metagenomics studies suggest that dysbiosis in the commensal microbiota is also associated with various cancer types adding microbiome as an additional hallmark (Rajagopala et al., 2017). The complexity of tumors represents a great challenge for therapeutic approaches, as experimental evidence exists that each core hallmark capability is regulated by partially redundant signaling pathways (Hanahan and Weinberg, 2011). Consequently, targeted therapy mediated by drugs that only act on one key pathway in a tumor may not be enough to "switch off" a hallmark capability completely. As a result some cancer cells can survive maintaining a basal function awaiting an adaptation of their progeny to the selective pressure imposed by the drug. This adaption can be accomplished by genetic changes, epigenetic reprogramming, or remodeling of the stromal microenvironment. All of these processes can contribute for restitution of the functional capability, allowing renewed tumor growth and consequently clinical relapse (Hanahan and Weinberg, 2011). Nevertheless, the drug resistance of tumor cell lines can also be mediated by other mechanisms, such as

drug efflux, suppression of drug activity, changes in cellular targets, enhanced DNA repair, inability to induce cell death and the epithelial-mesenchymal transition (Housman et al., 2014). Among all the treatments currently used in cancer (surgery, radiotherapy, hormonal treatment and immunotherapy, adjunct therapy, and chemotherapy), chemotherapy continues to play an extremely important role. However, its effectiveness is limited in some cases by the existence of drug resistance, making it necessary to define optimal combinations for therapeutic strategies that ensure an efficient elimination of the tumor. Moreover, in the last decades, with the continuous growth of cancer cases and concerns over toxicity, tumor cell resistance, the development of secondary cancers and the unwanted side effects observed with synthetic drugs, there has been an increased interest in exploiting NPs for cancer treatment (Newman and Cragg, 2012; Sawadogo et al., 2015).

ROLE OF THE MARINE CHEMICAL ECOLOGY IN THE PRODUCTION OF BIOACTIVE METABOLITES ON ALGAE

The oceans represent a vast area of the planet and play a fundamental role in its dynamic. Their physics, chemistry and biology are key elements in the functioning of the earth system, providing an interconnection between the different natural systems (terrestrial, freshwater, estuarine, coastal, and oceanic) and a range of valuable ecosystem services (Atkins et al., 2011; Halpern et al., 2012; Botana and Alfonso, 2015). Their essential role is further noted by the significant fraction of the Earth's biodiversity that oceans harbor (Brahmachari, 2015). According to the 33 animal phyla listed by Margulis and Chapman (2009), 32 of them are represented in aquatic environments, with 15 exclusively marine, 17 found in marine and non-marine environments (with 5 of these having more than 95% of their species only in marine environments), and only one exclusively non-marine (*Onychophora*). A recent study predicted the existence of ∼8.9 million eukaryotic species, of which ∼2.2 million are marine organisms, suggesting that around 86% of the species on the earth, and 91% in the ocean, have not yet been described (Mora et al., 2011; Cragg and Newman, 2013; Berkov et al., 2014). The existence of a huge diversity of life forms in the oceans is associated with the very exigent, competitive and aggressive surrounding that promotes specific and complex interactions, both inter-species and intra-species. During the evolutionary period, many species share a common environment establishing well-balanced associations between them. Inside of these communities several organisms survive and live in close association with other species, both macro (e.g., algae, sponges, and ascidians) and micro (e.g., bacteria, fungi, and actinomycetes) in order to ensure their survival (Da Cruz et al., 2012; Graça et al., 2013; Horta et al., 2014; Smith et al., 2018). Many of these complex interactions are mediated by chemical signals, which play a crucial role at the organizational level in the marine environment (Hay, 2009). These chemical cues constituting much of the language of sea life and are the utmost importance for several marine species which have not some senses such as vision and hearing; nevertheless even species that see and hear rely on chemical cues (Botana and Alfonso, 2015). Interactions mediated by chemical signals play a crucial and decisive role in ecological processes. These signals influence population structure, community organization and ecosystem function. In addition they are involved in the definition of escape strategies, commensal associations, partners and habitats, competitive interactions, feeding choices and energy and nutrients transfer within and among ecosystems (Hay, 2009).

Among marine organisms, algae are a clear example in which chemical signals play a fundamental role in ecological processes. These signs are involved in the growth and survival in extremely exigent conditions, giving them competitive advantages relative to other marine organisms including against predators and competitors. One such piece of evidence has recently been observed in coral reefs, which are in dramatic global decline, with algae commonly replacing them. Several studies have observed that algae damage corals directly or colonize opportunistically, suppressing coral recruitment through the production of specific chemical cues repulsing the recruits (Rasher and Hay, 2010, 2014; Dixson et al., 2014). Recently, Rasher et al. (2011) identified four compounds (two loliolide derivatives and two acetylated diterpenes) from two algae as potent allelochemicals which directly damage corals. Marine organisms sometimes face the dilemma of how to allocate the limited resources available, having to strategically adapt. Usually these decisions may have consequences on their growth, reproduction, or ability to counteract biological (e.g., predators, maintenance of unfouled surfaces, paralyzing their prey, etc.) and/or physical stress (e.g., UV light, temperature, nutrient availability, high pressure, salinity, oxygen content, etc.) (Winter et al., 2010; Botana and Alfonso, 2015). The production of these types of compounds has also been revealed to be an important weapon for the successful invasion of non-indigenous species into new ranges. For instance, the invasive red alga *Bonnemaisonia hamifera* has become one of the most abundant species in Scandinavian waters. According to Svensson et al. (2013) the high capacity of this alga to colonize these waters seems to be linked with the presence of a specific chemical compound (1,1,3,3-tetrabromo-2-heptanone). The production of this metabolite inhibit the settlement of propagules on its thallus and on surrounding surfaces, achieving a competitive advantage over native algae (Svensson et al., 2013). Chemical cues also play an important role in the symbiotic interactions established between algae and microorganisms. The cross-kingdom interactions between them are not restricted to the exchange of macronutrients, including vitamins and nutrients but also include the use of infochemicals with different functions, establishing a tight relationship and enabling them to interact as a unified functional entity (Egan et al., 2013; Wichard, 2015). For instance, associated microorganisms are responsible to produce compounds of utmost importance which mediate essential ecological functions in the development and growth of algae species including quorum sensing signaling molecules, compounds with biological activities, substances that promote the growth and other effective molecules compounds (Singh and Reddy, 2014). Some of

these compounds, such as bacterial morphogenetic compounds, dimethylsulfoniopropionate (DMSP), the amino acids proline and alanine, halogenated furanones and fucoxanthin, play important roles in the ecological function, interfering with the surface fouling of others organisms as well as with the vital functions performance of the algae. These compounds prevent the attachment of certain bacteria (e.g., *Cytophaga* sp.) and support the fixation of others (e.g., *Rheinheimera baltica*), controlling the community composition and abundance of the algae-associated bacteria (Saha et al., 2011, 2012; Spoerner et al., 2012; Egan et al., 2013). Marine organism interactions promote the production of a high diversity of marine NPs with quite specific and potent activities, representing an enormous source of new compounds with potential for biotechnology applications providing economic and human benefits.

MARINE NATURAL PRODUCTS AS A SOURCE OF NEW DRUGS AND CURRENT CLINICAL PIPELINE

Along of the evolution, marine organisms developed exceptional metabolic capacities through the production of compounds with quite specific and potent activities (Murray et al., 2013; Martins et al., 2014). These compounds, often defined as secondary metabolites, are generally limited to a particular taxonomic family, genus, species or even organism, characterized by their wide heterogeneity, and often constitutes a very small fraction of the total biomass of the organism (Ianora et al., 2006; Avila et al., 2008; Martins et al., 2014). Predominantly, their production occurs in sessile or slow-moving organisms (e.g., algae, sponges, cnidarians, tunicates and bryozoans) that, without effective escape mechanisms or structural protection, ensure their protection through chemical defense (Noyer et al., 2011; Botana and Alfonso, 2015). Nevertheless, many organisms have the capacity to sequester secondary metabolites from their diet and then derivatize them to more or less toxic forms can be these used for functions different from their roles in the original producer (Ianora et al., 2006; Kicklighter et al., 2011; Botana and Alfonso, 2015; Gotsbacher and Karuso, 2015). Moreover, since natural compounds released into the water are rapidly diluted, they need to be highly potent to retain their efficacy (Haefner, 2003). For these reasons, it is widely accepted that a huge number of NPs and novel chemical entities that exist in the oceans could be useful for providing sustainable economic and human benefits.

To date, different types of secondary metabolites (e.g., terpenoids, alkaloids, polyketides, peptides, shikimic acid derivatives, sugars, steroids, and a large mixture of biogenesis metabolites) were isolated from marine organisms and found to exhibit many biological activities (antimicrobial, antitumor, antidiabetic, anticoagulant, antioxidant, anti-inflammatory, antiviral, antimalarial, antitubercular, anti-aging antifouling, and antiprotozoal) with huge industrial and therapeutic potential (Blunt et al., 2013, 2014; Mayer et al., 2013, 2017; Agrawal et al., 2018). Marine NPs have exhibited rare and unique chemical structures, upon which the molecular modeling and chemical synthesis of new drugs can be based on (Dias et al., 2012; Botana and Alfonso, 2015). Since this kind of compounds are originated from nature present several advantages compared with synthetic compounds such as a chemical diversity, biochemical specificity, binding efficiency, and affinity to interact with biological systems, making them interesting structures for development of new drugs (Martins et al., 2014).

The marine biodiscovery and vision of marine-derived drugs on the market had their beginning in the early 1950s with Bergmann, who isolated and identified two nucleosides, spongouridine and spongothymidine, from the Caribbean sponge *Cryptotethya crypta* (previously known as *Tethya crypta*). These discoveries led researchers to synthesize analogs, *Ara-A* (*Vidarabine*®, *Vidarabin Thilo*®) and *Ara-C* (*Cytarabine*, *Alexan*®, *Udicil*®), the first marine derived compounds that have reached the market as antiviral and antitumor drugs, respectively, (Newman et al., 2009; Botana and Alfonso, 2015). Over the last 50 years was reported the isolation of more than 30,000 new compounds of marine origin (**Figure 1**) and the approval of more than 300 patents (Blunt et al., 2015; Botana and Alfonso, 2015).

Nevertheless, despite of the remarkable potential of marine NPs as source of new drugs their role have undergone several changes, having had an evident decline in the pharmaceutical R&D activities by the mid-1990s. After that decline, the larger research and development effort in the exploration of this niche was essentially assured by enterprising academics, mainly partnered with industry. In the last decade, this area seems to have benefited from a renaissance, since the number of new isolated marine compounds has increased when compared with the previous similar period (Molinski et al., 2009; Botana and Alfonso, 2015). This rebirth is directly associated with recent technological advances in analytical technology, spectroscopy, and high-throughput screening. Advances in "omics" techniques (genomics, metagenomics, proteomics), combinatorial biosynthesis, synthetic biology, selection methods, expression systems, and bioinformatics have contributed as powerful tools to discover new chemical entities

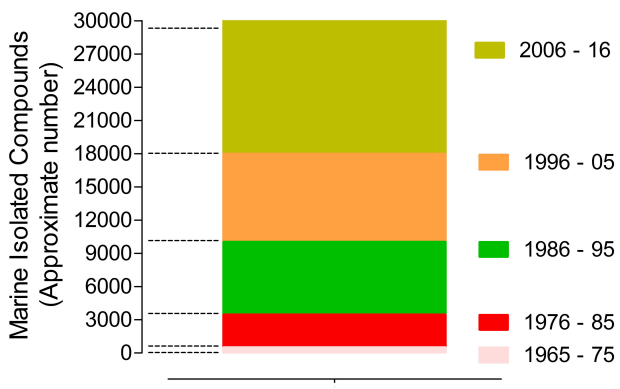


FIGURE 1 | Marine compounds isolated in the last 50 years (approximate number/10 years) (Faulkner, 1984, 1986, 1987, 1988, 1990, 1991, 1992, 1993, 1994, 1995, 1996, 1997, 1998, 1999, 2000, 2001, 2002; Blunt et al., 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013, 2014, 2015, 2016, 2017, 2018).

with pharmaceutical potential (Molinski et al., 2009; Bucar et al., 2013).

Over the last 30 years, great efforts have been made, showing productive and promising results, since it has been defined the major tendencies in secondary metabolism of several classes of marine organisms. Only in the last 20 years, more than 18,000 new marine compounds were described and six (**Figure 2**) out of the nine marine-derived drugs currently used in clinical therapy were approved, as well as one over-the-counter drug (OTC). Cytarabine (Cytosar-U®), Vidarabine (Vira-A®) (US discontinued), Ziconotide (Prialt®), Brentuximab Vedotin (Adcetris®), Eribulin Mesylate (Halaven®), Omega-3-acid ethyl esters (Lovaza®), Trabectedin (Yondelis®), Fludarabine Phosphate (Fludara®), and Nelarabine (Arranon®) were approved by the Food and Drug Administration (FDA) in the US Pharmacopeia and/ or by the European Agency for the Evaluation of Medicinal Products (EMA). Iota-carrageenan (Carragelose®), one OTC, was approved by EMA. However, it is expected that the number of newly approved drugs from marine origin will continue to increase, since 28 marine or marine-derived drugs are currently in clinical trials (six marine molecules—phase III; fourteen marine molecules—phase II; eight marine molecules—phase I) (**Figure 2**) (AndisInsight, 2018; Calado et al., 2018; EMA, 2018a,b; FDA, 2018a,b; Mayer, 2018).

Considering all the marine drugs available in the market, it is particularly interesting to see that six are used in cancer therapies (**Figure 3**), and the majority of the compounds that are in clinical trials are also for application in cancer therapy, which reveals the great potential of marine compounds as anticancer drugs (**Figure 2**).

Although not associated with cancer therapeutics, iota-carrageenan (Carragelose®) is the first product developed from algae in the market. It is a type of carrageenan, isolated from a red edible algae belonging a family of linear sulfated polysaccharides. Carragelose® has ability to block viral attachment to the host cells being effective against a broad spectrum of respiratory viruses (Ludwig et al., 2013; Calado et al., 2018).

ANTITUMOR POTENTIAL OF MARINE ALGAE-DERIVED COMPOUNDS

Among marine organisms, algae are one of the most important resources of the ocean, economically and ecologically (Kim, 2014). Their inclusion in the Asian diet has been associated with health benefits, where there has been observed a lower incidence of chronic diseases, such as hyperlipidaemia, coronary heart disease, diabetes and cancer, according to epidemiological studies comparing Japanese and Western diets (Brown et al., 2014; Bouga and Combet, 2015). Algae are valuable sources of protein, fiber, vitamins, polyunsaturated fatty acids, and macro-, and trace elements. More recently, they have also revealed to be an interesting source of useful bioactive components

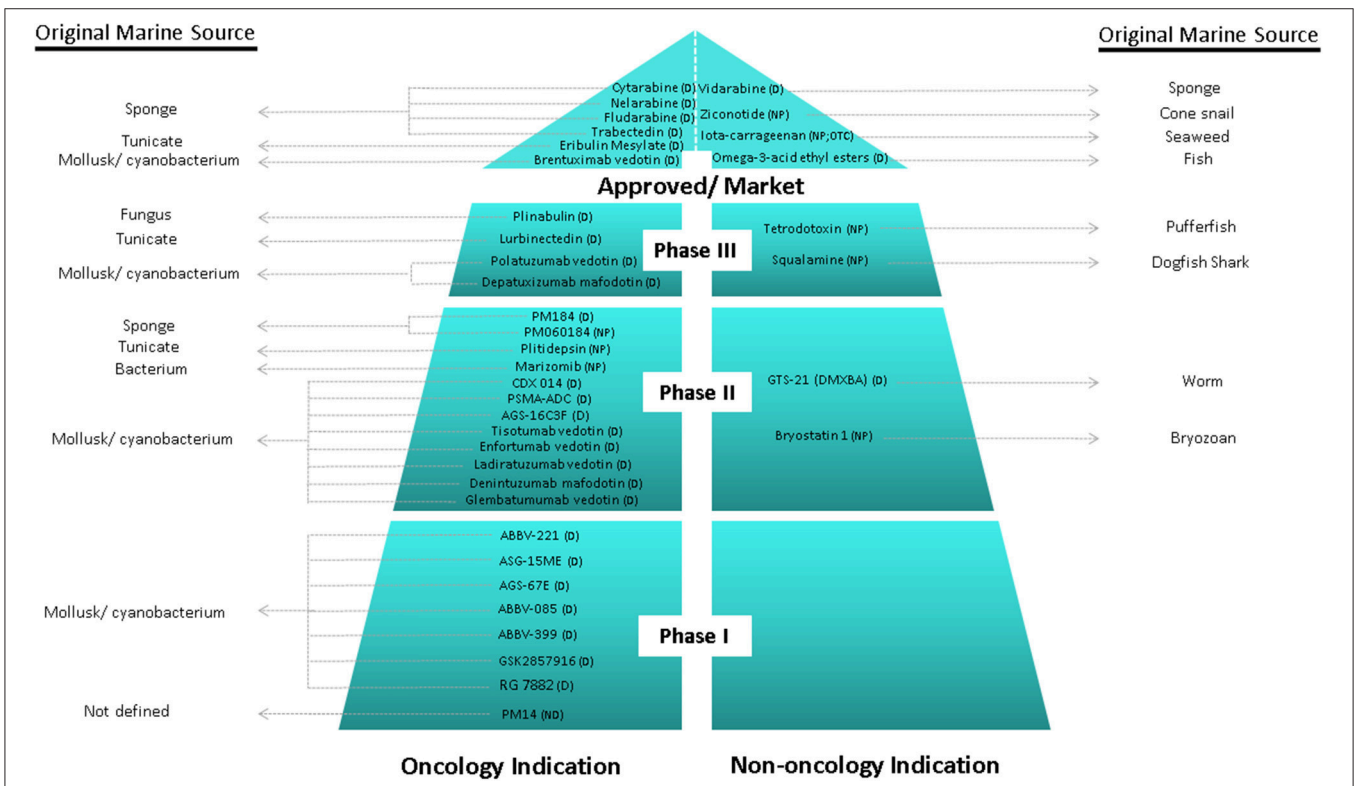


FIGURE 2 | Current clinical pipeline of marine-derived drugs and their original marine source. NP, Natural product; D, Derivate; OTC, Over-the-counter (AndisInsight, 2018; Calado et al., 2018; EMA, 2018a,b; FDA, 2018a,b; Mayer, 2018).

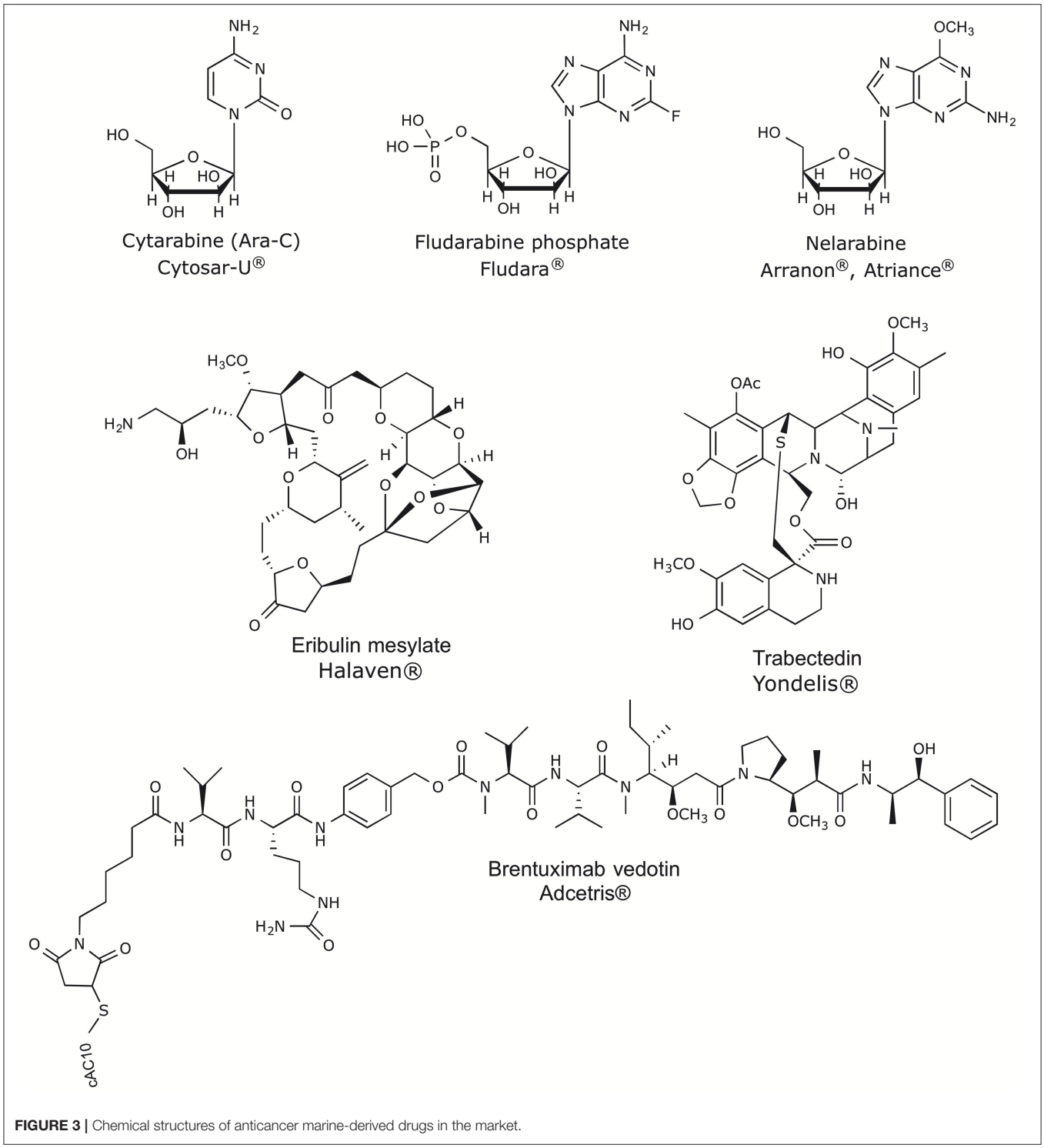


FIGURE 3 | Chemical structures of anticancer marine-derived drugs in the market.

such as antioxidants, phycocolloids, proteins, vitamins, minerals, carotenoids, soluble dietary fibers, polyunsaturated fatty acids, phycobilins, polysaccharides, sterols, tocopherols, terpenes, and phycocyanins. These compounds demonstrated to possess nutritional and functional value apart from their potential use as therapeutic agents in biomedical area (Chandini et al., 2008; Lordan et al., 2011; Mohamed et al., 2012; Alves et al., 2016b). Due to their unique structures and biochemical characteristics, the multifunctional properties of algae should be exploited in their fullness. In addition, the idea that algae are a promising prolific source of structurally unique NPs with biomedical potential is even more supported by the view that the number

of algae species identified around the world is more than 30,000 (Plouguerné et al., 2014). Moreover, algae have been revealed a major source of new compounds of marine origin, after sponges, microorganisms and phytoplankton (**Figure 4**).

Many of these algae-derived compounds have proven therapeutic properties associated with numerous health-promoting effects, including anti-obesity (Maeda et al., 2005; Kim et al., 2018), antidiabetic (Mayer et al., 2013), antihypertensive (Sivagnanam et al., 2015), antihyperlipidaemia (Sathivel et al., 2008), antioxidant (Magalhaes et al., 2011; Pinteus et al., 2017), anticoagulant (Magalhaes et al., 2011), anti-inflammatory (De Souza et al., 2009), immunomodulatory (Pérez-Recalde et al., 2014), anti-estrogenic (Skibola, 2004), thyroid-stimulating (Teas et al., 2007), neuroprotective (Pangestuti and Kim, 2011), anti-osteoarthritic (Moon et al., 2018), anti-osteoporosis (Deng et al., 2018), antiviral (Aguilar-Briseño et al., 2015), antimicrobial (Pinteus et al., 2015; Rodrigues et al., 2015), and antitumor (Moussavou et al., 2014; Rodrigues et al., 2015; Alves et al., 2016a). Among the well-documented bioactive compounds are brominated phenols, polysaccharides, and carotenoids, but especially a large diversity of terpenoids, several of them being halogenated compounds (Gribble, 2015; Rodrigues et al., 2015). Although many works have attempted to identify marine-derived compounds, detailed chemical characterization and identification of bioactive components are still largely lacking (Santos et al., 2015).

Along the last five decades, it is estimated that more than 3,000 NPs have been discovered from algae (Leal et al., 2013), and among all of the biological activities observed, the antitumor activity is one of the most promising. Despite several studies have shown the high cytotoxic potential of the compounds isolated from algae on different tumor cell lines, there are few studies that have characterized the intracellular signaling pathways involved in the process (**Table 1**). Currently, according to the National Cancer Institute (USA), different targeted therapies have been approved for use in cancer treatment, including hormonal therapies, inhibitors of signal transduction and angiogenesis, modulators of gene expression, inducers of apoptosis, immunotherapies and toxin delivery molecules.

In Vitro Antitumor Activities of Algae-Derived Compounds and Intracellular Signaling Pathways Activated

Analyzing the compounds isolated from algae with antitumor activity (**Table 1**) is possible to see that several of them mediate their activities in some of these target therapies mentioned previously. For example, dioxinodehydroeckol (Kong et al., 2009), sargachromanol E (Heo et al., 2011), EI-SP (Wang et al., 2014), siphonaxanthin (Ganesan et al., 2011), sulfated carrageenan (Murad et al., 2015), TDB (Lee et al., 2007), GLP (Thangam et al., 2014), mertensene (Tarhouni-Jabberi et al., 2017), TTB (Choi et al., 2017), DDSD (Velatooru et al., 2016), and clerosterol (Kim et al., 2013) induced apoptosis in different cell lines by similar intracellular signaling pathways, regulated by Caspase (−3, −9 or both) activation, downregulation of Bcl-xL or Bcl-2, upregulation of Bax and cleavage of PARP. Moreover, some of these compounds, such as EI-SP and clerosterol, also caused the loss of the mitochondrial membrane potential. The treatment of colon26 cells with *Eucheuma serra* agglutinin (Fukuda et al., 2006) also promoted an increase of Caspase-3 expression and translocation of phosphatidylserine in lectin-treated cells,

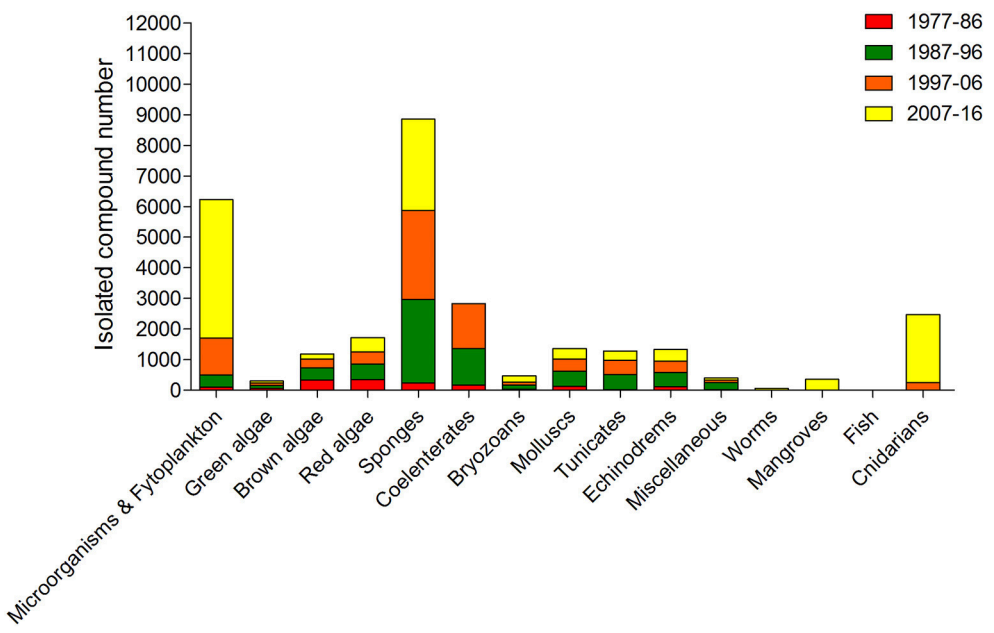


FIGURE 4 | Approximate numbers of new compounds isolated from different marine organism sources between 1977 and 2016 (Faulkner, 1984, 1986, 1987, 1988, 1990, 1991, 1992, 1993, 1994, 1995, 1996, 1997, 1998, 1999, 2000, 2001, 2002; Blunt et al., 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013, 2014, 2015, 2016, 2017, 2018).

TABLE 1 | Marine compounds isolated from algae with antitumor and cytotoxic activities and intracellular signaling pathways involved.

Algae	Compound	Chemical class	Intracellular signaling pathways	References
OCHROPHYTA (BROWN ALGAE)				
Ecklonia cava	Dieckol	Polyphenol	Induces a downregulation of FAK signaling pathway mediated by the scavenging of intracellular reactive oxygen species (ROS), influencing migration and invasion of HT1080 cells.	Park and Jeon, 2012
			Potent inhibitor for tumor promoter-mediated MAPK-signaling pathways, leading to Activator Protein 1 (AP-1) and Metalloproteinase (MMP)−9 activation by regulating cancer cell motility.	Oh et al., 2011
Ecklonia cava	6,6′-bieckol	Polyphenol	Acts as a suppressor of MMP-2 and MMP-9 expressions by downregulating Nuclear Factor kappa-light-chain-enhancer of Activated B cells (NF-κB) and inhibits the migration of HT1080 cells. In addition, cell morphology and shape are affected in 3D culture condition.	Zhang et al., 2010
Ecklonia Cava	Dioxinodehydroeckol	Phloroglucinol derivative	Induction of apoptosis through NF-κB family and NF-κB -dependent pathway.	Kong et al., 2009
Ecklonia cava	Fucodiphloroethol G	Phlorotannin	Promotes inhibition of AP-N, MMPs (-2,-9) and c-fos by blocking signal transduction of MAPK and Akt pathways in Vascular Endothelial Growth Factor (VEGF)-induced EVC304 and EA.hy926 cells.	Li et al., 2011
Eisenia bicyclis	Diphlorethohydroxycarmalol (DC)	Phlorotannin	Induces apoptosis on HL60 cells through the accumulation of sub-G1 cell population along with nuclear condensation, the reduction of Bcl-2 expression and the depletion of mitochondrial membrane potential ($\Delta\Psi m$).	Kang et al., 2012
Hizikia fusiformis	HFGP	Glycoprotein	Induces on HepG2 cells apoptosis and sub-G1 phase arrest. The expressions of Fas, Fas-associated death domain protein, Bax, and Bad were significantly upregulated in HFGP-treated cells. Moreover, HFGP induces the translocation of Bax to the mitochondria and the release of cytochrome c into the cytosol.	Ryu et al., 2012
Hydroclathrus clathratus	H3-a1	Sulfated polysaccharide	Induces significant arrest of sub-G1 phase on HL-60 and MCF-7 cells. *In vivo*, it inhibits tumor growth at doses of 20 and 50 mg Kg^{-1} in tumor-bearing BALB/c mice. Moreover, it suppresses ascitic sarcoma 180 tumor growth and prolongs the lifespan of the tumor-bearing mice by ∼30–40%. H3-a1 compound also increases the tumor necrosis factor-alpha (TNF-α) level in mouse serum.	Wang et al., 2010
Undaria pinnatifida	Fucoidan	Sulfated polysaccharide	Capable of suppress the proliferation of HLF cells by AMPK-associated inhibition of fatty acid synthesis and G1/S transition.	Kawaguchi et al., 2015
			Promotes apoptosis via ROS-mediated mitochondrial pathway on SMMC-7721 cells.	Yang et al., 2013
			Induces intrinsic and extrinsic apoptosis by stimulating ERK1/2 MAPK, deactivating P38 MAPK and PI3K/Akt signaling pathways and downregulating Wnt/β-catenin signaling pathway on prostate cancer cells (PC-3).	Boo et al., 2013
Undaria pinnatifida	Fucoxanthin	Carotenoid	Increases the efficiency of cisplatin treatment on HepG2 cell line. Reduces cell viability. Increases Bax/Bcl-2 ratio, probably through inhibition of NF-κB, and ERCC1 expression through ERK and PI3K/AKT pathways.	Liu et al., 2013
Cladosiphon okamuranus Tokida	Fucoxanthinol	Carotenoid	Inhibits Akt and Activator protein-1 pathways that influenced the suppression of cell growth, migration and invasion and the induction of apoptosis on osteosarcoma cells.	Rokkaku et al., 2013
Sargassum siliquastrum	Sargachromanol E	Meroditerpenoid	Induction of apoptosis on HL-60 cells mediated by Caspase-3 activation. Apoptosis accompanied by downregulation of Bcl-xL, upregulation of Bax, activation of Caspase−3, and cleavage of poly (ADP-ribose) polymerase (PARP).	Heo et al., 2011
Ascophyllum nodosum	Ascophyllan	Sulfated polysaccharide	Reduces N-Cadherin levels and increases E-Cadherin, which lead to the inhibition of migration and adhesion of B16 cell line.	Abu et al., 2015
Laminaria digitata	Laminarin	Polysaccharide	Induces apoptosis and cell cycle arrest at sub-G1 and G2/M phases on human colon cancer cells (HT-29) and suppresses ErbB signaling pathway activation.	Park et al., 2013

(Continued)

TABLE 1 | Continued

Algae	Compound	Chemical class	Intracellular signaling pathways	References
Laminaria japonica	LJGP	Glycoprotein	Supresses cell proliferation and induces apoptosis on HT-29 cells mediated through Fas signaling pathway, mitochondrial pathway and cell cycle arrest.	Go et al., 2010
Sargassum horneri	SHPSA	Polysaccharide	Inhibits the proliferation of human colon cancer cells (DLD) by increasing the accumulation of cells at G2/M phase and inducing the apoptosis of DLD cells.	Wang S. et al., 2015
Sargassum vulgare	PSV1	Sulfated polysaccharide	It blocks tubulogenesis and VEGF secretion on rabbit aorta endothelial cells using Matrigel. Inhibitory effect on angiogenesis.	Guerra Dore et al., 2013
Leathesia nana	Bis(2,3-Dibromo-4,5-dihydroxybenzyl) ether (BDDE)	Bromophenol	Induces apoptosis on K562 cells by a mitochondrial mediated pathway. Induces ROS generation and arrests cell cycle in S phase. Interacts with the minor groove of DNA and inhibits Topoisomerase I activity.	Liu et al., 2012
			Displays *in vitro* anti-angiogenic activity by suppressing significantly vascular endothelial cells (HUVEC) proliferation, migration, and tube formation, without any effect on the preformed vascular tube. Decreases the levels of VEGF and VEGFR proteins and inhibits the VEGF downstream signaling molecules, including mTOR and Src, while activates Akt and ERK. On zebrafish embryos, blocks sub-intestinal vessel formation and exhibits toxicity when used in higher concentrations (*in vivo*).	Qi et al., 2015
Sargassum siliquastrum	9′-*cis*-(6′*R*) fucoxanthin (FcA) 13′-*cis*-(6′*R*) fucoxanthin complex (FcB)	Carotenoid	Both compounds reduce MMP-2, MMP-9 and mRNA levels, and the migration of HT1080 cells. Moreover, increase the expression of MMP inhibition factors (MMP-1) and suppress significantly the transcriptional activity of NF-κB, c-Jun N-terminal kinase (JNK), as well as p38 mitogen-activated protein kinase activity.	Nguyen et al., 2014
Sargassum stenophyllum	SargA	Sulfated polysaccharide	*In vitro*, induces a decrease of B16F10 cells migration and viability. *In vivo*, causes the inhibition of tumor growth with no systemic toxicity and exhibits an anti-angiogenic effect.	Dias et al., 2005
Sargassum macrocarpum	Tuberatolide B (TTB)	Meroterpenoid	TTB reduces the cell viability of several cancer cells lines (MDA-MB-231, MDA-MB-453, MCF-7, A549, H1299, HCT-116, SW620, CT26, PC-3, and DU145) by apoptosis decreasing Bcl2 expression and increasing the Caspase-3 and PARP cleavage. Promotes γH2AX foci formation and phosphorylation of several proteins (Chk2 and H2AX) related to DNA damage. In addition TTB promotes the production of ROS inhibiting STAT3 activation, which result in the decrease of the levels of cyclin D1, MMP-9, survivin, VEGF, and IL-6. Its activity seems to be mediated by ROS production and consequently inhibition of STAT3 signaling.	Choi et al., 2017
Stoechospermum marginatum	5(*R*), 19-diacetoxy-15,18 (*R* and *S*), dihydro spata-13, 16(*E*)-diene (DDSD)	Spatane diterpenoid	*In vitro* DDSD induces cell cycle arrest at the S-phase and cell death by apoptosis on B16F10 melanoma cells. This compound promotes the generation of ROS, and consequently alterations in the ratio of Bax/Bcl-2 and in the mitochondrial transmembrane potential ($\Delta\Psi$m), phosphatidylserine externalization, release of cytochrome c to the cytoplasm, Caspase activation, nuclear condensation, and fragmentation of DNA. Moreover, the results suggest that DDSD induces apoptosis through deregulating PI3K/AKT signaling pathway. *In vivo*, DDSD inhibits tumor growth (volume and weight) without evident toxic effects on C57BL/6 mice bearing B16F10 melanoma.	Velatooru et al., 2016
Not identified by the authors	MSP	Sulfated polysaccharide	Exhibits anti-metastatic ability, both *in vitro* and *in vivo*. Induces regulatory effects on Actin dynamics in an FAK/ERK1/2-dependent manner, which might be further attributed to its binding to FN and, consequently, FN-induced tumor adhesion, and migration.	Tang et al., 2006
Not identified by the authors	Not defined	Sulfated polysaccharide	Induces apoptosis and cell arrest at G2/M phase of MKN45 cells via ROS/JNK signaling pathway. In addition, it promotes ROS production and mediate the phosphorylation of several proteins, including Jun N-terminal kinase (JNK), p53, Caspase-9, and -3.	Xie et al., 2016

(Continued)

TABLE 1 | Continued

Algae	Compound	Chemical class	Intracellular signaling pathways	References
RHODOPHYTA (RED ALGAE)				
Lophocladia sp.	Lophocladines B	Alkaloid	Cell cycle analysis on MDA-MB-435 cells showed arrest at G2/M phase and induction of microtubule depolymerization on A-10 cells.	Gross et al., 2006
Laurencia viridis	Polyether triterpenoid dehydrothyrsiferol	Terpenoid	Induces apoptosis on breast cancer cells by estrogen-depend and independent pathways.	Pec et al., 2003
Eucheuma serra	*Eucheuma serra* agglutinin (ESA)	Lectin	Increases Caspase-3 expression and translocation of phosphatidylserine in lectin-treated colon26 cells, suggesting that cell death is mediated by apoptosis. *In vivo* is observed a significant growth inhibition of Colon26-induced tumors on BALB/c mice. DNA fragmentation in tumor cells after intravenous injection with ESA is also detected.	Fukuda et al., 2006
Gracilaria verrucosa	(*E*)-9- oxooctadec-10-enoic acid (C10)	Enone fatty acid	Angiogenesis and NF-κB activation in HUVECs cells stimulated by VEGF are blocked as well as their proliferation and migration. This is also observed *in vivo* model of angiogenesis using mouse cornea. Moreover, the neovascularization induced by VEGF is significantly suppressed.	Furuno et al., 2011
Grateloupia filicina	GFP08	Sulfated polysaccharide	In the chicken chorioallantoic membrane assay, reduced new vessel formation. In mice decreases the weight of sarcoma-180 cells-induced tumor in a dose-dependent manner. Also decreased Tissue Factor (TF) expression without affecting the activities of MMP-2 and−9.	Yu et al., 2012
Laurencia intricata	Laurenditerpenol	Diterpene	Inhibits hypoxia-inducible factor-1 (HIF-1) mediated hypoxic signaling in breast tumor cells.	Mohammed et al., 2004
Laurencia papillosa	Sulfated carrageenan (ESC)	Sulfated polysaccharide	Inhibits MDA-MB-231 cell proliferation and induces cell death through nuclear condensation and DNA fragmentation. Cell death is induced by apoptosis as result of activation of the extrinsic apoptotic Caspase-8 gene. The apoptotic signaling pathway is regulated through the Caspase-3, Caspase-9, p53, Bax, and Bcl-2 proteins.	Murad et al., 2015
Laurencia majuscula	Hexadecyl-1-*O*-α-l-arabinopyranoside	Arabinopyranoside	Decreases significantly CDK1 and Cyclin A expression, with slight changes in Cyclin B1; arrests cell cycle at G2/M.	Du et al., 2010
Callophycus serratus	Bromophycolide A	Diterpene–benzoate macrolides	Induces apoptosis on A2780 human ovarian cells; arrests G1 phase of the cell cycle, consistent with decreased number of cells from the S and G2/M phases.	Kubanek et al., 2005
Chondrus ocellatus	λ-Carrageenan	Sulfated galactan	Conjugation with 5-Fluorouracil (5-FU) enhanced antitumor activity and mitigated immunocompetence damage of 5-FU.	Zhou et al., 2006
Laurencia microcladia	Elatol	Sesquiterpene	Induces cell cycle arrest at G1 and sub-G1 phases, leading cells to undergo apoptosis. Reduces the expressions of Cyclin-D1, Cyclin-E, Cyclin-dependent kinase (Cdk)2 and Cdk4. It is also observed increases in Bak, Caspase-9 and p53 expressions and a decrease in Bcl-xl expression. *In vivo* elatol treatment reduces tumor growth on C57Bl6 mice.	Campos et al., 2012
Laurencia thyrsifera	Thyrsiferol	Triterpene	Supresses HIF-1 activation on T47D human breast tumor cells and blocks mitochondrial respiration at complex I.	Mahdi et al., 2011
Champia feldmannii	Cf-PLS	Sulfated polysaccharide	*In vivo* antitumor activity without marked toxicity. Enhances the efficacy of 5-FU, while preventing immunocompetence hindrance by 5-FU.	Lins et al., 2009
Porphyra haitanensis	Porphyran	Sulfated galactan	Conjugation with 5-FU enhanced its antitumor activity and mitigated immunocompetence damage.	Wang and Zhang, 2014
Porphyra yezoensis	PY-D2	Polysaccharide	Blocks cell cycle at G0/G1 or G2/M check-points on different cell lines (SMMC-7721, HO-8910, MCF-7, K562 cells).	Zhang et al., 2011
Porphyra yezoensis	Sulfoquinovosyldiacylglycerol (SQDG)	Sulfolipids	Inhibits significantly telomerase activity.	Eitsuka et al., 2004
Grateloupia elliptica	Pheophorbide a (Pa)	Chlorophyll	Induces cytostatic activity on glioblastoma cells (U87 MG). The cell cycle distribution showed that U87 MG cells are arrested at G0/G1 phase.	Nguyen et al., 2014

(Continued)

TABLE 1 | Continued

Algae	Compound	Chemical class	Intracellular signaling pathways	References
Grateloupia longifolia	GLP	Polysaccharide	Prevents the proliferation of HMEC-1 and HUVEC cells, suppresses the formation of intact tube networks and decreases migration. Decreases vessels density and new vessels formation in the chick chorioallantoic membrane assay and also, by intravenous administration decreases tumor weight and vascular density without showing toxicity in mice bearing sarcoma-180-cells-induced tumors.	Zhang et al., 2006
Rhodomelaceae confervoides	Bis-(2,3-dibromo-4,5-dihydroxy-phenyl)-methane (BDDPM)	Bromophenol	Inhibits several biological processes associated with angiogenesis, including endothelial cell sprouting, migration, proliferation, and tube formation.	Wang B. et al., 2015
Symphyacladia latiuscula	2,3,6-tribromo-4,5-dihydroxybenzyl methyl ether (TDB)	Bromophenol	Inhibits MCF-7 breast cancer cells growth and induces DNA fragmentation by apoptosis, accompanied by a downregulation of Bcl-2 protein expression and PARP cleavage by Caspase-3. This treatment increases the level of p21 WAF1/CIP1 protein in a p53-dependent manner.	Lee et al., 2007
Pterocladiella capillacea	Mertensene	Halogenated monoterpene	Induces apoptosis on HT-29 cells accompanied by Caspase-3 activation and PARP cleavage. Decreases the phosphorylated forms of several proteins (p53, Rb, Ccd2, Chkp2) and the levels of cyclin-dependent kinases CDK2 and CDK4, and increases the levels of death receptor-associated protein TRADD. In addition it seems to promote the activation of MAPK ERK-1/-2, Akt and NF-κB pathways.	Tarhouni-Jabberi et al., 2017
CHLOROPHYTA (GREEN ALGAE)				
Avrainvillea nigricans	Nigricanosides A (NA)	Glycolipid	Arrests MCF-7 breast cancer cells in mitosis. Cells exhibit disorganized microtubule spindles. *In vitro* induces polymerization of Tubulin and inhibition of both MCF-7 and HCT-116 cells proliferation.	Williams et al., 2007
Caulerpa spp.	Caulerpin	Alkaloid	Acts as an inhibitor of the transportation of electrons to mitochondrial complex III, interfering with the mitochondrial ROS-regulated HIF-1 activation and HIF-1 downstream target genes expression.	Liu et al., 2009
Caulerpa taxifolia	Caulerpenyne	Sesquiterpenoid	An early shift into synthesis phase (S) along with a blockade at G2/M phase is observed on colorectal cancer cells.	Fischel et al., 1994
Codium fragile	Siphonaxanthin	Carotenoid	Induces apoptosis on HeLa cells accompanied by a decrease of Bcl-2 expression and subsequently activation of Caspase-3 and increase of the expression of GADD45α and the Death Receptor 5 (DR5).	Ganesan et al., 2011
Ulva intestinalis	EI-SP	Sulfated polysaccharide	Induces apoptosis on HepG2 cells accompanied by changes in mitochondrial membrane potential, release of cytochrome c to the cytosol, decrease and increase of Bcl-2 and Bax expression, respectively and cleavage of Caspase-3 and Caspase-9, as well as cleavage of PARP.	Wang et al., 2014
Ulva intestinalis	DAEB	Sulfated polysaccharide	Exhibits low toxicity *in vitro*. *In vivo* DAEB reduces tumor mass and increases thymus and spleen mass. Tumor growth inhibition is ascribed to increase levels of TNF- α, NO, and ROS.	Jiao et al., 2009
Capsosiphon fulvescens	Cf-GP	Glycoprotein	Inhibits AGS cells proliferation and migration by a decrease of Integrin expression via the TGF-β 1-activated FAK/PI3K/AKT pathways.	Boo et al., 2013
Capsosiphon fulvescens	Cf-PS	Polysaccharide	Inhibits cell proliferation and induces apoptosis by inhibiting IGF-IR signaling and the PI3K/Akt pathway.	Kwon and Nam, 2007
Codium fragile	Clerosterol	Sterol	Induces apoptosis accompanied by changes in mitochondrial membrane potential, an increase and a decrease of Bax and Bcl-2 expression, respectively, and activation of Caspase-3 and Caspase-9.	Kim et al., 2013
Codium decorticatum	GLP	Glycoprotein	GLP induces apoptosis on MDA-MB-231 breast cancer cells by mitochondria-mediated intrinsic pathway promoting changes in the mitochondrial membrane potential and Bax/Bcl-2 ratio, cytochrome c release, and Caspases-3 and 9 activation.	Thangam et al., 2014

suggesting that cell death was mediated by apoptosis.. On the other hand, algae-derived compounds such as lophocladines B (Gross et al., 2006), hexadecyl-1- *O*-α-l-arabinopyranoside (Du et al., 2010) and caulerpenyne (Fischel et al., 1994) affected the intracellular signaling pathways linked with regulation of the cell cycle. Lophocladines B showed a marked reduction of MDA-MB-435 cells at the G1 and S phases, with an accumulation of cells at G2/M, indicating a G2/M cell cycle arrest. This compound also induced microtubule depolymerization on A-10 cells. Du et al. (2010) isolated from the alga *Laurencia majuscula* a new arabinopyranoside compound designed as hexadecyl-1-*O*-α-l-arabinopyranoside, which exhibited significant antitumor activity in different cancer cell lines. The active compound arrested cell lines at G2/M phase of the cell cycle by decreasing the expression of CDK1 and Cyclin A proteins, which are critical for the G2/M-phase transition. Caulerpenyne induced cell cycle arrest in colorectal cancer cells that exhibited an early shift into the S phase followed by a blockade at the G2/M phase (Fischel et al., 1994).

Nevertheless, most of the intracellular signaling pathways activated by these compounds are simultaneously linked with the regulation of cell cycle and apoptosis. Park et al. (2013) demonstrated that laminarin, extracted from brown alga *Laminaria digita*, induced apoptosis on HT-29 colon cancer cells and increased the percentage of cells in the sub-G1 and G2/M phases. The observed decrease in cellular proliferation was found to be dependent on ErbB, followed by subsequent activation of c-Jun N-terminal kinase. In the same way, Liu et al. (2012) verified that a bromophenol compound, bis(2,3-dibromo-4,5-dihydroxybenzyl) ether, induced apoptosis on K562 cells by a mitochondria-mediated pathway, as well as the arrest of the cell cycle at the S phase. Additionally, this compound interacted with the minor groove of DNA and inhibited Topoisomerase I activity. On A2780 human ovarian cells, bromophycolide A induced the arrest at the G1 phase of the cell cycle and a consequent and consistent loss of cells from the S and G2/M phases, while simultaneously induced apoptosis (Kubanek et al., 2005). Similar effects were induced by elatol, a compound isolated from the alga *Laurencia microcladia*, which induced cell cycle arrest in the G1 and sub-G1 phases, leading the cells to undergo apoptosis. It influenced the expression of several proteins (cyclins, Bax, Bcl-xl, caspases, p53) that play important roles in these biological processes (Campos et al., 2012). Diphlorethohydroxycarmalol, isolated by Kang et al. (2012), induced apoptosis by the accumulation of the sub-G1 cell population and nuclear condensation, depletion of mitochondrial membrane potential ($\Delta\Psi m$) and regulation of the expression of the pro-survival and pro-apoptotic Bcl-2 family members. HFGP (Ryu et al., 2012) and LJGP (Go et al., 2010) glycoproteins induced apoptosis on HepG2 and HT-29 cells, which was mediated by Fas signaling and mitochondrial pathway, and cell cycle arrest. On the other hand, Cf-PS polysaccharide (Kwon and Nam, 2007) inhibited the cell proliferation and induced apoptosis by inhibiting IGF-IR signaling and the PI3K/Akt pathway, which are involved in the regulation of cell growth, proliferation, differentiation, motility, survival, metabolism and protein synthesis (Chen et al., 2014).

Other examples such as dieckol (Oh et al., 2011; Park and Jeon, 2012), 6,6′-bieckol (Zhang et al., 2010), ascophyllan (Abu et al., 2015), 9′-*cis*-(6′*R*) fucoxanthin (FcA) (Nguyen et al., 2014), fucoxanthinol (Rokkaku et al., 2013), and 13′-*cis*-(6′*R*) fucoxanthin complex (FcB) (Nguyen et al., 2014), SargA (Dias et al., 2005), MSP (Tang et al., 2006), and Cf-GP (Boo et al., 2013) showed the capacity to inhibit the motility, migration, adhesion or invasion on different *in vitro* and *in vivo* models using distinct intracellular signaling pathways, as described in **Table 1**.

Currently, one of the targets of cancer treatment, especially in solid tumors, is angiogenesis, which is responsible for the formation of new blood vessels and is a requirement for the sustained growth and proliferation of solid tumors. Accordingly, the search for inhibitors of this process has become a leading line of investigation in anticancer research, with the consequent release of several drugs on the market that have clearly improved outcomes in patients with different tumor types and metastatic disease (Marín-Ramos et al., 2015). The compounds PSV1 (Guerra Dore et al., 2013), SargA (Dias et al., 2005), BDDE (Qi et al., 2015), GFP08 (Yu et al., 2012), GLP (Zhang et al., 2006), Fucodiphloroethol G (Li et al., 2011), C10 (Furuno et al., 2011), and BDDPM (Wang B. et al., 2015) also demonstrated interesting anti-angiogenic activities. For example, the sulfated polysaccharide, PSV1, inhibited tubulogenesis in RAEC cells in Matrigel and VEGF secretion (Guerra Dore et al., 2013); SargA induced a marked dose-dependent inhibition of capillary networks development (Dias et al., 2005). As to fucodiphloroethol G, this compound inhibit angiogenesis on ECV-304 and EA.hy926 cells when induced with VEGF, as well as the transcriptional factor c-fos and its targets AP-N, MMP-2, by MAPK, and Akt signaling pathways inhibition (Li et al., 2011). However, one of the most interesting and promising compounds is BDDPM, reported to inhibit various biological processes associated with angiogenesis, including endothelial cell sprouting, migration, proliferation, and tube formation (Wang B. et al., 2015). Kinase assays revealed that BDDPM is a potent selective but multi-target receptor tyrosine kinase (RTKs) inhibitor (VEGFR, PDGFR, FGFR, and EGFR). However, other compounds have not only evidenced *in vitro* activity but also *in vivo* as described in the section Preclinical and Clinical Evidence of Antitumor Activities of Algae-Derived Compounds of the present review.

Compounds such as laurenditerpenol (Mohammed et al., 2004), caulerpin (Liu et al., 2009), thyrsiferol (Mahdi et al., 2011), phlorofucofuroeckol-A (Lee et al., 2012), SQDG (Eitsuka et al., 2004), and DAEB (Jiao et al., 2009) showed antitumor activity by activating other intracellular signaling pathways. For example, laurenditerpenol, thyrsiferol, and caulerpin showed the capacity to inhibit the transcription factor HIF-1 by blocking the induction of the oxygen-regulated HIF-1α protein, which promotes tumor cell adaptation and survival under hypoxic conditions (Ke and Costa, 2006). Lee et al. (2012) observed that phlorofucofuroeckol-A, a compound isolated from the edible brown alga *Eisenia bicyclis*, is a potent inhibitor of the aldo-keto reductase family 1 B10 (AKR1B10), a member of the NADPH-dependent aldo-keto reductase (AKR) superfamily, considered to be a potential cancer therapeutic target. In the same way, the

compound SQDG showed a marked inhibition of the telomerase activity, which is an enzyme that drives the uncontrolled division and replication of cancer cells (Eitsuka et al., 2004).

Some of the compounds isolated from algae can also be used as co-adjuvants to improve the efficiency of the drugs currently used as therapeutics. For instance, the pre-treatment of HepG2 cells with fucoxanthin allowed to improve the therapeutic effect of cisplatin (Yang et al., 2013). According to Liu et al. (2013), these effects were associated with NFκB expression inhibition and an increase in the Bax/Bcl-2 mRNA ratios regulated by NFκB. Moreover, the decrease of the DNA repair systems regulated by ERK, p38, and PI3K/AKT seems also be associated with these effects. Additionally, the conjugation of λ-carrageenan (Zhou et al., 2006), Cf-PLS (Lins et al., 2009), and porphyran (Wang and Zhang, 2014) compounds with 5-FU drug enhanced its antitumor activity. According to previous studies, the conjugation of these compounds with 5-FU increased the antitumor activities of the drug and mitigated the immunocompetence damage induced by 5-FU (Zhou et al., 2006; Lins et al., 2009; Wang and Zhang, 2014).

Many of the studies discussed above have identified compounds, such as polysaccharides, polyphenols, carotenoids, alkaloids, terpenes and others, that mediate specific inhibitory activity on a number of key cellular processes, including apoptosis pathways, angiogenesis, migration and invasion processes, in different *in vitro* models revealing their potential use as anticancer drugs (**Figure 5**).

Preclinical and Clinical Evidence of Antitumor Activities of Algae-Derived Compounds

Despite the antitumor activities of algae-derived compounds have majorly been described on *in vitro* human tumor models,

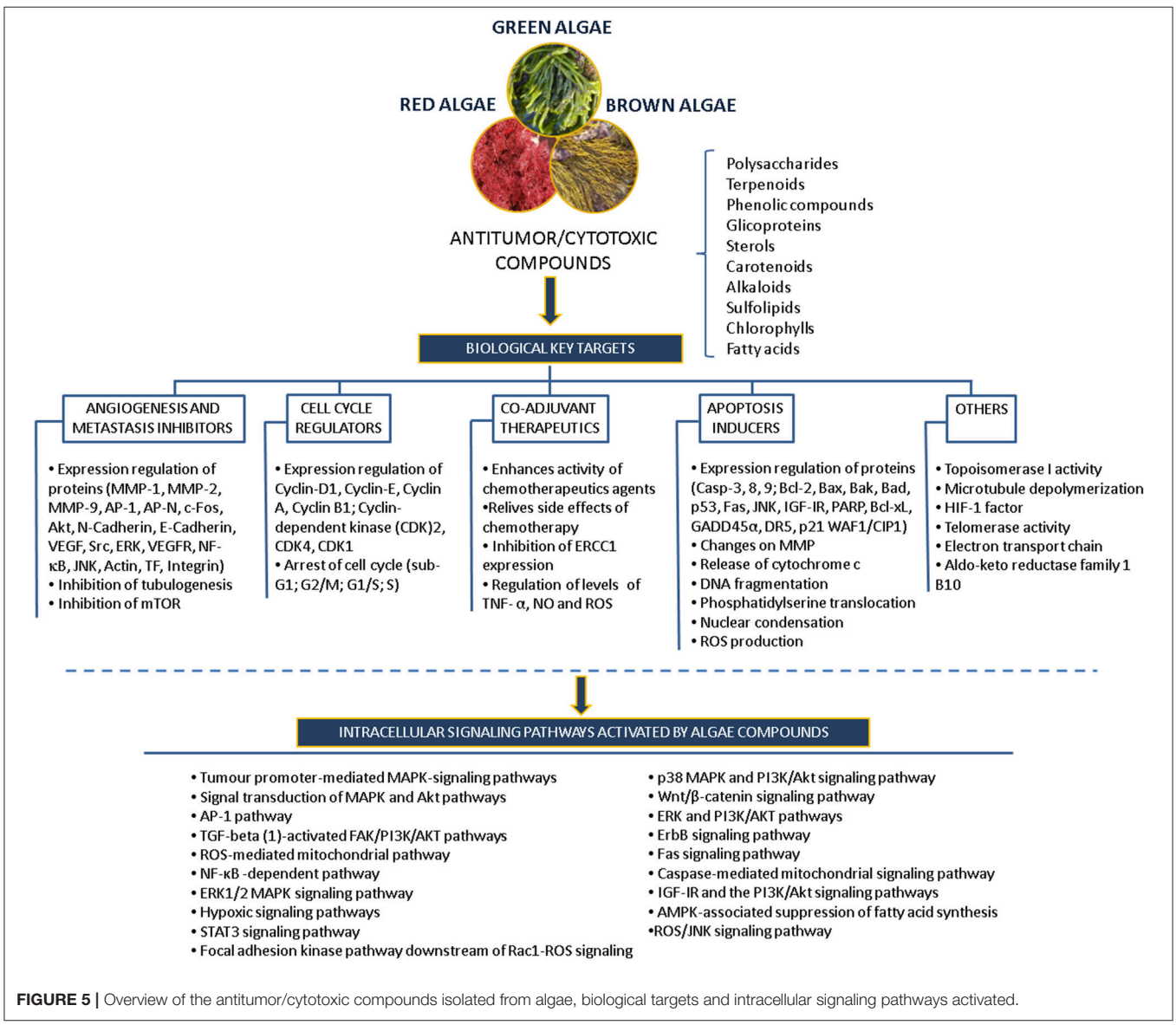


FIGURE 5 | Overview of the antitumor/cytotoxic compounds isolated from algae, biological targets and intracellular signaling pathways activated.

there are several studies in preclinical and clinical trials demonstrating the potential of these compounds as antitumor and/or co-adjuvants drugs with capacity to act in distinct intracellular signaling pathways.

Among the chemical structures derived from algae, the highest number of *in vivo* studies were conducted with sulfated polysaccharides, revealing their potential to be used in antitumor therapies. For instance, the sulfated polysaccharides H3-a1 (Wang et al., 2010) and DAEB (Jiao et al., 2009) revealed antitumor activity by immune system enhancement, through the increase of the tumor necrosis factor-alpha (TNF-α) and ROS levels and by the activation of peritoneal macrophages leading to the secretion of TNF-α and NO. In addition, when administrated in tumor-bearing BALB/c mice at doses of 20 and 50 mg/Kg, the sulfated polysaccharide H3-a1 exhibited capacity to suppress the ascitic sarcoma 180 tumor growth, increasing the lifespan of the tumor-bearing mice in ∼30–40%. On the other hand, the treatment with the sulfated polysaccharide DAEB reduced the thymus and spleen tumor growth in mice promoting the increase of immune organs weight.

Due to increasing evidences of the antitumor potential of algae derived sulfated polysaccharides, several studies have been conducted to deepen their antitumor potential, including studies addressing the possibility to be used as co-adjuvants drugs, to increase treatments efficiency and also as an attempt to reduce undesirable side-effects. As an example, the sulfated polyssacharide Cf-PLS extracted from the red alga *C. feldmannii* exhibited capacity to reduce the tumor growth on mice transplanted with sarcoma 180 tumor cells and to increase the leukocytes number in the peritoneal cavity and neutrophil migration. In addition, its co-administration with the chemotherapeutic agent 5-FU on tumor-bearing animals allowed to significantly increase the tumor growth inhibition, comparing with single administration, also preventing the immunocompetence hindered by 5-FU (Lins et al., 2009).

As described above, one of the targets for cancer treatment is angiogenesis. In these studies, several compounds have exhibited promising activities on *in vivo* models. The treatment of mice bearing sarcoma-180 cells with GLP polysaccharide promoted the reduction of tumor weight around 52% and the vascular density of the tumor (Zhang et al., 2006). In the same model, the GFP08 compound decreased the tumor weight by 68.9% and inhibited the formation of new vessels in the chicken chorioallantoic membrane assay (*ex vivo*) (Yu et al., 2012). By other side, the bromophenol BDDE also exhibited anti-angiogenesis properties by inhibiting sub-intestinal vessel formation in zebrafish embryos *in vivo* (Qi et al., 2015). Similarly to the bromophenol BDDE, the compound C10 inhibited markedly the neovascularization induced by VEGF in the mouse cornea (Furuno et al., 2011). On the other hand, the compound SargA inhibited the vascularization of Gelfoam skin implants and exhibited antitumor properties on melanoma cells with doses of 1.5 and 150 μg/animal without inducing deaths or body weight loss (Dias et al., 2005).

The MSP sulfated polysaccharide mediated a potent inhibitory effect on the metastasis of Lewis lung carcinoma reducing significantly the number of pulmonary metastatic colonies. The reduction of colony-formation rate was between 70 and 93.6%. Moreover, compared with cisplatin treatment, this compound promoted a significant amelioration of alveolar structures without inducing weight loss (Tang et al., 2006). DDSD (4, 10, and 15 mg/Kg) and Elatol [(oral (3, 10, 30 mg/Kg) or intraperitoneal (1, 3, 10 mg/Kg)] demonstrated significantly reduction of the tumor growth without evident toxic effects (Campos et al., 2012; Velatooru et al., 2016). The lectin ESA extracted from red alga *Eucheuma serra*, injected in the tail vein of BALB/c mice with colon26 cells, promoted a significant delay of the tumors growth inducing the cells death by apoptosis as observed *in vitro*. Additionally the treatment with ESA compound did not promoted weight loss or animal death (Fukuda et al., 2006). These studies become more relevant since the antitumor activities mediated by many of these compounds are not linked with toxic effects.

Other of the compounds widely studied on *in vivo* models, including clinical trials, is the sulfated polyssacharide fucoidan. This compound exhibits multi-targets acting in different signaling pathways, including the activation of the intrinsic and extrinsic pathways of apoptosis, suppression of angiogenesis, increase immune response, and mobilization of haematopoietic progenitor cells (Kwak, 2014; Moghadamtousi et al., 2014). The co-administration of low-molecular –weight fucoidan with standard drugs in patients with metastatic colorectal cancer improved the disease control rate suggesting its potential application as additional therapy (Tsai et al., 2017). In addition when administrated in conjugation with the standard hormonal drugs letrozole and tamoxifen, in patients with breast cancer, revealed to be well tolerated and without influence in the steady-state plasma concentrations of these drugs (Tocaciu et al., 2018). Despite de great potential demonstrated by fucoidans on clinical trials, to be used as suplementary therapy, its use as anticancer drugs is being studied, since fucoidan preparations obtained from different sources have induced different anticancer activities *in vivo*. These differential responses seem to be associated with their different structural properties. Therefore, it will be important to determine the structural characteristics of fucoidan responsible for the verified *in vivo* antitumor activities to ensure its potential use as therapeutic agent (Kwak, 2014).

The marine-derived cyclic depsipeptide kahalalide F was the first compound found in algae that achieved the phase II of clinical trials (Murphy et al., 2014). Kahalalide F is a potent cytotoxic compound produced by the green alga *Bryopsis pennata* and found in the mollusk *Elysia rufescens* (Miguel-Lillo et al., 2015; Sable et al., 2017). It advanced through five clinical trials and completed the safety evaluation in phase I in patients with distinct advanced solid tumors. Nevertheless, kahalalide F dropped in phase II due to lack of efficacy, short half-life, limited spectrum of activity and a poor response in patients. However, due the high potential of this compound as cytotoxic, it inspired the development of several synthetic analogs to overcome its limitations increasing its potency and half-life time (Wang B. et al., 2015).

In the last years nanotechnology has emerged as a promising solution to be used in drug delivery systems to suppress some of these limitations, being considered as one of the next-generation platform for cancer therapy (Sun et al., 2014; Xin et al., 2017).

Therefore the production of nano-formulations of drugs derived from algae-derived compounds can be an interesting approach to potentiate anticancer properties. In addition the use of nano-formulations can also be useful to overcome some limitations that arise from specific characteristics of each compound, such as nonspecific biodistribution, low water solubility, lack of targeting capability, systemic toxicity, weak therapeutic effect, and limited bioavailability (Sun et al., 2014; Blanco et al., 2015). For instance one of the most promising compounds isolated from algae with interesting antitumor proprieties is fucoxanthin. However, the poor solubility, chemical instability, and low bioavailability of this carotenoid limit its use in cancer therapeutics. To overcome these drawbacks several approaches have been assessed such as the inclusion of fucoxanthin in nano-emulsions (Huang et al., 2017), nano-suspensions (Muthuirulappan and Francis, 2013) and nano-gels (Ravi and Baskaran, 2015). However as described by Bajpai et al. (2018) for the microalgae, the published data on nano-formulations using compounds derived from macroalgae is also scarce suggesting a new area to be explored that can potentiate the capacity of these compounds in cancer therapeutics.

According to the interesting activities mediated by algae-derived compounds, it is expected that in the next few years some of them may reach the clinical trials stages or inspire the development of new compounds allowing their translation into clinically useful drugs in the future. Moreover, the use of some those compounds as co-adjuvant in pre-existent therapeutics regimes appears to be a valid approach to improve the therapeutic effects of the antitumor drugs and decrease their side-effects.

The Therapeutic Potential of *Sphaerococcus coronopifolius* Compounds—Case Study

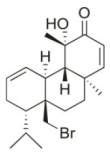
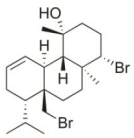
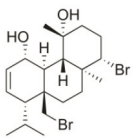
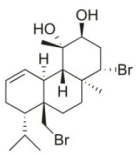
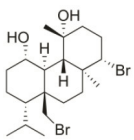
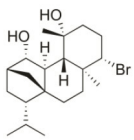
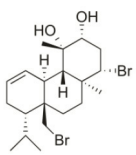
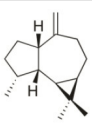
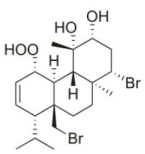
Sphaerococcus coronopifolius is a red alga belonging to the Rhodophyta phylum, which is narrow, compressed, two-edged, cartilaginous, scarlet fronds and main axes that are dark brownish-red. The habitat of this species is rarely on rocks in the lower littoral, but it is common in the shallow sublittoral to a 15 m depth. They are distributed in the East Atlantic (Ireland and Britain to Canary Islands) and Mediterranean and Black Seas (Guiry and Guiry, 2015). Since its first chemical analysis in 1976, *S. coronopifolius* has demonstrated to be an interesting source of brominated cyclic diterpenes, most of them containing one or two bromine atoms (Rodrigues et al., 2015). Although more than 40 compounds have been isolated and described from *S. coronopifolius*, in the last four decades, few studies characterized their biological activities. According to previous studies, some of the compounds isolated evidenced great biological activities, including antifouling (Piazza et al., 2011), antimalarial (Etahiri et al., 2001) and antimicrobial (Etahiri et al., 2001; Smyrniotopoulos et al., 2010b,c; Rodrigues et al., 2015). Another interesting bioactivity exhibited by these compounds is their antitumor potential. Several compounds revealed interesting cytotoxic activities in different *in vitro* models (**Table 2**). For example, 14*R*-Hydroxy-13,14-dihydro-sphaerococcenol-A decreased significantly the viability of NSCLC-N6-L16 and A549 human lung cancer cell lines with an IC_{50} of 5 and 4 μg/mL, respectively. Moreover, spirosphaerol and corfusphaeroxide showed moderate cytotoxic activity against the malignant cell lines A549, Hs683 and MCF-7. Smyrniotopoulos et al. (2010a) isolated several metabolites and evaluated their cytotoxic activity toward four human apoptosis-resistant (U373, A549, SK-MEL-28, OE21) and two human apoptosis-sensitive (PC-3, LoVo) cancer cell lines with IC_{50} values ranging from 3 to 100 μM. In a study performed by Rodrigues et al. (2015), the compound Sphaerococcenol A exhibited the highest anti-proliferative activity on HepG2 cells with an IC_{50} of 42.87 μM, being more potent than cisplatin (75.41 μM). Although some compounds isolated from *Sphaerococcus coronopifolius* have exhibited interesting biological activities, its potential remains understudied since the intracellular mechanisms associated with the observed effects are yet uncovered. *S. coronopifolius* is therefore a clearly example of the potential of algae as source of cytotoxic compounds, still under explored, suggesting that the potential of algae as source of compounds for the development of new antitumor drugs is probably much higher than previously thought.

CONCLUSIONS AND FINAL REMARKS

Over the last several decades, marine organisms revealed to be an interesting source of both pre-existing and unrecognized compounds with the potential for providing sustainable economic and human benefits. Many of these compounds demonstrated great potential for therapeutic applications, exhibiting specific and potent activities against different diseases, including cancer. Their potential as a source of antitumor drugs has been proven by the current pipeline. Six of nine drugs from marine origin currently in the market are used in cancer treatment, and several compounds originated or derived from marine organisms are undergoing clinical trials with indications for oncologic therapeutics. However, the number of compounds in the market or in clinical trials is very low compared with the total number of isolated compounds with antitumor potential. This is mainly due to the different constraints between discovery and commercialization such as the discontinuation of "candidates" in clinical trials or preclinical tests due to difficulties on harvesting the organism and on the isolation and purification procedures, low yields, insufficient investment by pharmaceutical companies, environmental policies, high toxicity and low efficiency of the active compounds. Nevertheless, considering the approved marine drugs, it is also possible to observe that six of them were approved in the last 10 years. This fact is probably directly associated with the development of high-throughput screening technologies that allowed faster and more accurate results. Thus, it is expected that the number of antitumor drugs of marine origin will increase in the next few years.

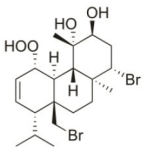
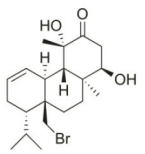
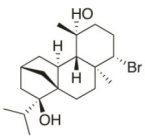
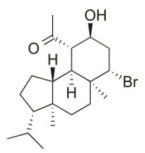
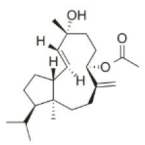
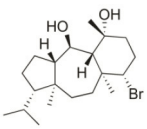
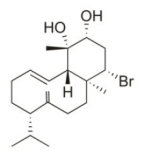
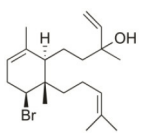
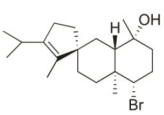
Among the marine organisms, many crude extracts, enriched fractions and compounds obtained from algae displayed

TABLE 2 | Cytotoxic compounds isolated from *Sphaerococcus coronopifolius*.

Compound name	First report	Locale of collection	Chemical structure	Cytotoxic activities (IC_{50} in μM or μg/mL)	References
Sphaerococcenol A	1976	La Escala, Spain		U373 (IC_{50}:3.2 μM)*; A549 (IC_{50}:3.7 μM); SK-MEL-28 (IC_{50}:5.2 μM); OE21 (IC_{50}:3 μM); PC-3 (IC_{50}:3.7 μM); LoVo (IC_{50}:2.8 μM); HepG2 (IC_{50}:42.87 μM)	Fenical et al., 1976; Smyrniotopoulos et al., 2010a; Rodrigues et al., 2015
Bromosphaerol	1976	Italy		U373 (IC_{50}:30 μM); A549 (IC_{50}:35 μM); SK-MEL-28 (IC_{50}:34 μM); OE21 (IC_{50}:28 μM); PC-3 (IC_{50}:30 μM); LoVo (IC_{50}:23 μM); HepG2 (IC_{50}:203.33 μM)	Fattorusso et al., 1976; Smyrniotopoulos et al., 2010a; Rodrigues et al., 2015
Bromosphaerodiol	1977	Portopalo, Sicily, Italy		U373 (IC_{50}:22 μM); A549 (IC_{50}:24 μM); SK-MEL-28 (IC_{50}:31 μM); OE21 (IC_{50}:15 μM); PC-3 (IC_{50}:26 μM); LoVo (IC_{50}:20 μM)	Smyrniotopoulos et al., 2010a
12*S*-Hydroxy-bromosphaerol	1982	Bay of Salerno, Italy		HepG2 (IC_{50}:291.42 μM); U373 (IC_{50}:16 μM); A549 (IC_{50}:19 μM), SK-MEL-28 (IC_{50}:22 μM); OE21 (IC_{50}:19 μM); PC-3 (IC_{50}:12 μM); LoVo (IC_{50}:9 μM)	Cafieri et al., 1982; Smyrniotopoulos et al., 2010a; Rodrigues et al., 2015
1*S*-Hydroxy-1,2-dihydro-bromosphaerol	1982	Bay of Salerno, Italy		U373 (IC_{50}:25 μM); A549 (IC_{50}:28.6 μM); OE21 (IC_{50}:20 μM); SK-MEL-28 (IC_{50}:26 μM); PC-3 (IC_{50}: 25 μM); LoVo (IC_{50}: 23 μM)	Cafieri et al., 1982; Smyrniotopoulos et al., 2010a
Bromotetrasphaerol	1986	Bay of Napoles, Massalubrense, Italy		U373 (IC_{50}:34 μM); A549 (IC_{50}:38 μM); OE21 (IC_{50}:33 μM); SK-MEL-28 (IC_{50}:43 μM); PC-3 (IC_{50}:43 μM); LoVo (IC_{50}:56 μM)	Cafieri et al., 1986; Smyrniotopoulos et al., 2010a
12*R*-Hydroxy-bromosphaerol	1987	Bay of Naples, Massalubrense, Italy		HepG2 (IC_{50}:104.83 μM); U373 (IC_{50}:25 μM); A549 (IC_{50}:28 μM); OE21 (IC_{50}:25 μM); SK-MEL-28 (IC_{50}:29); PC-3 (IC_{50}: 26 μM); LoVo (IC_{50}: 26 μM)	Cafieri et al., 1987; Smyrniotopoulos et al., 2010a; Rodrigues et al., 2015
Alloaromadendrene	1988	Plomin, Croatia		U373 (IC_{50}:71 μM); A549 (IC_{50}:79 μM); OE21 (IC_{50}:83 μM); PC-3 (IC_{50}: 35 μM); LoVo (IC_{50}: 63 μM)	de Rosa et al., 1988; Smyrniotopoulos et al., 2010a
1*S*-Hydroperoxy-12*R*-hydroxy-bromosphaerol-B	2008	Palaiokastritsa bay, Corfu Island, Greece		NSCLC-N6-L16 (IC_{50}: 9.5 μg/mL); A549 (IC_{50}:12 μg/mL); U373 (IC_{50}:32 μM); A549 (IC_{50}:40 μM); OE21 (IC_{50}:25 μM); SK-MEL-28 (IC_{50}:31 μM); PC-3 (IC_{50}: 30 μM); LoVo (IC_{50}: 22 μM)	Smyrniotopoulos et al., 2008, 2010a

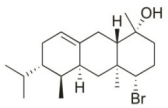
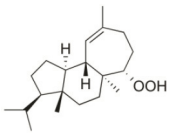
(Continued)

TABLE 2 | Continued

Compound name	First report	Locale of collection	Chemical structure	Cytotoxic activities (IC_{50} in μM or μg/mL)	References
1*S*-Hydroperoxy-12*S*-hydroxy-bromosphaerol-B	2008	Palaiokastritsa bay, Corfu Island, Greece		NSCLC-N6-L16 (IC_{50}: 6 μg/mL); A549 (IC_{50}:5 μg/mL); U373 (IC_{50}:22 μM); A549 (IC_{50}:26 μM); OE21 (IC_{50}:27 μM); SK-MEL-28 (IC_{50}:28 μM); PC-3 (IC_{50}: 28 μM); LoVo (IC_{50}: 28 μM)	Smyrniotopoulos et al., 2008, 2010a
14*R*-Hydroxy-13,14-dihydro-sphaerococcenol-A	2008	Palaiokastritsa bay, Corfu Island, Greece		NSCLC-N6-L16 (IC_{50}:5 μg/mL); A549 (IC_{50}: 4 μg/mL); U373 (IC_{50}:7.2 μM); A549 (IC_{50}:18 μM); OE21 (IC_{50}:8.4 μM); SK-MEL-28 (IC_{50}:21 μM); PC-3 (IC_{50}: 8.1 μM); LoVo (IC_{50}: 5.3 μM)	Smyrniotopoulos et al., 2008, 2010a
4*R*-Hydroxy-1-deoxy-bromotetrasphaerol	2010	Palaiokastritsa bay, Corfu Island, Greece		U373 (IC_{50}:75 μM); A549 (IC_{50}:63 μM); OE21 (IC_{50}:64 μM); PC-3 (IC_{50}: 43 μM); LoVo (IC_{50}: 56 μM)	Smyrniotopoulos et al., 2010a,c
Coronone	2010	Palaiokastritsa bay, Corfu Island, Greece		U373 (IC_{50}:31 μM); A549 (IC_{50}:42 μM); SK-MEL-28 (IC_{50}:38 μM); OE21 (IC_{50}:30 μM); PC-3 (IC_{50}:30 μM); LoVo (IC_{50}:28 μM)	Smyrniotopoulos et al., 2010a
Sphaerollane-I	2009	Palaiokastritsa bay, Corfu Island, Greece		U373 (IC_{50}:20 μM); A549 (IC_{50}:44 μM); SK-MEL-28 (IC_{50}:57 μM); OE21 (IC_{50}:34 μM); PC-3 (IC_{50}:34 μM); LoVo (IC_{50}:23 μM)	Smyrniotopoulos et al., 2009, 2010a
Sphaerostanol	2010	Palaiokastritsa bay, Corfu Island, Greece		U373 (IC_{50}:85 μM); A549 (IC_{50}:97 μM); SK-MEL-28 (IC_{50}:96 μM); OE21 (IC_{50}:60 μM); PC-3 (IC_{50}:74 μM); LoVo (IC_{50}:64 μM)	Smyrniotopoulos et al., 2010a
10*R*-Hydroxy-bromocorodienol	2010	Palaiokastritsa bay, Corfu Island, Greece		U373 (IC_{50}:60 μM); A549 (IC_{50}:64 μM); SK-MEL-28 (IC_{50}:62 μM); OE21 (IC_{50}:33 μM); PC-3 (IC_{50}:48 μM); LoVo (IC_{50}:24 μM)	Smyrniotopoulos et al., 2010a
Sphaerodactylomelol	2015	Berlenga Nature Reserve, Peniche, Portugal		Inhibition of cell proliferation (IC_{50}: 280 μM); Cytotoxicity (IC_{50}: 720 μM) on HepG2 cells	Rodrigues et al., 2015
Spirosphaerol	2015	Liapades Bay, Corfu, Greece		A549 (IC_{50}:69 μM); Hs683 (IC_{50}:56 μM); MCF-7 (IC_{50}:67 μM); B16F10 (IC_{50}:65 μM)	Smyrniotopoulos et al., 2015

(Continued)

TABLE 2 | Continued

Compound name	First report	Locale of collection	Chemical structure	Cytotoxic activities (IC_{50} in μM or μg/mL)	References
Anthrasphaerol	2015	Liapades Bay, Corfu, Greece		A549 (IC_{50}:90 μM); Hs683 (IC_{50}:93 μM); MCF-7 (IC_{50}:85 μM); B16F10 (IC_{50}:63 μM)	Smyrniotopoulos et al., 2015
Corfusphaeroxide	2015	Liapades Bay, Corfu, Greece		A549 (IC_{50}:67 μM); Hs683 (IC_{50}:63 μM); MCF-7 (IC_{50}:60 μM); U373 (IC_{50}:81 μM); SK-MEL-28 (IC_{50}:75 μM); B16F10 (IC_{50}:46 μM)	Smyrniotopoulos et al., 2015

**Induce cytostatic activity on U373 cells inhibiting cell entrance into mitosis (3 μM). For the others compounds the possible intracellular signaling pathways were not characterized.*

interesting antitumor potential along the years. These effects are mediated by compounds from different chemical classes including polysaccharides, terpenoids, phenolic compounds, glicoproteins, sterols, carotenoids, alkaloids, sulfolipids, chlorophylls, and fatty acids. Despite the high number of reports evidencing the cytotoxic and cytostatic properties of algae-derived compounds, few studies have characterized the intracellular signaling pathways underlying their effects. This view is clearly evident with *Sphaerococcus coronopifolius*, since some of its bioactive compounds exhibited interesting cytotoxic activities on different cell lines, but the intracellular signaling pathways involved in its activities have still not been deeply characterized. Thus, there is a need to design and perform studies to evaluate these type of molecules in more complex biological systems, including *in vivo* models. Another important tool that still remain unexplored is the use of 3D chemical structural modeling techniques to find new biochemical targets for the algae-derived compounds. Moreover, the idea of using algae-derived compounds as co-adjuvants in therapeutics should also be evaluated.

One of the major challenges in this area is the sustainable production of these compounds to "supply" sufficient quantity for preclinical, clinical and future commercialization, since the algae's slow growth and seasonality together with low extraction yields are significant limitations. In line with this requirement, total chemical synthesis can not only guarantee the sustainable and continuous production of the bioactive molecules but also improve or enhance their functional zones. Aquaculture can also be an interesting approach for the continuous supply of algae. However, more studies are needed to understand if algae continue to produce the desired compounds under artificial conditions.

The applications of new techniques such as nanotechnology to develop new nano-formulations can potentiate the development of anticancer drugs from algae origin leading to the suppression of limitations and improve the capabilities of some algae-derived compounds. However further technologies are needed to validate the improvement of their antitumor activities when applied in nanoformulations. According with this search this area was not yet explored. By other side, the creation/establishment of interdisciplinary teams (including for example chemists, pharmaceuticals, biotechnologists and biologists) can contribute a faster bioscreening process, from the isolation and identification of algae-derived compounds to full validation of their antitumor capabilities. The studies gathered in the present review clearly demonstrate that algae have great potential as a source of antitumor compounds with the capacity to hamper different key pathway targets involved in cancer development including cell cycle regulation, apoptosis, angiogenesis, migration and invasion processes. Even though, to date algae have not been fully exploited, it is expected that in a few years, their translation to clinical use can be a reality.

AUTHOR CONTRIBUTIONS

CA and RP conceived the research topic and the design the review. CA, JS, and SP did the bibliographic research. HG and MA critically contributed to discuss the cancer threat and current marine clinical pipeline. LB critically contributed to discuss the antitumor potential of marine algae-derived compounds. All authors performed the literature review and participated in the drafting and revision of the manuscript, thus making a direct and intellectual contribution to the work.

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Annex II

Chapter 2 article

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Article

Antitumor and Antimicrobial Potential of Bromoditerpenes Isolated from the Red Alga, *Sphaerococcus coronopifolius*

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Abstract: Cancer and infectious diseases continue to be a major public health problem, and new drugs are necessary. As marine organisms are well known to provide a wide range of original compounds, the aim of this study was to investigate the bioactivity of the main constituents of the cosmopolitan red alga, *Sphaerococcus coronopifolius*. The structure of several bromoditerpenes was determined by extensive spectroscopic analysis and comparison with literature data. Five molecules were isolated and characterized which include a new brominated diterpene belonging to the rare dactylomelane family and named sphaerodactylomelol (**1**), along with four already known sphaerane bromoditerpenes (**2**–**5**). Antitumor activity was assessed by cytotoxicity and anti-proliferative assays on an *in vitro*

model of human hepatocellular carcinoma (HepG-2 cells). Antimicrobial activity was evaluated against four pathogenic microorganisms: *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans*. Compound **4** exhibited the highest antimicrobial activity against *S. aureus* (IC_{50} 6.35 μM) and compound **5** the highest anti-proliferative activity on HepG-2 cells (IC_{50} 42.9 μM). The new diterpene, sphaerodactylomelol (**1**), induced inhibition of cell proliferation (IC_{50} 280 μM) and cytotoxicity (IC_{50} 720 μM) on HepG-2 cells and showed antimicrobial activity against *S. aureus* (IC_{50} 96.3 μM).

Keywords: red algae; *Sphaerococcus*; diterpenes; sphaerane; dactylomelane; HepG-2; pathogenic microorganisms

1. Introduction

Cancer and infectious diseases continue to be one of the major public health concerns, and consequently, there is a perpetual need for new chemotherapeutics to fight new diseases and drug resistance. In the last few decades, nature has played a significant role as a source of new drugs, and recent trends in drug research emphasize that the marine environment has a high potential for the discovery of new pharmaceuticals [1–3]. Marine ecosystems are among the richest and most complex ones in terms of biodiversity. Original chemical and physical conditions in such an environment provide conditions for the production of quite specific and potent active molecules. Among other reasons, marine organisms have been found to produce original and bioactive substances because they are living in an exigent, competitive and aggressive environment [4,5]. These characteristics render marine organisms ideal candidates as novel sources of both preexisting and unrecognized high value-added biomolecules with potential for providing sustainable economic and human benefits [6]. Marine algae have been one of the richest and promising sources of bioactive specialized metabolites that probably have diverse simultaneous functions for their producer and can act, for example, as antimicrobial, antifouling and herbivore deterrents or as ultraviolet-screening agents [7–9]. These defensive strategies can result in a high level of structural and chemical diversity for the metabolites, originating from different metabolic pathways with great pharmaceutical and biomedical potential [10,11]. Marine algae-originated compounds have been found to be associated with numerous health-promoting effects, including, in particular, anti-oxidative, anti-inflammatory, antiviral, antimicrobial or anticancer effects [10,12]. Well-documented bioactive metabolites of marine algae include mainly brominated phenols, polysaccharides, but especially, a large variety of terpenoids, several of them being halogenated [13–16].

Since its first chemical analysis in 1976, the cosmopolitan red alga, *Sphaerococcus coronopifolius*, has yielded a large number of interesting brominated cyclic diterpenes, most of them containing one or two bromine atoms [17–40]. Concerning the biological activities of these compounds, some of them have already demonstrated antibacterial activity against Gram-positive bacteria [33], and others have been assayed for their cytotoxicity against human lung cancer cell lines [37] or their antibacterial activity against multidrug-resistant and methicillin-resistant *Staphylococcus aureus* strains [38,39]. Several

bromoditerpenes have also been screened against the model organism, *Amphibalanus amphitrite*, in order to evaluate their antifouling properties.

The aim of the present study was to address the antitumor and antimicrobial bioactivity characterization of the major brominated diterpenes isolated from samples of *S. coronopifolius* collected in the Atlantic, while most of the chemically-studied specimens of this algal species were collected from the Mediterranean or the Adriatic seas.

2. Results and Discussion

2.1. In Vitro Bioactivity-Guided Fractionation

The screening of antitumor and antimicrobial activities of compounds isolated from *S. coronopifolius* was performed for the methanol (MeOH) and dichloromethane (CH_2Cl_2) extracts of this alga. The CH_2Cl_2 extract exhibited the highest anti-proliferative and antimicrobial activities (Tables 1 and 2, respectively). Consequently, in order to isolate and identify the compounds responsible for these biological activities, this extract was further studied. It was first fractionated by normal phase vacuum liquid chromatography (VLC) on silica with eluents of increasing polarities (from cyclohexane to EtOAc), yielding five fractions (F1–F5 through a 25% step). In the first bioassay, fraction F2 exhibited the strongest cytotoxicity (IC_{50} 104 µg/mL) and anti-proliferative (19.8 µg/mL) activity (Table 1). On the other hand, fractions F1, F3, F4 and F5 demonstrated much more potency in the inhibition of HepG-2 cell proliferation than in the reduction of their viability. In antimicrobial assays (Table 2), the highest growth inhibition was measured against *S. aureus* for fractions F2 and F3 with IC_{50} of 5.10 and 5.39 µg/mL, respectively. In the antifungal assay, the highest activity was exhibited against *C. albicans* by fraction F2 with an IC_{50} of 53.9 µg/mL.

Table 1. Cytotoxicity and anti-proliferative (IC_{50}) effects induced on HepG-2 cells by crude extracts and VLC fractions of *S. coronopifolius*. IC_{50} values are expressed as the means of eight independent experiments with 95% confidence intervals.

		IC_{50} (µg/mL)	
		Cytotoxicity	**Anti-Proliferative**
Crude extracts	MeOH	470.6 (310.7–712.6)	646.5 (398.4–1049.0)
	CH_2Cl_2	14.13 (8.12–24.60)	32.32 (22.37–46.70)
VLC fractions	F1	>1000	102.5 (68.08–154.2)
	F2	104.3 (81.82–132.9)	19.78 (13.79–28.38)
	F3	>1000	70.17 (38.78–127.0)
	F4	>1000	36.68 (23.37–57.55)
	F5	>1000	39.32 (25.89–59.71)

According to these results, several VLC fractions showed interesting effects against different targets, and it was decided to purify and structurally characterize the main components of fractions F2 and F3, due to their high anti-proliferative and antimicrobial effects, respectively.

Table 2. Antimicrobial activities (IC_{50}) against *E. coli*, *P. aeruginosa*, *S. aureus* and *C. albicans* of crude extracts and VLC fractions obtained from *S. coronopifolius*. IC_{50} values are expressed as the means of eight independent experiments with 95% confidence intervals.

		IC_{50} (μg/mL)			
		E. coli	***P. aeruginosa***	***S. aureus***	***C. albicans***
Crude Extracts	MeOH	>1000	>1000	73.65 (58.52–92.69)	>1000
	CH_2Cl_2	267.1 (231.5–308.1)	363.1 (207.0–637.1)	25.15 (13.47–46.96)	435.9 (285.6–665.5)
VLC Fractions	F1	107.0 (92.02–124.5)	338.7 (248.3–461.9)	16.49 (10.19–26.66)	78.61 (58.12–106.3)
	F2	228.4 (188.7–276.4)	141.5 (117.6–170.4)	5.10 (4.50–5.78)	538.9 (308.4–941.6)
	F3	>1000	599.9 (307.4–1171.0)	5.39 (4.19–6.93)	>1000
	F4	433.9 (358.5–525.1)	436.4 (184.1–1035.0)	6.45 (5.16–8.05)	>1000
	F5	757.0 (614.5–932.6)	422.8 (291.9–612.4)	13.16 (9.98–17.35)	>1000

2.2. Isolation and Structure Elucidation of the Major Compounds of Fractions F2 and F3

The HPLC purifications of the selected VLC fractions, F2 and F3, followed by the structural identification of the major compounds by NMR and MS led to the identification of five diterpenes belonging to two distinct families: one new dactylomelane (**1**) and four known sphaeranes (**2**–**5**) (Figure 1). It was not surprising to find compounds belonging to the sphaerane class of diterpenes in our samples. In comparison with literature data, we were thus able to identify, unambiguously, bromosphaerol (**2**) [31], 12*S*-hydroxybromosphaerol (**3**) [34], 12*R*-hydroxybromosphaerol (**4**) [34] and sphaerococcenol (**5**) [31] as additional major metabolites of the CH_2Cl_2 extract of *S. coronopifolius* harvested on the Portuguese coast.

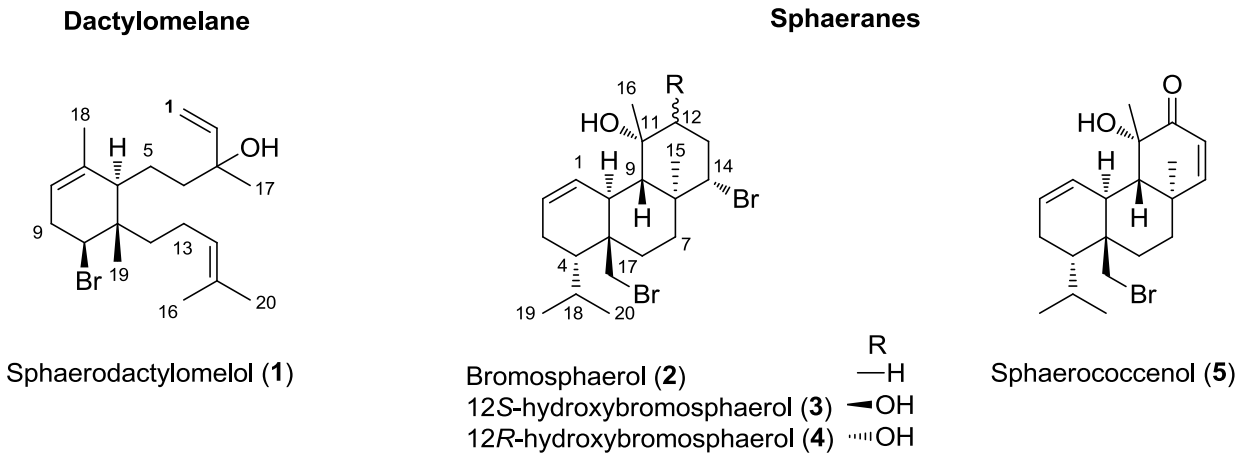

Figure 1. Chemical structure of the five bromoditerpenes, **1**–**5**, isolated from *S. coronopifolius.*

Compound **1** was isolated as an optically-active oil, and its (+)-HRESIMS spectrum (see Supplementary Information) revealed an isotopic pattern characteristic of the presence of one bromine atom at *m/z* 351.17117 and 353.16891 (intensities 1:1) that was consistent with the molecular formula $C_{20}H_{32}Br^+$. The ^{1}H NMR spectrum (see Supplementary Information) confirmed the presence of a diterpene with five characteristic methyl signals at δ_H 0.89 (s, H_3-19), 1.30 (s, H_3-17), 1.63 (s, H_3-16), 1.69 (s, H_3-20) and 1.70 (s, H_3-18), the chemical shifts of the last three revealing three methyls substituted on olefinic double bonds (Table 3). A terminal monosubstituted double bond was evidenced by the vinylic signals at δ_H 5.92 (dd, J = 17.2 and 10.8 Hz, H-2), 5.22 (dd, J = 17.2 and 1.2 Hz, H-1a) and 5.09 (dd, J = 10.8 and 1.2 Hz, H-1b). Two additional trisubstituted double bonds were inferred from the NMR signals at δ_H 5.22–5.17 (m, H-8), δ_C 120.5 (CH, C-8) and 137.2 (qC, C-7) and at δ_H 5.12–5.08 (m, H-14), δ_C 124.3 (CH, C-14) and 131.8 (qC, C-15).

Table 3. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of sphaerodactylomelol (**1**).

Atom n°	δ_H in ppm, mult. (J in Hz)	δ_C in ppm, mult.
1	5.09, dd (10.8, 1.2) 5.22, dd (17.2, 1.2)	112.3, CH_2
2	5.92, dd (17.2, 10.8)	144.9, CH
3	-	73.6, qC
4	1.83, td (13.1, 4.7) 1.46 m	44.2, CH_2
5	1.63, tdd (13.1, 4.7, 2.0) 1.38–1.29, m	23.3, CH_2
6	2.11–2.06, m	45.0, CH
7	-	137.2, qC
8	5.22–5.17, m	120.5, CH
9	2.62–2.49, m	35.3, CH_2
10	4.31, dd (10.2, 6.2)	61.5, CH
11	-	41.4, qC
12	1.57, ddd (14.4, 12.4, 5.1) 1.43 m	38.6, CH_2
13	2.03, dd (12.9, 5.8) 1.90–18.1, m	21.5, CH_2
14	5.12–5.08, m	124.3, CH
15	-	131.8, qC
16	1.63, s	17.9, CH_3
17	1.30, s	27.9, CH_3
18	1.70, s	22.4, CH_3
19	0.89, s	16.8, CH_3
20	1.69, s	25.9, CH_3

It appeared that the terminal double bond was connected to a quaternary carbon, as evidenced by the absence of further scalar coupling from the vinylic protons. The H-1 and H-2/C-3 HMBC correlations indicated that the chemical shift of the quaternary carbon was δ_C 73.6 (qC, C-3), which was characteristic of an allylic alcohol (Figure 2a). The conclusion on the presence of an alcohol and not a bromine atom at this position was also given by the observation of a second heteroatom-substituted carbon at δ_H 4.31

(dd, J = 10.2 and 6.2 Hz, H-10), δ_C 61.5 (CH, C-10), with chemical shifts fully consistent with a brominated methine. Consequently, the isotopic pattern obtained by HRESIMS was the result of dehydration of the allylic and tertiary alcohol at C-3 $[M - H_2O + H]^+$, and the molecular formula of **1** was then determined to be $C_{20}H_{33}BrO$. In view of the NMR data, from the four unsaturations corresponding to this molecular formula, the last one was assigned to a cycle. As described in Figure 2a, full analysis of ^1H-^1H COSY and HMBC spectra (see Supplementary Information) allowed the establishment of the carbon skeleton of compound **1**. More precisely, the closing of the precursor geranylgeranyl chain through the C-6/C-11 bond was ascertained by the key H_3-19, H-10, H_2-12/C-6 and H_3-19, H-10, H-9/C-11 HMBC correlations, thus leading to the six-membered ring. The key H_2-12/C-6, C-11, C-19 HMBC correlations placed the last H-12/H-13/H-14 spin coupled system at C-11, thus revealing the full planar structure of **1**. We relied on the NOESY spectrum (see Supplementary Information) for the elucidation of the relative configuration of **1**. H-10/H-6 nOe placed the bromine on the same side as the linear alkyl chain at C-6, while the second alkyl chain was placed on the other side, thanks to H_3-18/H_2-4 and H-10/H_2-13 nOes (Figure 2b). The absolute configuration was not determined, due to the lack of simple assessment. However, we assume the same stereochemistry as the one determined for the known analogues, **2**, **3** and **4**.

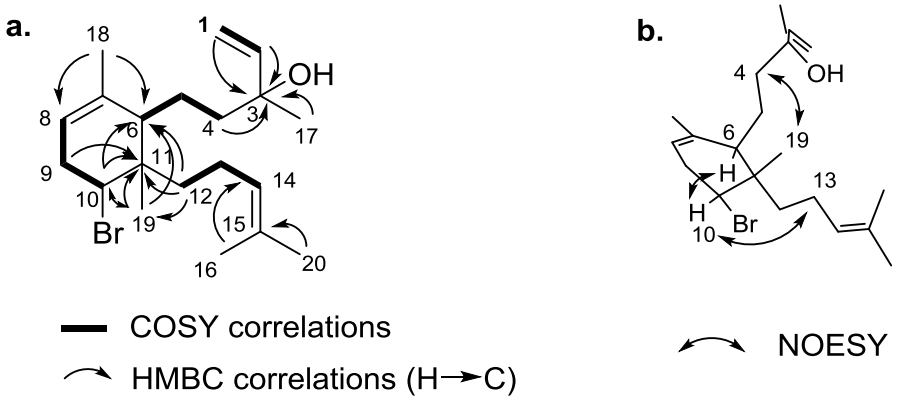

Figure 2. Key COSY, HMBC (**a**) and NOESY (**b**) correlations for sphaerodactylomelol (**1**).

This compound does not belong to the usual sphaerane family found in this species, but it is another rare example of the dactylomelane family (Figure 1) [41]. To date, there are nine compounds of this class of diterpenes described in the literature. Eight of them are characterized by a bridged oxide between C-7 and C-10, thus leading to a 7-oxabicyclo[2.2.1]heptane moiety. These compounds were isolated from sea hares of the genus *Aplysia*, but also from a red alga of the genus *Laurencia*, thus confirming the diet of the sea hare [41–43]. The last compound of this chemical class was isolated from the same species, *S. coronopifolius*, harvested also along the Atlantic coast [33]. Its chemical structure is very similar to that of compound **1**, differing by the presence of an allylic alcohol at C-14 instead of a dimethyl-substituted double bond. Interestingly, all of the compounds isolated from samples of *S. coronopifolius* collected in the Mediterranean and Adriatic seas belong to the sphaerane family, suggesting that the presence of dactylomelanes would be restricted to Atlantic specimens.

2.3. Antitumor and Antimicrobial Activities of Purified Compounds

The results of the anti-proliferative experiments demonstrated that all isolated compounds have anti-proliferative activity on HepG-2 cells at sub-toxic concentrations (Table 4). The highest inhibition of cell proliferation was exhibited by compound **5** and then compound **4** with IC_{50} of 42.9 and 105 μM, respectively. Sphaerococcenol A (**5**) has already demonstrated high cytotoxic activities against A549, OE21, PC-3 and LoVo cell lines, with LC_{50} of 3.7, 3.0, 3.7 and 2.8 μM, respectively [37]. However, this is the first report of activity against HepG-2 cells. In the same report, 12*R*-hydroxybromosphaerol (**4**) also showed cytotoxic activity against the four cell lines with LC_{50} of 28, 25, 26 and 26 μM, respectively. The absence of cytotoxicity was verified in the case of NSCLC-N6 (L16) and A549 cell lines [34]. On the other hand, the major reduction of HepG-2 cell viability was induced by the new compound **1** with an IC_{50} of 720 μM, which also showed an anti-proliferative activity (IC_{50} 280 μM).

Table 4. Cytotoxicity and anti-proliferative effects (IC_{50}) induced on HepG-2 cells by bromoditerpenes **1**–**5** isolated from *S. coronopifolius* and drugs (positive controls). IC_{50} values are expressed as the means of eight independent experiments with 95% confidence intervals.

		IC_{50} (μM)	
		Cytotoxicity	**Anti-Proliferative**
Bromoditerpenes	**1**	719.85 (519.79–996.81)	279.93 (206.78–378.74)
	2	>1000	203.33 (90.65–456.18)
	3	>1000	291.42 (206.22–411.83)
	4	>1000	104.83 (55.27–198.89)
	5	>1000	42.87 (22.76–78.88)
Drugs (+)	Cisplatin	454.6 (388.9–531.3)	75.41 (61.78–92.05)
	Tamoxifen	>1000	45.68 (31.84–65.57)

Regarding antimicrobial activity (Table 5), the highest growth inhibition against *S. aureus* was obtained for compounds **4** (IC_{50} 6.35 μM), **2** (IC_{50} 22.4 μM) and **1** (IC_{50} 96.3 μM). This is the first report for an antimicrobial activity of 12*R*-hydroxybromosphaerol (**4**). For sphaerococcenol A (**5**), a previous report showed an antimalarial activity against the chloroquine-resistant *Plasmodium falciparum* FCB1 strains with an IC_{50} of 1 μM, but no results has been published on antimicrobial activities [33].

3. Experimental Section

3.1. General Experimental Procedures

UV and CD spectra were measured using a Jasco J-810 spectropolarimeter. Optical rotation was measured on a Perkin-Elmer 343 polarimeter, using a 100-mm microcell. IR spectra were recorded on a Bruker Tensor 27 spectrophotometer. NMR spectra were measured on a Bruker Avance 500-MHz spectrometer. Chemical shift values (δ) were reported in parts per million (ppm) relative to the appropriate internal solvent standard, and coupling constants (*J*-values) are given in Hertz and referenced to residual solvent signals ($CDCl_3$ at δ_H 7.26 and δ_C 77.16). HPLC purifications were carried out on a Jasco LC-2000 system equipped with a PU-2087 Plus preparative pumping system and a UV-2075 Plus

detector. HRESIMS data were conducted on an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA).

Table 5. Antimicrobial activities (IC_{50}) against *E. coli*, *P. aeruginosa*, *S. aureus* and *C. albicans* of bromoditerpenes **1**–**5** isolated from *S. coronopifolius* and drugs (positive controls). IC_{50} values are expressed as the means of eight independent experiments with 95% confidence intervals.

		IC_{50} (μM)			
		E. coli	***P. aeruginosa***	***S. aureus***	***C. albicans***
Bromoditerpene	**1**	>100	>100	96.30 (84.60–109.61)	>100
	2	>100	>100	22.42 (15.44–32.57)	>100
	3	>100	>100	>100	>100
	4	>100	>100	6.35 (4.78–8.42)	>100
	5	>100	>100	>100	>100
Drugs (+)	Ampicillin	6.42 (1.86–22.26)	-	0.11 (0.08–0.15)	-
	Bacitracin	>100	-	2.85 (2.36–3.44)	-
	Chloramphenicol	>100	-	80.49 (58.99–109.86)	-
	Oxytetracycline	1.12 (0.65–1.89)	2.13 (1.65–2.76)	0.87 (0.59–1.32)	-
	Amphotericin b	-	-	-	>100
	Flumequine	-	-	-	>100

3.2. Sampling, Identification and Treatment of Algal Material

Sphaerococcus coronopifolius samples were collected freshly from Berlenga Nature Reserve (39°24'44.8"N 9°30'29.5"W), Peniche (Portugal), in June 2012, and immediately transported to the laboratory. The alga was then cleaned and washed with sea water to remove epiphytes, detritus and encrusting material, and the resulting algal material was freeze-dried (Scanvac Cool Safe, LaboGene, Lynge, Denmark). The dry algal material was ground and stored at −80 °C until further use.

3.3. Extraction and Fractionation of Algal Extract by Vacuum Liquid Chromatography (VLC)

Freeze-dried samples of *S. coronopifolius* (470 g) were sequentially extracted in a 1:4 biomass:solvent ratio with MeOH and then CH_2Cl_2 at constant stirring for 12 h. Liquid-liquid extraction was additionally performed for the MeOH extract, using *n*-hexane to remove fats. The CH_2Cl_2 extract (5.2 g) was further concentrated and subjected to normal phase vacuum liquid chromatography on silica gel 60 (0.06–0.2 mm), using cyclohexane with increasing amounts (25%) of ethyl acetate (EtOAc) as the mobile phase (5 fractions, each one with 400 mL of eluent). The dried fractions were stored at −20 °C until further use (F1 287 mg, F2 412 mg, F3 796 mg, F4 442 mg, F5 159 mg).

3.4. Purification of Bromoditerpenes

Fraction F2 (412 mg) was further purified by preparative reversed-phase HPLC (XSelect CSH Phenyl-Hexyl 19 mm × 250 mm, 5 μm, Waters) at a flow rate of 12 mL/min and with a mixture of eluents H_2O/CH_3CN: isocratic from 0 to 5 min (25:75) and then a linear gradient from 5 to 20 min (from 25:75 to 15:85). This first purification afforded 6 subfractions, F2f1–F2f6, from which only

compound **2** (F2p6, t_R 18.8 min, 23 mg) was pure. Fraction F2f1 (t_R 12.6 min, 80 mg) was subjected to a second purification step on a semi-preparative reversed-phase column (Synergi Fusion-RP 80 A, 10 mm × 250 mm, 4 µm, Phenomenex, Torrance, CA, USA) with an isocratic mode H_2O/CH_3CN (27:73) and a flow rate of 4.5 mL/min to afford pure compounds **3** (F2f1p1, t_R 17.5 min, 10 mg) and **5** (F2f1p2, t_R 19.0 min, 26 mg). Subfraction F2f3 (t_R 14.2 min, 9 mg) was purified on the same column with an isocratic mode H_2O/CH_3CN (20:80) at a flow rate of 5 mL/min to yield new compound **1** (F2f3p1, t_R 18.0 min, 4.5 mg).

The purification of fraction F3 (796 mg) was performed with the same procedure previously used for fraction F2, starting with a preparative reversed-phase HPLC separation. The elution was performed using mixtures of H_2O/CH_3CN under the following conditions: isocratic step from 0 to 5 min (30:70) and then a linear gradient until 25 min (from 30:70 to 15:85). This purification afforded pure compound **4** (F3p1, t_R 13.4 min, 19 mg), along with the four compounds previously purified from fraction F2.

*3.5. Sphaerodactylomelol (**1**)*

Colorless oil; $[\alpha]_D^{20}$ −33.3 (*c* 0.15, $CHCl_3$); UV (DAD) λ_{max} 255 nm; ECD (*c* 2.68 × 10^{-4} M, CH_2Cl_2) λ_{max} ($\Delta\varepsilon$) 228 (0.95), 277 (−0.13) nm; IR (neat) σ 2968, 2927, 2855, 1457, 1437, 1377 cm^{-1}; ^1H and ^{13}C NMR data, see Table 3; HRESIMS *m/z* 351.17117 and 353.16891 $[M - OH]^+$ (50:50, calcd. for $C_{20}H_{32}Br^+$, 351.16819 and 353.16614, Δ −8.5 and −7.8 ppm).

3.6. Biological Activities

3.6.1. Cytotoxicity and Anti-Proliferative Activities

The anti-proliferative activities were performed on an *in vitro* carcinoma model of a human hepatocellular cancer (HepG-2), acquired in the American Type Culture Collection (ATCC). HepG-2 cells were cultured in RPMI 1640 (Sigma, Saint-Louis, MO, USA) medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) supplemented with 100 U/mL penicillin G, 0.25 µg/mL amphotericin B and 100 µg/mL streptomycin (Sigma, USA). The cells medium was changed every 3 days, and the cells reached confluence after 5–6 days of initial seeding in plates of a 25-cm^2 growth area at a concentration of 42.2 × 10^6 cells/ plate. For the subculture, cells were dissociated with trypsin-EDTA (Sigma, Saint-Louis, MO, USA), split into a 1:3 ratio and subcultured in Petri dishes with a 25-cm^2 growth area. Cells were maintained in controlled conditions: 95% of humidified atmosphere, 5% of CO_2 and 37 °C. Cells were seeded in 96-well plates, at a concentration of 4.4 × 10^4 cells/well, for the cytotoxicity and anti-proliferative assays.

Cytotoxicity was evaluated after the cells reached total confluence and anti-proliferative activity after 36 h of initial seeding. Cells were incubated with crude extracts, VLC fractions and purified compounds, previously sterile filtered (0.2 µm, Whatman, Little Chalfont, UK), during 24 h at 1 mg/mL. For the samples with the highest activity, dose-response assays were done (10–1000 µg/mL; 24 h). Cisplatin (Sigma, St. Loiu, MO, USA) and tamoxifen (Sigma, Shanghai, China) were used as positive controls. The effects were estimated by the colorimetric assay based on the conversion of tetrazolium dye (MTT) (Sigma, Seelze, Germany) into a blue formazan product by living mitochondria [44]. After the treatment with bromoterpenes compounds, the cells medium was removed, and cells were washed with Hank's

medium (medium composition, in mM: NaCl 137, KCl 5, $MgSO_4$ 0.8, Na_2HPO_4 0.33, KH_2PO_4 0.44, $CaCl_2$ 0.25, $MgCl_2$ 1.0, Tris HCl 0.15 and sodium butyrate 1.0, pH = 7.4). Cells were then incubated with MTT (1.2 mM), previously dissolved in Hank's medium, during 4 h at 37 °C. The formazan products were dissolved in isopropanol (Panreac, Barcelona, Spain), contained 0.04 N HCl and were determined by the absorbance at 570 nm.

Results were expressed as IC_{50}, defined as the concentration causing a 50% reduction or inhibition of cell viability and cell proliferation, respectively.

3.6.2. Antimicrobial Activities

Antimicrobial activities were evaluated by the capacity to inhibit *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *S. aureus* (ATCC 25923) and *C. albicans* (ATCC 10231) growth ($OD\ 600_{nm}$). The crude extracts, VLC fractions, purified compounds and positives controls (ampicillin, bacitracin, chloramphenicol, oxytetracycline, amphotericin b and flumequine, from Sigma Aldrich, Canada) were prepared with sterile-filtered dimethylsulfoxide and stored at −20 °C. Tests were performed in 96-well plates at 37 °C for bacteria and 30 °C for fungi. Antimicrobial activity was expressed as IC_{50}, defined as the concentration causing a 50% reduction of microorganism growth.

3.6.3. Data Analysis

IC_{50} were calculated from the analysis of non-linear regression using GraphPad Prism program even $Y = 100/(1 + 10\ (X - Log\ IC_{50}))$ equation.

4. Conclusions

In the present study, the chemical characterization of the major brominated diterpenes isolated from samples of *S. coronopifolius* collected in the Atlantic revealed five major compounds, including a new brominated dactylomelane diterpene, named sphaerodactylomelol (**1**), and four already known sphaerane bromoditerpenes, **2**–**5**. These molecules exhibited interesting high antimicrobial and antitumor activities against different targets and open new therapeutic potential. To the best of our knowledge, this is the first study on the isolation, identification and bioactive screening of bromoditerpenes isolated from *S. coronopifolius* collected from the Atlantic. Nonetheless, future studies will deeply describe the antitumor and antimicrobial activities and the mechanisms of action of the isolated compounds.

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Author Contributions

The authors Daniel Rodrigues and Celso Alves contributed equally to this work and did the main experiments (purification, identification, cytotoxicity, anti-proliferative, antimicrobial assays and drafted the manuscript). Joana Silva, André Horta and Susete Pinteus have been involved in the antimicrobial, cytotoxicity and antiproliferative assays. Gérald Culioli and Olivier Thomas designed and coordinated the purification and identification of compounds. Rui Pedrosa designed and coordinated the antimicrobial, cytotoxicity and anti-proliferative experiments and conceived the study.

Conflicts of Interest

The authors declare no conflict of interest.

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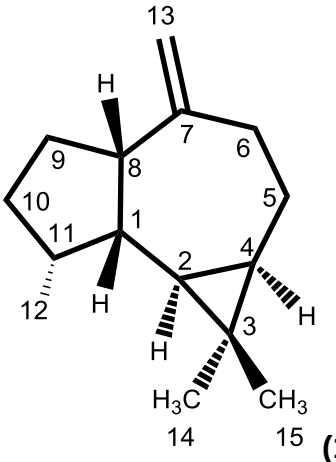
Annex III

NMR spectra of known compounds

Table 1. ^{1}H (400 MHz) and ^{13}C (125 MHz) NMR data of alloaromadendrene (**1**).



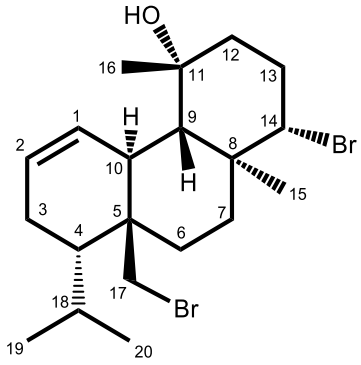
(1)

	Compound (1)		**Alloaromadendrene[1]**		**Alloaromadendrene [2]**		
Atom n°	**$\delta^{1}H$**	**$\delta^{13}C$**	**$\delta^{1}H$**	**$\delta^{13}C$**	**$\delta^{1}H$**	**$\delta^{13}C$**	**Mult.**
1	1.86 *m*	42.13	1.80-1.90 *m*	42.3	1.86 *m*	42.24	CH
2	0.24 *dd* (10.6, 9.6)	23.48	0.25 *dd* (11.0, 9.6)	23.7	0.24 *dd* (10.9; 9.9)	23.59	CH
3	-	17.21	-	17.3	-	17.23	C
4	0.55 *m*	24.76	0.56 *m*	25.0	0.55 *m*	24.87	CH
5	1.84 *m* 1.25 *m*	22.11	1.80-1.90 *m* 1.26 *m*	22.2	1.84 *m*(α) 1.24 *m*(β)	22.20	CH_2
6	2.30 *m*	35.68	2.32 *m*	35.8	2.34 *m*(α) 2.28 *m*(β)	35.76	CH_2
7	-	152.58	-	152.2	-	152.32	CH_2
8	2.68 *m*	50.76	2.67 *m*	51.0	2.65 *m*	50.84	CH
9	1.89 *m* 1.71 *m*	28.19	1.73 *m*	28.3	1.88 *m* 1.73 *m*	28.27	CH_2
10	1.74 *m* 1.32 *m*	31.17	1.80-1.90 *m*	31.3	1.73 *m*(β) 1.32 *m*(α)	31.26	CH_2
11	2.07 *m*	37.77	2.07 *m*	37.8	2.07 *m*	37.86	CH
12	0.94 *d* (7.2)	16.39	0.94 *d* (7.2)	16.4	0.94 *d* (8.0)	16.44	CH_3
13	4.73 *m* 4.71 *m*	109.64	4.73 *m* 4.71 *m*	109.6	4.74 *m* 4.71 *m*	109.78	CH_2
14	1.00 *s*	28.60	1.00 *s*	28.7	1.01 *m*	28.65	CH_3
15	0.96 *s*	15.85	0.96 *s*	15.8	0.96 *m*	15.89	CH_3

[1] NMR data (500MHz, $CDCl_3$) from Rosa *et al.* (1988). Terpenes from the red alga *Sphaerococcus coronopifolius* of the north Adriatic Sea. *Phytochemistry* 27(6)**,** 1875-1878

[2] NMR data (400MHz, $CDCl_3$) from Faure, R. *et al.* (1991). Two-dimensional nuclear magnetic resonance of sesquiterpenes. 4 - Application to complete assignment of 1H and 13C NMR spectra of some aromadendrene derivatives. *Magnetic Resonance Chemistry* 29(9), 969-971.

Table 2. ^{1}H (400 MHz) and ^{13}C (125 MHz) NMR data of bromosphaerol (**3**).



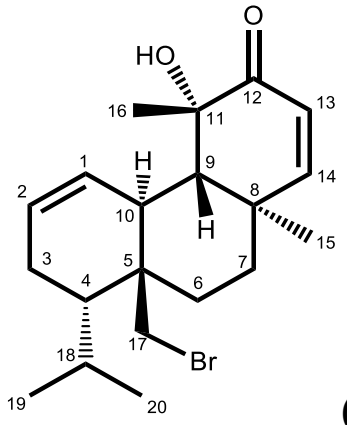
(3)

	Compound (3)		Bromosphaerol[1]		
Atom n°	**$\delta^{1}H$**	**$\delta^{13}C$**	**$\delta^{1}H$**	**$\delta^{13}C$**	**Mult.**
1	6.01 *br d* (10.6)	128.59	6.01 *br d* (10.5)	128.65	CH
2	5.69 *m* (10.6)	126.96	5.69 *dm* (10.5)	126.88	CH
3	2.14 *m*	21.88	2.13 *m*	21.91	CH_2
	1.93 *m*		1.92 – 2.00 *m*		
4	1.76 *m*	42.56	1.65 – 1.88 *m*	42.64	CH
5	-	40.72	-	40.76	C
6	1.81 *m*	24.97	1.28 *m*	36.52[3]	CH_2
	1.52 *m*		1.65 – 1.88 *m*		
7	1.85 *m*	36.44	1.65 – 1.88 *m*	25.04[3]	CH_2
	1.25 *m*		1.46 – 1.54 *m*		
8	-	41.87	-	41.89	C
9	1.48 *d* (10.9)	50.60	1.46 – 1.54 *m*	50.67	CH
10	2.98 *dm* (10.9)	37.34	2.97 *br d* (10.2)	37.41	CH
11	-	72.31	-	72.75	C
12	1.58-1.68 *m*	46.21	1.65 – 1.88 *m*	46.18	CH_2
13	2.41 *qd* (13.0,	30.10	2.41 *ddd* (13.3, 12.4, 4.1)	30.16	CH_2
	4.4)		1.92 – 2.00 *m*		
14	3.99 *dd* (12.5,	68.82	4.00 dd (12.4, 3.4)	68.80	CH
	3.5)				
15	1.30 *s*	14.02	1.30 *s*	14.07	CH_3
16	1.38 *s*	35.04	1.38 *s*	35.02	CH_3
17	3.61 *brd* (10.5)	40.37	3.61 d (10.5)	40.36	CH_2
	3.93 *d* (10.5)		3.93 d (10.5)		
18	1.95 *m*	25.85	1.92 – 2.00 *m*	25.88	CH
19*	0.90 *d* (6.9)	19.84	0.90 *d* (6.8)	19.78	CH_3
20*	0.97 *d* (6.8)	26.05[4]	0.97 *d* (6.8)	20.01	CH_3

[1] NMR (500MHz, $CDCl_3$) data from De Rosa *et al.* (1988) Terpenes from the red alga *Sphaerococcus coronopifolius* of the North Adriatic Sea. *Phytochemistry* 27, 1875-1878.

The carbones C6 and C7 were incorrectly assigned in De Rosa *et al.* (1988). In Table 2 is shown the correct assignment of those carbons made by the HMBC correlations observed (1.30s/36.44 H-15/C-7; 3.93d/24.97 H-17/C-6).

Table 3. ^{1}H (400 MHz) and ^{13}C (125 MHz) NMR data of sphaerococcenol A (**4**).



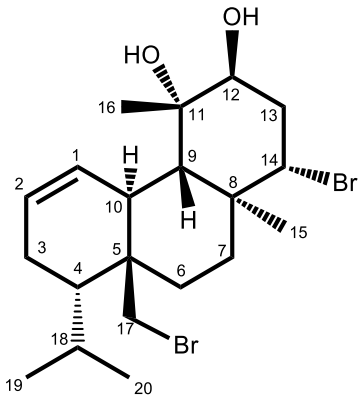
(4)

	Compound (4)		**Sphaerococcenol A[1]**		
Atom n°	**$\delta^{1}H$**	**$\delta^{13}C$**	**$\delta^{1}H$**	**$\delta^{13}C$**	**Mult.**
1	6.05 *br d* (10.4)	128.20	6.05 *br d* (10.3)	128.31	CH
2	5.75 *m*	127.79	5.74 *dm* (10.3)	127.67	CH
3	2.16 *m* 1.99 *m*	22.35	2.16 *m* 1.90 – 2.00 *m*	22.38	CH_2
4	1.78 *brd* (6.6)	42.04	1.78 *m*	42.16	CH
5	-	40.25	-	40.31	C
6	1.59 *m* 1.85 *m*	24.52[4]	1.46 *m* 1.68 *m*	33.21	CH_2
7	1.45 *dm* (12.8) 1.85 *td* (13.5, 3.7)	33.05	1.59 *m* 1.76 *m*	24.61	CH_2
8	-	36.87	-	36.90	C
9	1.93 *d* (12.0)	45.50	1.46-1.54 *m*	45.75	CH
10	2.90 *br d* (12.0)	35.50	2.90 *br d* (13.0)	35.61	CH
11	-	75.37	-	75.36	C
12	-	203.49	-	203.34	C
13	6.07 d (9.8)	124.47	6.07 d (9.8)	124.51	CH
14	6.83 d (9.8)	162.12	6.83 d (9.8)	161.94	CH
15	1.09 *s*	21.43	1.09 *s*	21.07	CH_3
16	1.33 *s*	31.40	1.33 *s*	31.38	CH_3
17	3.72 br*d* (10.7) 3.89 *d* (10.7)	39.91	3.72 *d* (10.7) 3.89 *d* (10.7)	39.82	CH_2
18	1.93-2.01 *m*	25.85	1.90 – 2.00 *m*	25.95	CH
19*	0.92 *d* (6.8)	19.52	0.92 *d* (6.8)	19.45	CH_3
20*	0.97 *d* (6.8)	25.98	0.97 *d* (6.8)	26.00	CH_3
OH	2.93 *s*[3]	-	-	-	OH

[1] NMR data (500MHz, $CDCl_3$) from De Rosa *et al.* (1988) Terpenes from the red alga *Sphaerococcus coronopifolius* of the North Adriatic Sea. *Phytochemistry* 27, 1875-1878.

The carbones C6 and C7 were incorrectly assigned in De Rosa *et al.* (1988). In Table 2 is shown the correct assignment of those carbons made by the HMBC correlations observed (1.05/33.05 e 3.93/24.52).

Table 4 - ^{1}H (400 MHz) and ^{13}C (125 MHz) NMR data of 12*S*-hydroxy-bromosphaerol (**5**).

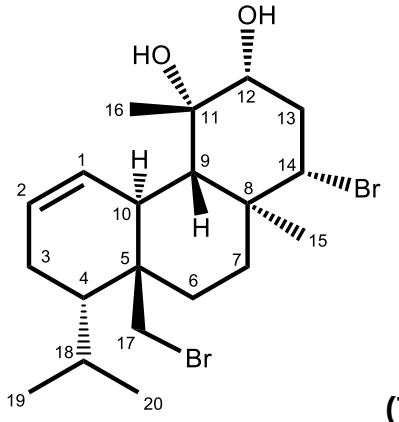


(5)

	Compound (5)		**12*S*-Hydroxy-bromosphaerol[1]**		
Atom n°	**δ^{1}H**	**δ^{13}C**	**δ^{1}H**	**δ^{13}C**	**Mult.**
1	5.99 *brd* (10.6)	128.46	5.97 *br d* (10.4)	128.5	CH
2	5.71 *m*	127.54	5.69 *ddt* (10.4, 5.0, 2.5)	127.5	CH
3	1.95 *m* 2.14 *m*	21.93	α 1.93 *m* β 2.10 *m*	21.9	CH_2
4	1.74 *m*	42.62	1.72 *br s*	42.6	CH
5	-	41.07	-	41.1	C
6	1.80 *m* 1.53 *m*	24.87	α 1.75 *m* β 1.51 *m*	24.9	CH_2
7	1.84 *m* 1.31 *m*	36.42	α 1.81 *m* β 1.29 *m*	36.4	CH_2
8	-	41.78	-	41.8	C
9	1.80 *m*	45.95	1.78 *m*	45.9	CH
10	2.99 *dm* (11.2)	36.82	2.97 *dm* (11.2)	36.8	CH
11	-	74.83	-	74.8	C
12	3.47 *m*	79.41	3.45 *br s*	79.4	CH
13	2.16 *dt* (13.8, 3.7) 2.72 *td* (13.7, 2.7)	37.38	β 2.14 *dt* (13.7, 3.7) α 2.70 *ddd* (13.7, 12.8, 2.9)	37.4	CH_2
14	4.49 *dd* (12.6, 3.8)	63.36	4.46 *dd* (12.8, 3.7)	63.4	CH
15	1.29 *s*	14.87	1.27 *s*	14.9	CH_3
16	1.46 *s*	31.84	1.44 *s*	31.8	CH_3
17	3.62 *dd* (10.6, 1.7) 3.95 *d* (10.6)	40.54	3.60 *dd* (10.8, 2.1) 3.93 *d* (10.8)	40.5	CH_2
18	1.96 *m*	25.90	1.93 *m*	25.9	CH
19	0.91 *d* (6.8)	19.71	0.89 *d* (7.0)	19.7	CH_3
20	0.97 *d* (6.8)	25.93	0.95 *d* (7.0)	25.9	CH_3
OH					OH

[1] NMR data (500MHz, $CDCl_3$) from Smyrniotopoulos *et al*. (2008) Cytotoxic bromoditerpenes from the red alga *Sphaerococcus coronopifolius*. *Tetrahedron* 64, 5184-5190.

Table 5. ^{1}H (400 MHz) and ^{13}C (125 MHz) NMR data of 12*R*-hydroxy-bromosphaerol (**7**).



(7)

	Compound (7)		**12*R*-Hydroxy-bromosphaerol[1]**		
Atom n°	**$\delta^{1}H$**	**$\delta^{13}C$**	**$\delta^{1}H$**	**$\delta^{13}C$**	**Mult.**
1	6.01 *br d* (10.6)	128.79	5.99 *br d* (10.4)	128.8	CH
2	5.69 *m*	126.41	5.67 *ddt* (10.4, 5.8, 2.9)	126.4	CH
3	2.14 *m* 1.93 *m*	21.80	β : 2.12 *m* α : 1.91 *m*	21.8	CH_2
4	1.77 *m*	42.63	1.73 *m*	42.5	CH
5	-	40.47	-	40.5	C
6	1.81 *m* 1.52 *m*	24.94	α : 1.77 *m* β : 1.50 *m*	24.9	CH_2
7	1.82 *m* 1.22 *m*	35.97	α : 1.78 *m* β : 1.18 *m*	36.0	CH_2
8	-	41.97	-	42.0	C
9	1.40 *d* (10.7)	48.71	1.38 *d* (10.8)	48.7	CH
10	3.03 *dm* (10.7)	37.49	3.02 *dm* (10.8)	37.5	CH
11	-	73.46	-	73.5	C
12	3.37 *dd* (11.7, 5.2)	76.94	3.34 *dt* (11.6, 5.4)	76.9	CH
13	2.23 *ddd* (12.4, 5.2, 3.2) 2.35 *q* (12.4)	38.03	β : 2.21 *ddd* (12.4, 5.4, 3.3) α : 2.33 *ddd* (12.8, 12.4, 11.6)	37.9	CH_2
14	3.90 *dd* (12.8, 3.2)	62.96	3.88 *dd* (12.8, 3.3)	63.0	CH
15	1.26 *s*	13.64	1.25 *s*	13.6	CH_3
16	1.43 *s*	30.86	1.41 *s*	30.9	CH_3
17	3.91 *d* (10.3) 3.60 br*d* (10.3)	40.28	3.89 *d* (10.4) 3.59 *dd* (10.4, 1.7)	40.3	CH_2
18	1.94 *m*	25.83	1.93 *m*	25.8	CH
19*	0.91 *d* (6.8)	19.94	0.89 *d* (7.1)	19.9	CH_3
20*	0.98 *d* (6.8)	26.13	0.95 *d* (7.1)	26.1	CH_3

[1] NMR data (500MHz, $CDCl_3$) from Smyrniotopoulos *et al*. (2008) Cytotoxic bromoditerpenes from the red alga *Sphaerococcus coronopifolius*. *Tetrahedron* 64, 5184-5190.

Annex IV

NMR spectra of sphaerodactylomelol

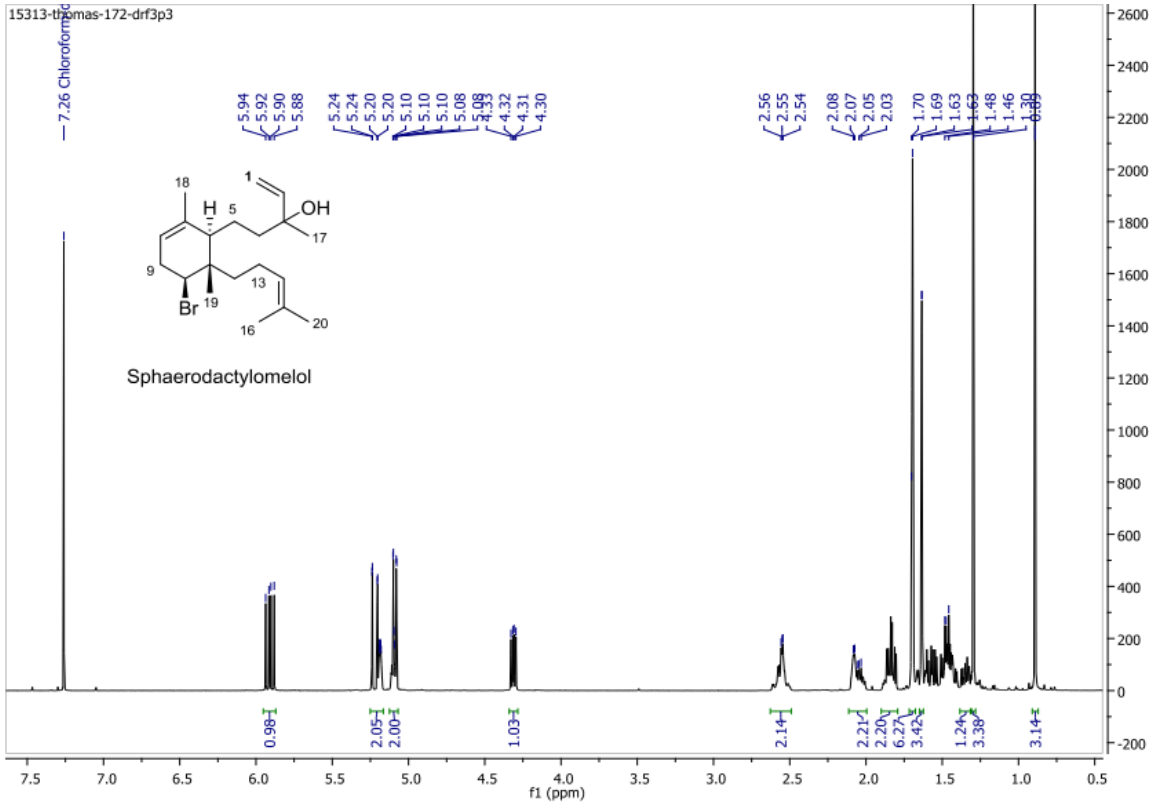


Figure 1. ^{1}H NMR spectrum of sphaerodactylomelol (500 MHz) in $CDCl_3$.

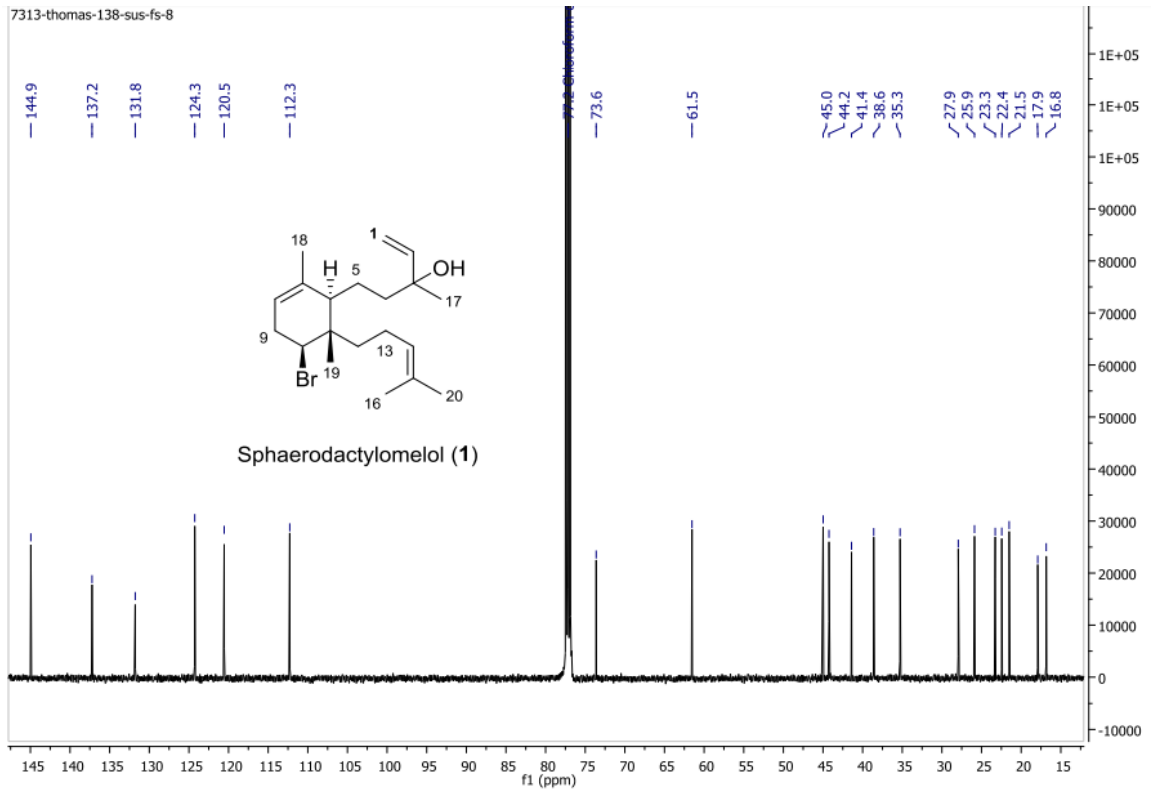


Figure 2. ^{13}C NMR spectrum of sphaerodactylomelol (125 MHz) in CD_3OD.

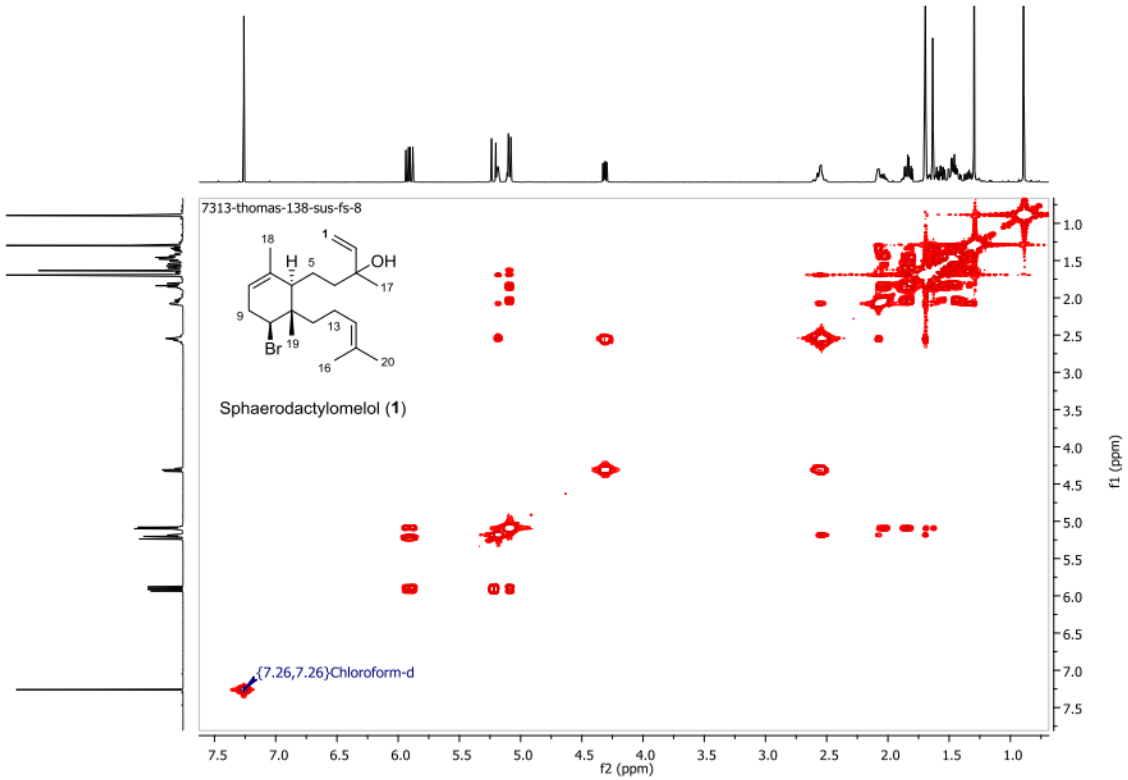


Figure 3. COSY spectrum of sphaerodactylomelol in $CDCl_3$.

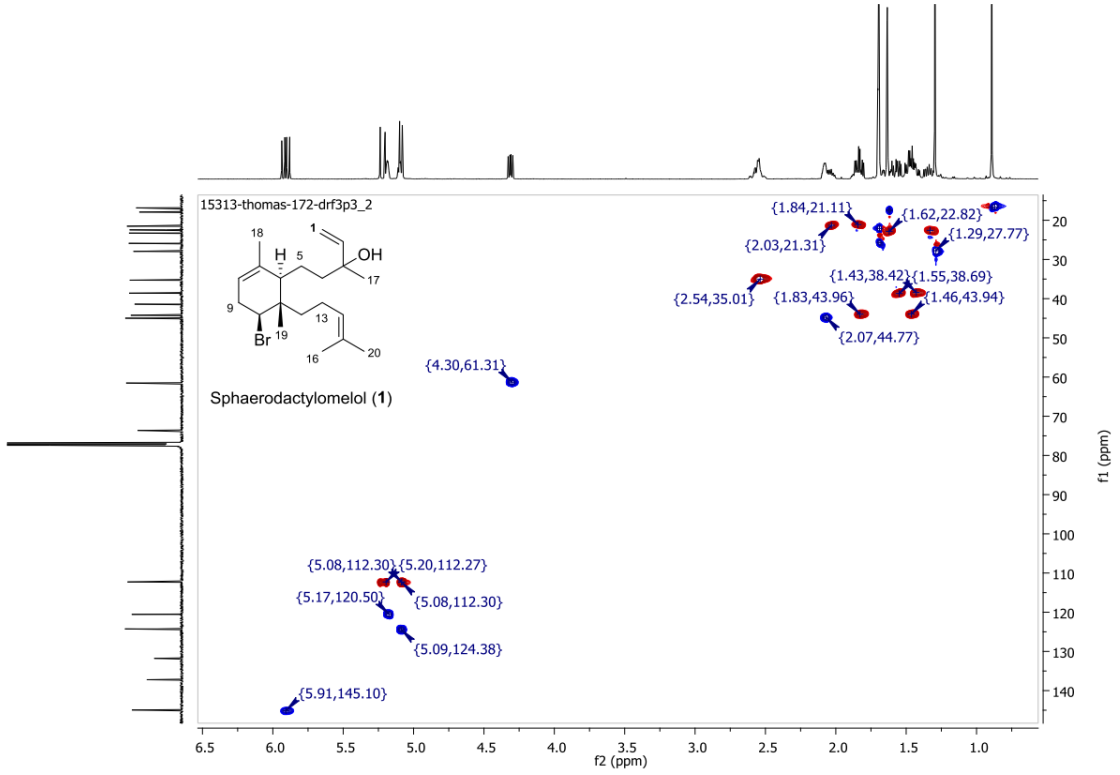


Figure 4. HSQC spectrum of sphaerodactylomelol in $CDCl_3$.

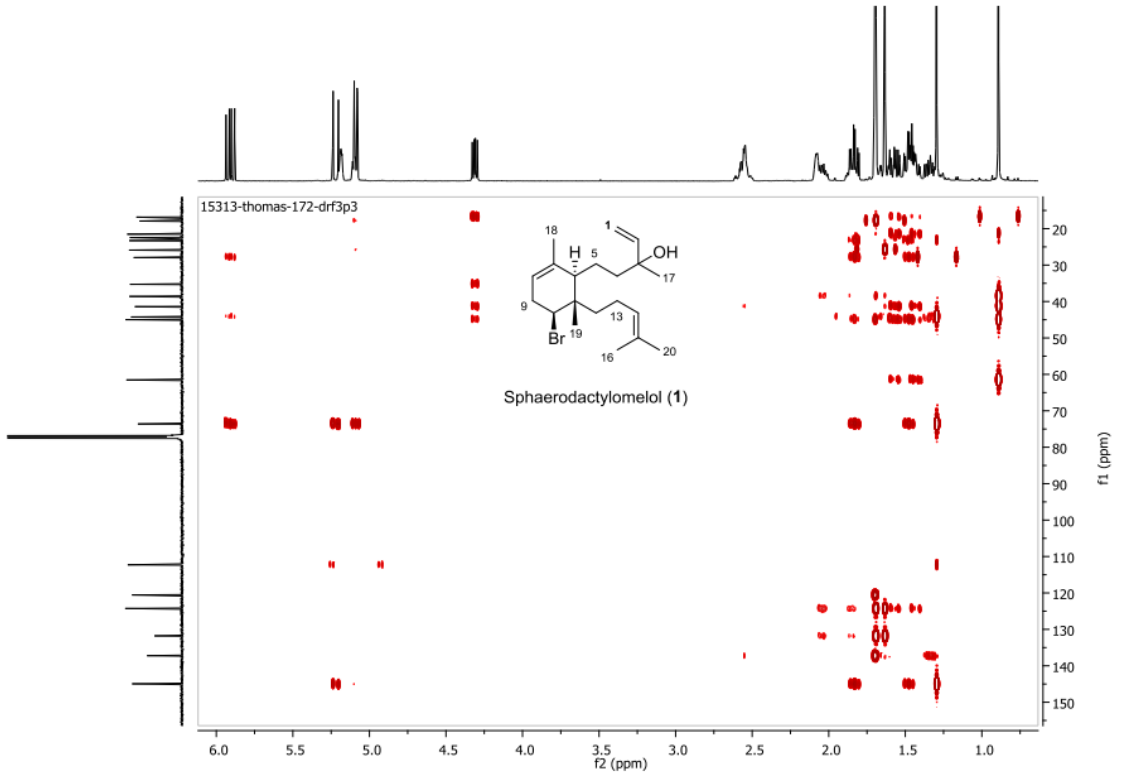


Figure 5. HMBC spectrum of sphaerodactylomelol in $CDCl_3$.

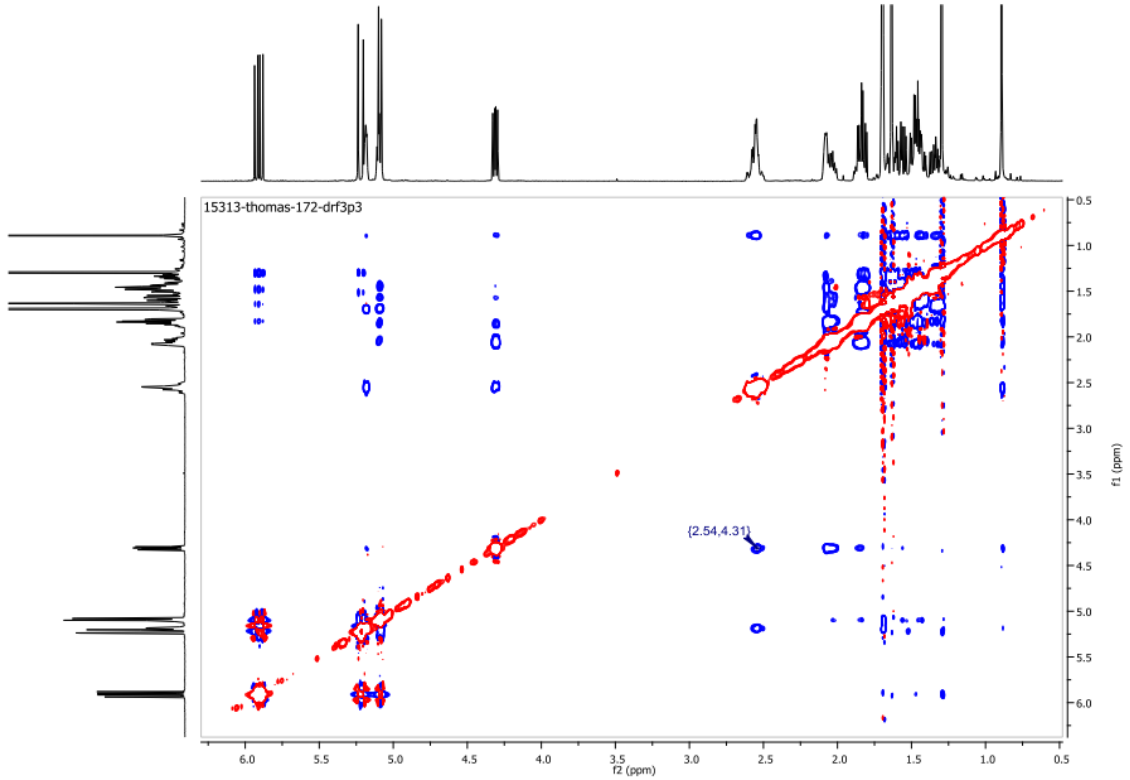


Figure 6. NOESY spectrum of sphaerodactylomelol in $CDCl_3$.

Annex V

NMR spectra of 6-acetyl-sphaeroeudesmanol

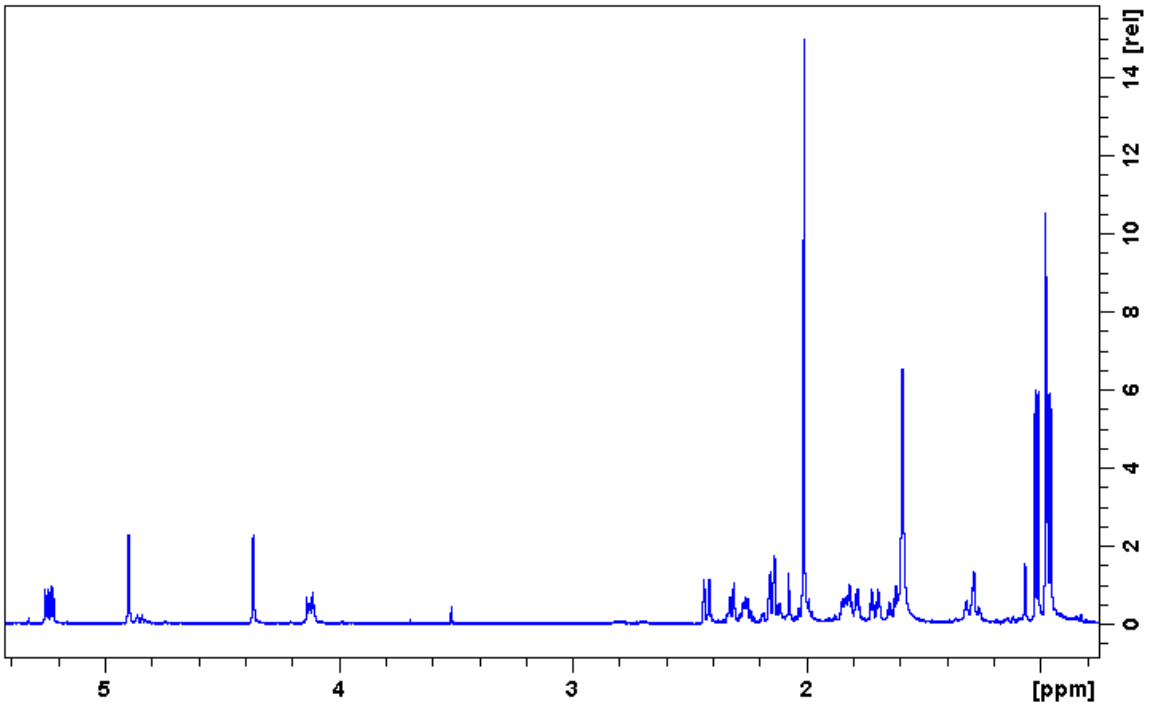


Figure 1. ^{1}H NMR spectrum of 6-acetyl-sphaeroeudesmanol (500 MHz) in $CDCl_3$.

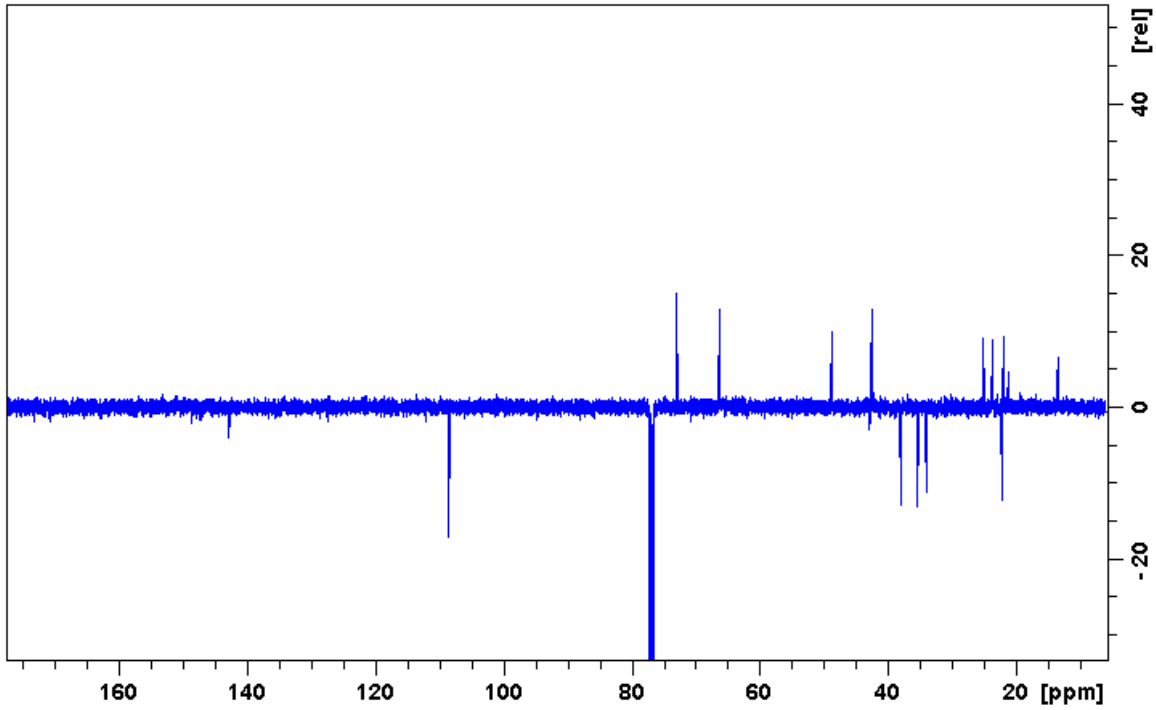


Figure 2. ^{13}C APT NMR spectrum of 6-acetyl-sphaeroeudesmanol (125 MHz) in $CDCl_3$.

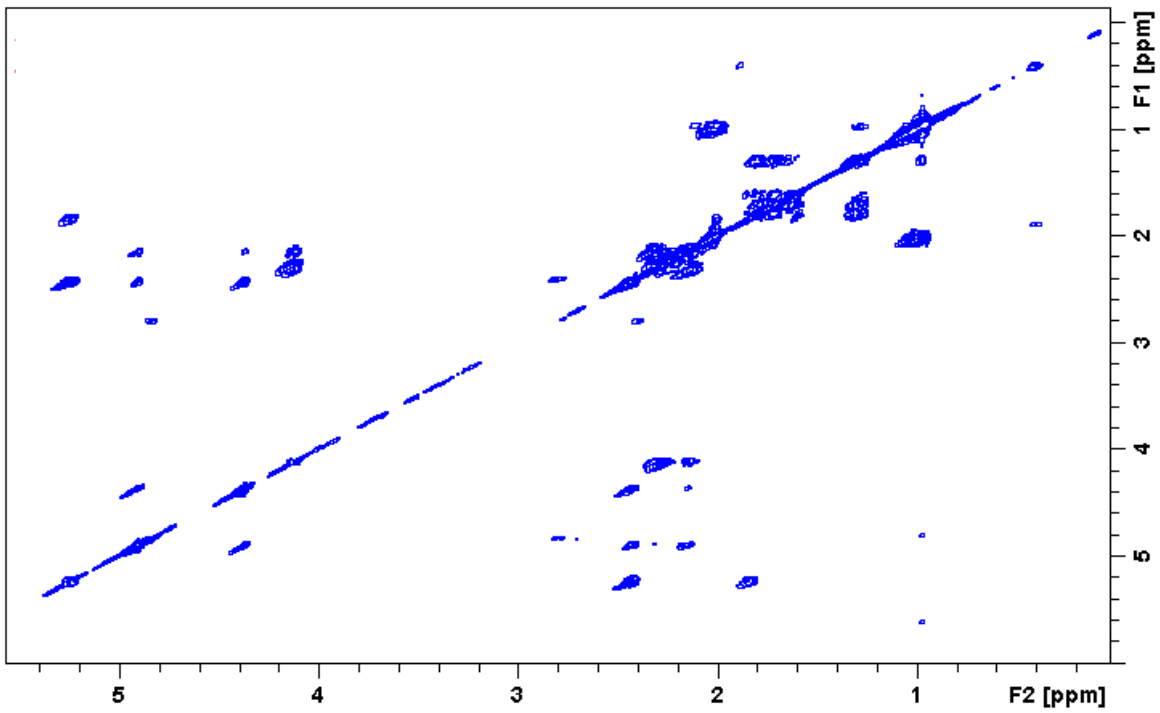


Figure 3. COSY spectrum of 6-acetyl-sphaeroeudesmanol in $CDCl_3$.

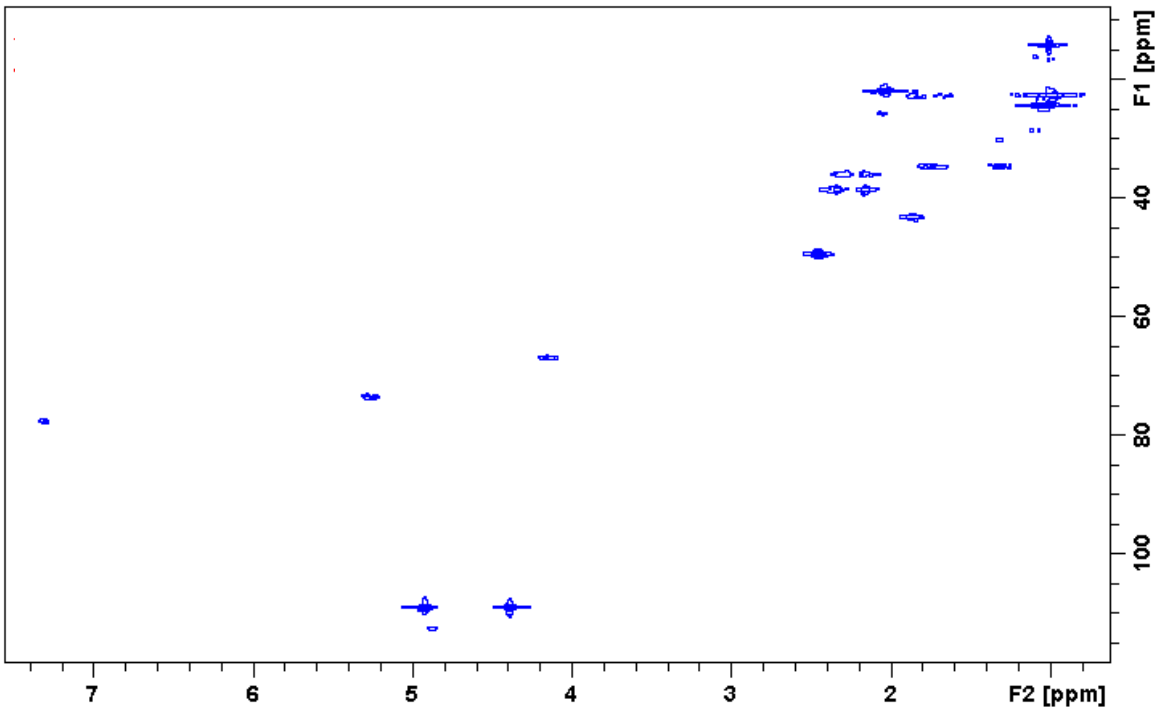


Figure 4. HSQC spectrum of 6-acetyl-sphaeroeudesmanol in $CDCl_3$.

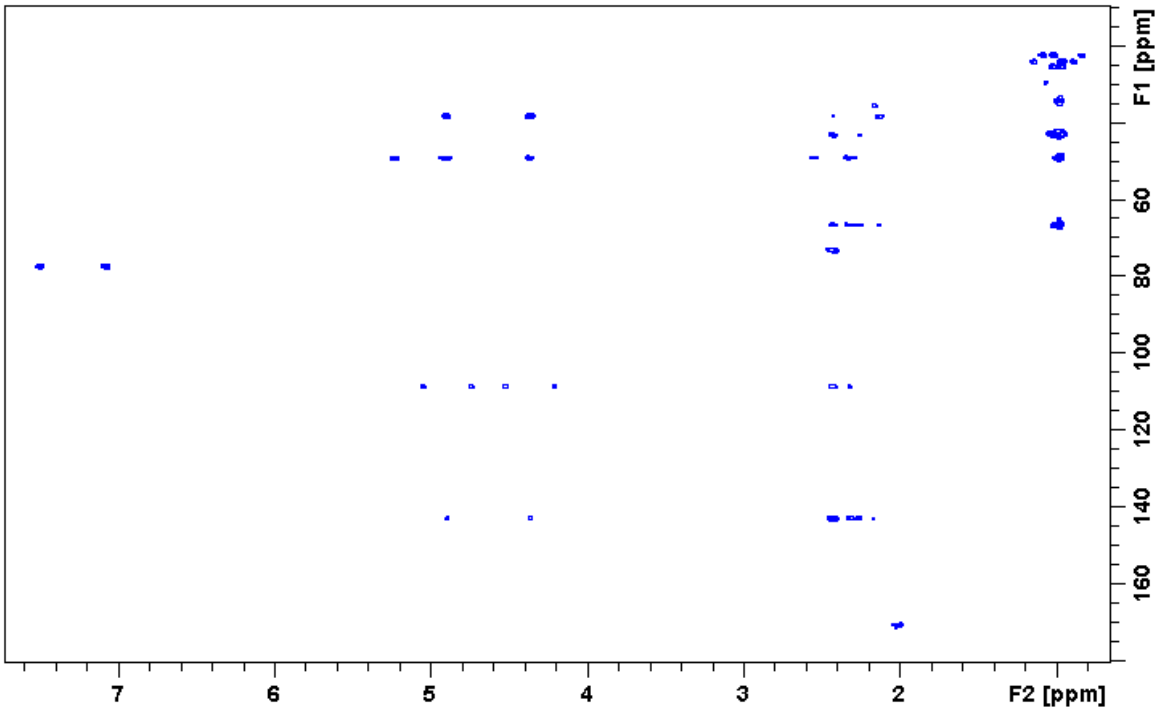


Figure 5. HMBC spectrum of 6-acetyl-sphaeroeudesmanol in $CDCl_3$.

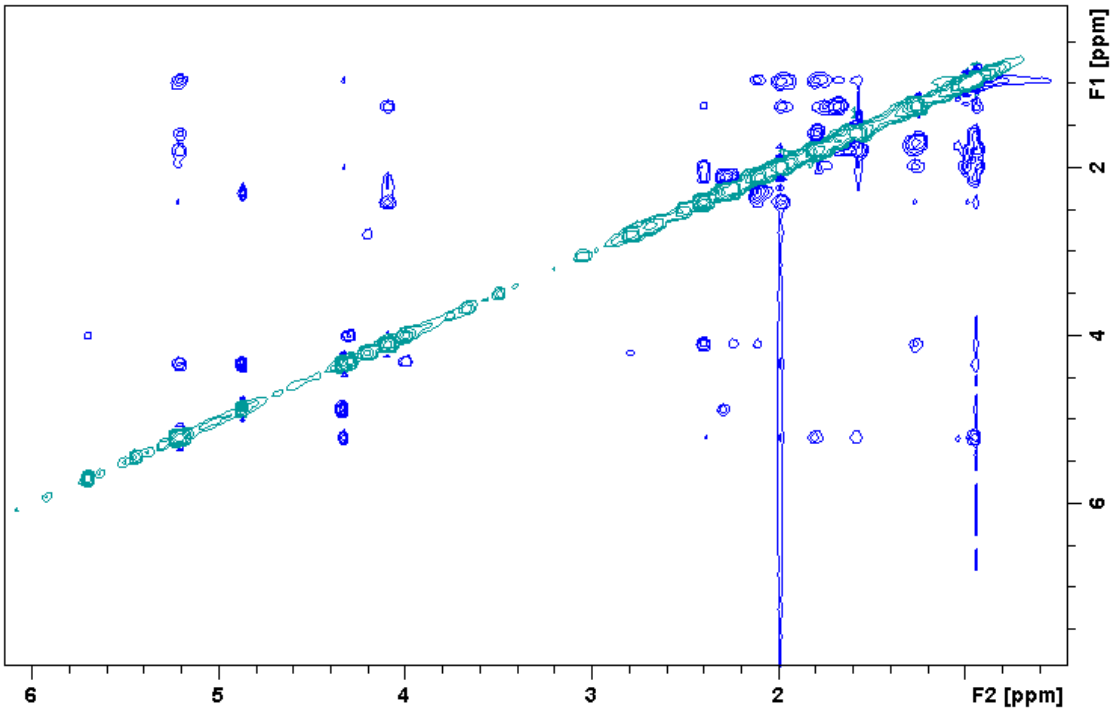


Figure 6. NOESY spectrum of 6-acetyl-sphaeroeudesmanol in $CDCl_3$.