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ANTICANCER ACTIVITY OF MARINE-DERIVED FUNGI EXTRACTS AND ISOLATED COMPOUNDS IN HUMAN CANCER CELL LINES

MARIA PRATA E CASTRO DE SENA ESTEVES

DISSERTAÇÃO DE MESTRADO APRESENTADA

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Abstract

The marine environment is a prolific and largely unexplored source for the prospection of bioactive compounds. The isolation of such substances from marine microorganisms has gained increasing interest over the last decade. Marine fungi are known to produce a great variety of secondary metabolites with unique chemical structures that have appealing bioactivities.

Cancer is currently one of the most globally prevalent diseases. Furthermore, the incidence of cancer is steadily increasing, accompanying the ageing and growth of populations, as well as imbalanced life-styles and declining environmental conditions. The treatment of cancer is commonly (partially or solely) based on chemotherapy, however, resistance to common chemotherapeutic agents and hazardous side effects highlight the importance of the search for safer, more efficient and tumour-specific anticancer drugs.

The first part of this study aimed to assess the *in vitro* anticancer activity of the crude ethyl extracts of three marine sponge-derived fungi, namely, *Aspergillus similanensis* KUFA 0013 (**E1**), *Neosartorya paulistensis* KUFC 7897 (**E2**) and *Talaromyces trachsypermus* KUFC 0021 (**E3**), and of one sea fan-derived fungi *Neosartorya siamensis* KUFA 0017 (**E4**) in a panel of seven cancer cell lines, glioblastoma (U251), malignant melanoma (A375), non-small cell lung cancer (A549), hepatocellular carcinoma (HepG2), colon carcinoma (HCT116 and HT29) and breast adenocarcinoma (MCF-7). Extracts **E2** and **E4** significantly decreased cell proliferation in HepG2, A375 and HCT116 cancer cells, while extract **E2** also decreased long-term cell proliferation in all three cell lines, and extract **E4** in HepG2 and HCT116 cell lines, as observed by the clonogenic assay. Both extracts also managed to induce cell death in HCT116 and HepG2 cells. No genotoxic effect was observed, thus the observed cell death induction does not seem related to the induction of DNA damage, namely by the induction of DNA strand breaks.

The second part of this study focused on nine compounds isolated from *Neosartorya siamensis* KUFA 0017 (**E4**), namely, 2,4-dihydroxy-3-methylacetophenon (**C1**), nortryptoquivaline (**C2**), chevalone C (**C3**), tryptoquivaline H (**C4**), fiscalin A (**C5**), *epi*-fiscalin C (**C6**), *epi*-neofiscalin A (**C7**), *epi*-fiscalin A (**C8**) and tryptoquivaline F (**C9**), which were also screened for *in vitro* anticancer activity in the three cell lines in which extract **E4** presented most activity, namely in HepG2, A375 and HCT116 cells. Results showed that compounds **C2**, **C3**, **C5**, **C6**, **C7** and **C8** presented a significant anti-proliferative effect against the selected cell lines, with IC_{50} values ranging from 24 to 153 μM. The induction

of cell death was observed in HepG2 cells by compounds **C2**, **C5**, **C7** and **C8**, and in HCT116 cells by compounds **C2**, **C3** and **C5**. The induction of cell death is once more possibly unrelated to induction of DNA damage — at least to a point of induction of DNA strand breaks — as none of the compounds exhibited genotoxic activity assessed by the comet assay.

In summary, crude ethyl extracts of the marine-derived fungi *Neosartorya paulistensis* and *Neosartorya siamensis* were shown to have anticancer activity against hepatocellular carcinoma, colon carcinoma and malignant melanoma cell lines. The activity of compounds isolated from *N. siamensis* was in most cases greater than that of the extract, presenting appreciable IC_{50} values, ranging from 24 to 153 μM, and considerable induction of cell death, with compounds exhibiting a range of 5 to 12% increase of cells presenting nuclear condensation. Further studies should be undertaken to elucidate the underlying mechanisms of action and molecular targets.

Resumo

O meio marinho é um ambiente altamente prolífico e ainda parcamente explorado na procura de compostos bioactivos. O isolamento de compostos bioactivos provenientes de microorganismos marinhos tem adquirido particular relevância na última década. Os fungos marinhos são já reconhecidos pela produção de uma grande variedade de metabolitos secundários com estruturas químicas singulares, apresentando bioactividade de interesse.

O cancro é actualmente uma das doenças com maior prevalência a nível global. A incidência do cancro tem aumentado consistentemente, acompanhando o envelhecimento e crescimento das populações, bem como estilos de vida pouco equilibrados e condições ambientais prejudiciais. O tratamento do cancro é habitualmente baseado, em parte ou exclusivamente, na quimioterapia, contudo, o aumento da resistência associada a agentes quimioterapêuticos de uso comum, acompanhado de efeitos secundários nefastos, revela a importância na pesquisa de drogas anticarcinogénicas mais seguras, eficientes e específicas.

A primeira parte deste estudo baseou-se na avaliação da actividade anticarcinogénica *in vitro* de extractos de acetato de etilo de três fungos marinhos associados a esponjas, nomeadamente, *Aspergillus similanensis* KUFA 0013 (**E1**), *Neosartorya paulistensis* KUFC 7897 (**E2**) e *Talaromyces trachsypermus* KUFC 0021 (**E3**), e de um fungo associado a gorgónias, *Neosartorya siamensis* KUFA 0017 (**E4**), num painel de sete linhas celulares de cancro, nomeadamente, glioblastoma (U251), melanoma maligno (A375), carcinoma de pulmão de não-pequenas células (A549), carcinoma hepatocelular (HepG2), carcinoma do cólon (HCT116 e HT29) e adenocarcinoma da mama (MCF-7). Os extractos **E2** e **E4** levaram a uma diminuição significativa da proliferação celular em células HepG2, A375 e HCT116, enquanto que o extracto **E2** diminuiu também a proliferação celular a longo termo nas mesmas três linhas celulares, sendo que o extracto **E4** teve efeito similar em HepG2 e HCT116, tal como observado pelo ensaio clonogénico. Ambos os extractos induziram morte celular em células HCT116 e HepG2. Não foi observado qualquer efeito genotóxico, consequentemente é possível sugerir que a indução de morte celular observada não aparenta estar relacionada com a indução de danos no ADN, nomeadamente indução de quebras nas cadeias de ADN.

A segunda parte deste estudo focou-se em nove compostos isolados de *Neosartorya siamensis* KUFA 0017, nomeadamente, 2,4-dihydroxy-3-methylacetophenon (**C1**),

nortryptoquivaline (**C2**), chevalone C (**C3**), tryptoquivaline H (**C4**), fiscalin A (**C5**), *epi*-fiscalin C (**C6**), *epi*-neofiscalin A (**C7**), *epi*-fiscalin A (**C8**) e tryptoquivaline F (**C9**), que foram também analisados em termos de actividade anticarcinogénica *in vitro* nas três linhas celulares onde o extracto **E4** apresentou maior actividade, especificamente em células HepG2, A375 e HCT116. Os resultados demonstraram que os compostos **C2**, **C3**, **C5**, **C6**, **C7** e **C8** apresentaram um efeito anti-proliferativo significativo contra as linhas celulares seleccionadas, com valores de IC_{50} a variar entre 24 a 153 μM. A indução de morte celular foi observada em HepG2 por via dos compostos **C2**, **C5**, **C7** e **C8**, enquanto que em HCT116, por via dos compostos **C2**, **C3** e **C5**. Mais uma vez, a indução de morte celular parece não estar relacionada com a indução de danos no ADN nomeadamente indução de quebras nas cadeias do ADN, uma vez que nenhum dos compostos exibiu actividade genotóxica observável pelo ensaio cometa.

Em suma, os extractos de acetato de etilo dos fungos marinhos *Neosartorya paulistensis* e *Neosartorya siamensis* demonstraram possuir uma actividade anticarcinogénica relevante contra linhas celulares de carcinoma hepatocelular, carcinoma do cólon e melanoma maligno. A actividade dos compostos isolados de *N. siamensis* suplantou na sua maioria a actividade do extracto, apresentando valores de IC_{50} apreciáveis, numa gama de valores de IC_{50} entre 24 a 153 μM, e uma indução de morte celular considerável, onde os compostos induziram um aumento de 5 a 12% de células com núcleos condensados. Estudos futuros deverão procurar elucidar quais os mecanismos e alvos moleculares associados.

Index

Abbreviations

A375 – Human Malignant Melanoma Cell Line

A549 – Human Non-small Lung Cancer Cell Line

Apaf-1 – Apoptosis Protease Activating Factor-1

BGC823 – Human Gastric Cell Line

CSC – Cancer Stem Cell

CTC – Cancer Transitioning Cell

DAPI – 4',6-diamidino-2-phenylindole

DISC – Death Inducing Signaling Complex

DMEM – Dulbecco's Modified Eagle Medium

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

ED_{50} – Half Maximal Effective Concentration

EDTA – Ethylenediaminetetraacetic acid

EMA – European Medicines Agency

EU – European Union

Fas – First Apoptosis Signal

FBS – Fetal Bovine Serum

FDA – Food and Drug Administration

GI_{50} – Half Maximal Growth Inhibitory Concentration

IAP – Inihibitor of Apotosis Proteins

NCI-H1975 – Non-small cell lung cancer

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HBV – Hepatitis B Virus

HCC – Hepatocellular Carcinoma

HCT116 – Human Colorectal Carcinoma Cell Line

HCV – Hepatitis C Virus

HepG2 – Human Hepatocellular Carcinoma Cell Line

HL-60 – Human Promyelocytic Leukemia Cell Line

Hs683 – Human Oligodendroglioma Cell Line

Huh-7 – Human Hepatocellular Carcinoma Cell Line

HT29 – Human Caucasian Colon Adenocarcinoma Grade II Cell Line

IC_{50} – Half Maximal Inhibitory Concentration

K562 – Human Erythromyeloblastoid Leukemia Cell Line

LMP – Low Melting Point Agarose

MCF-7 – Human Breast Adenocarcinoma Cell Line

MEM – Minimum Essential Medium Eagle

MOLT4 – Human Acute Lymphoblastic Leukemia Cell Line

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

NCI-H460 – Human Non-small Lung Cancer Cell Line

NMP – Normal Melting Point Agarose

P388 – Murine Lymphocytic Leukemia Cell Line

PBS - Phosphate Buffered Saline

PFA – Paraformaldehyde

PLC – Primary Liver Cancer

ROS – Reactive Oxygen Species

RPMI – Roswell Park Memorial Institute Medium

SK-MEL-28 – Human Malignant Melanoma Cell Line

SMAC – Small Mitochondria-derived Activator of Caspases

TNF – Tumour Necrosis Factor

U251 – Human Glioblastoma Astrocytoma Cell Line

U373 – Human Glioblastoma Cell Line

U937 – Human Leukemic Monocyte Lymphoma Cell Line

Preface

In the last few decades, there has been an increasing demand for novel compounds with pharmaceutical applications. The marine environment has become a leading hotspot for the bio-prospection of bioactive compounds, and due to the notable diversity and unique chemical structures, marine sourced natural products possess interesting properties that deserve further research and development into biotechnological applications.

This work was developed in the context of the project MARBIOTECH in the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR) of the University of Porto, which aims to obtain bioactive compounds from marine organisms, with the objective of screening these compounds for a diversity of biological activities, such as allelopathic, antimicrobial, antifouling and anticancer activity.

In this context, the aim of this study was to screen several extracts obtained from marine invertebrate-associated fungi for *in vitro* anticancer activity in a panel of cancer cell lines. The screening of anticancer activity was based on the observation of anti-proliferative activity, cell death induction and genotoxic activity by induction of DNA damage (single strand breaks and alkali-labile sites). The following goal was to select extracts with demonstrated bioactivity and proceed to the subsequent isolation of compounds, and to screen these compounds for *in vitro* anticancer activity.

In summary, this work is organized in four chapters. Chapter I is a brief review to the themes of cancer and bioactive compounds of marine origin. Chapter II and III are comprised by original manuscripts, of which the first has been submitted and the latter is to be submitted to a peer-reviewed international journal. Chapter IV presents an overall conclusion of the work and future perspectives, while the Appendix displays the protocols used during this work to a more extensive detail.

List of publications

The elaboration of this Master thesis, and cooperation with other ongoing related works, rendered data sets that were included both in presentations in international meetings, with published abstracts, and in original papers, as follows:

1. Articles under submission or to be submitted to international peer-reviewed journals

- Ramos A. A.*, **Prata-Sena M.***, Castro-Carvalho B., Dethoup T., Buttachon S., Kijjoa A. & Rocha E. (2014) Testing the potential of four marine-derived fungi extracts as anti-proliferative and cell death-inducing agents in seven human cancer cell lines. *Submitted.*

- Ramos A. A.*, Castro-Carvalho B.*, **Prata-Sena M.**, Dethoup T., Buttachon S., Kijjoa A. & Rocha E. (2014) Extracts from *Neosartorya* (fungi) species exhibit anti-proliferative activity with cell death induction in colon, breast and skin cancer cell lines. *Submitted.*

- **Prata-Sena M.**, Ramos A. A., Castro-Carvalho B., Dethoup T., Buttachon S., Kijjoa A. & Rocha E. (2014) Cytotoxic activity of compounds isolated from marine-derived fungi *Neosartorya siamensis* in human cancer cells. *To be submitted.*

- Castro-Carvalho B., Ramos A. A., **Prata-Sena M.**, Dethoup T., Buttachon S., Kijjoa A. & Rocha E. (2014) Marine-derived fungi extracts and isolated compounds enhance the anticancer activity of doxorubicin in non-small cell lung cancer cells. *To be submitted.*

2. *Abstracts published in international peer-reviewed journals*

- **Prata-Sena, M.**, Ramos, A., Castro-Carvalho, B., Dethoup, T., Buttachon, S., Kijjoa, A., & Rocha, E. (2014). Anti-proliferative and pro-apoptotic activities of two marine sponge-derived fungi extracts in HepG2, HCT116 and A375 cancer cell lines. *Planta Medica, 80*(16), P1N3. doi: 10.1055/s-0034-1394594

- Castro-Carvalho, B., Ramos, A., **Prata-Sena, M**., Dethoup, T., Buttachon, S., Kijjoa, A., & Rocha, E. (2014). Extracts from the marine fungus *Neosartorya tsunodae* and the soil fungus *Neosartorya fischeri* exhibit anti-proliferative and pro-apoptotic effects in human cancer cell lines. *Planta Medica, 80*(16), P1N32. doi: 10.1055/s-0034-1394622

- Ramos, A., Moreira, M., Castro-Carvalho, B., **Prata-Sena, M.**, Dethoup, T., Buttachon, S., Kijjoa, A., & Rocha, E. (2014). Marine-derived fungi extracts increase doxorubicin's cytotoxic effect in lung cancer cells. *Planta Medica, 80*(16), P1N25. doi: 10.1055/s-0034-1394615

- Ramos, A., Malhão, F., Ferreira, A., Alves, Â., Castro-Carvalho, B., **Prata-Sena, M.**, Gargiulo, D., Dethoup, T., Buttachon, S., Lobo-da-Cunha, A., Kijjoa, A., Rocha, E. (2014) Marine and soil fungi extracts with anti-proliferative activity induce morphological alterations in breast cancer cells. *Microscopy and Microanalysis (in press)*

Note: 1) Abstracts published in *Planta Medica* concern posters presented at the 62nd International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research – GA2014, between the 31st of August and 4th of September 2014, in Guimarães; and 2) extended abstract to appear in *Microscopy and Microanalysis* was derived from a poster presented at the INCOMAM'14 — International Conference on Microscopy and Microanalysis, XLVIII Congress of the Portuguese Microscopy Society, from the 6th to the 7th of November 2014, in Porto.

CHAPTER I

GENERAL INTRODUCTION

1.1. CANCER

Cancer is the general denomination for a series of diseases associated to regulatory abnormalities in cell growth and homeostasis. Overall, more than 100 distinct types of cancer have been described (Hanahan & Weinberg, 2000).

The etymology of the term “cancer” originates from the greek word *karkinos*, and its coinage is attributed to Hippocrates (460-370 B.C.), who made an analogy of the disease and its lesions to a moving, clasping crab. This early observation and appellation comes as no surprise, considering that in spite of cancer usually being considered a disease of the ‘modern ages’, it has accompanied both animals and mankind since time immemorial. The oldest description of cancerous lesions, in this case of breast cancer, dates back to Egypt in 3000 B.C. (Hajdu, 2011).

Cancer currently represents a high economic and societal burden for both developed and developing countries. Several factors have been pinpointed as causative factors of cancer. Environmental factors account for around 90-95% of all cancers, while inherited genetic factors are involved in 5-10% of the cases (Anand et al., 2008). Examples of environmental factors that have been correlated with the onset of cancer are, for example, smoking tobacco, alcohol consumption, diet, obesity, infectious agents, environmental pollution, radiation and physical inactivity (Jemal et al., 2011). Ultimately, this disease is best fought by prevention, where prophylactic measures such as annual cancer screenings in critical age-groups, vaccination for viral-induced cancers, the consumption of fruit, vegetables, wholegrain foods, teas and spices, vitamins, physical exercise and caloric restrictions are deemed essential for the maintenance of good health and lowering of cancer risk factors (Anand et al., 2008).

1.1.1. Demographics of cancer

In the 2012 GLOBOCAN project, it was estimated that there was a worldwide incidence of 14.1 million new cancer cases, 32.6 million people living with cancer and 8.2 million deaths due to cancer. Over half of all cancer cases and cancer-related deaths occurred in developing countries. The five most common cancers are lung, breast, colorectal, prostate and stomach cancer. All the aforementioned cancers in the exception of prostate, and

including liver cancer, are considered to be the deadliest cancers, with the highest annual mortality rates. Considering the ageing and growth of the global populations, projections based on this study suggest an increase of 19.3 million new cases of cancer per annum by the year 2025 (Ferlay et al., 2013).

1.1.1.1. Colorectal carcinoma

Colorectal cancer is one of the most commonly diagnosed cancers on a global scale, arising each year 1.23 million new cases (Ferlay et al., 2010). Around 55% of the total colorectal cancer cases arise in more developed regions (Ferlay et al., 2013). Notwithstanding its decreasing tendency in developed countries due to increased awareness and anticipated detection, shifts in key risk factors such as smoking, alcohol abuse, obesity, high caloric intake, low vegetable and fruit intake and physical inactivity have lead to an increase in the number of cases (Ferlay et al., 2010; LeMarchand et al., 1997). An important factor, however, is the hereditary factor involved in the development of colorectal cancer (Jasperson et al., 2010).

1.1.1.2. Hepatocellular carcinoma

The most current annual estimate for the incidence of primary liver cancers (PLC) is a total of approximately 780.000 cases diagnosed worldwide (Ferlay et al., 2013). The annual worldwide mortality rate is just as high, and in 2004 it was estimated that PLC was the cause of 1% of all deaths (Blachier et al., 2013; Nordenstedt et al., 2010). Consequently, in 2011 primary liver cancer held fifth and second place in the list of worldwide most common cancers and cause of cancer death, respectively (Jemal et al., 2011). Hepatocellular cancer (HCC) represents around 85-90% of all PLC cases. This type of cancer is most prevalent amongst men, and its distribution is most centered in South-East and East Asia and West and Middle Africa populations (Jemal et al., 2011). This may be in part explained by the risk factors associated to hepatocellular carcinoma, which are mainly attributed to chronic infection by hepatitis B virus (HBV) and hepatitis C virus (HCV), accounting for 80 to 90% of all cases. Other risk factors that have been suggested as influential in the development of HCC carcinogenesis are alcohol, non-

alcoholic fatty liver disease, diabetes, tobacco, oral contraceptives, obesity and exposure to dietary aflatoxin (El-Serag & Rudolph, 2007; Nordenstedt et al., 2010).

1.1.1.3. Malignant melanoma

Malignant melanoma is a type of skin cancer that origins from pigment-producing epidermal melanocytes. Common risk factors include exposure to ultraviolet light in sunbeds and sunlamps, history of sunburn, chronic sun exposure and familial genetic factors (Choi & Fisher, 2014; Ferlay et al., 2010; Shi et al., 2014). Melanoma of the skin was estimated to have caused 232.130 new cases and 55.488 deaths worldwide in 2012 (Ferlay et al., 2013). Fortunately, primary melanomas have a high cure rate, with 98.3% of patients presenting a 5-year rate survival, however, when the metastatic process is initiated, the cancer becomes highly aggressive and seriously undermines the 5-year survival rate to about 16% (Garraway & Chin, 2011).

1.1.2. The biology of cancer

1.1.2.1. Carcinogenesis

The process of carcinogenesis is defined as that by which normal cells gradually acquire a malignant, invasive profile. Cell division is an essential process that ensures the renovation and repopulation of tissues and organs. The cells involved in this proliferative activity are stem cells, which are able to divide and differentiate. Although every individual goes through the process of cell renewal daily, the total amount of cells in the body is maintained (Bertram, 2000). This occurs due to intricate control mechanisms that determine the extent of cell proliferation, but also govern cell death. The programmed death of cells is vital for the renewal of important tissues and removal of defective or damaged cells. However, the process of cell proliferation is dependent on several factors, such as the microenvironment cells are surrounded by, influence of exogenous factors and the accurate functioning of DNA control and repair mechanisms. The DNA molecule is inherently instable, so damage to the DNA can occur spontaneously due to replicative errors, errors in repair, or even by chemical induction (Cohen & Arnold, 2008). DNA

damage may also occur when induced by environmental carcinogens, which can be of chemical or physical nature (Bertram, 2000). Normal cells constantly ensure the maintenance of their DNA, by sensing and responding to DNA damage when it occurs and adequately repairing it. However, when a cell suffers a critical alteration in its DNA, which it is unable to repair, the cell usually enters a process of programmed cell death by apoptosis. Notwithstanding, when this does not occur and the damage to the DNA is maintained, the potential beginning of carcinogenesis is observed (Kryston et al., 2011). If this damage remains unrepaired and several other specific mutations appear, which may vary from a few dozen to thousands, remaining unchecked and allowed to accumulate, then the cell acquires a malignant profile (Greaves & Maley, 2012). These changes may take several years to take place. Mutations that promote oncogene expression and inhibit tumor suppressor gene expression are of critical importance (Babashah & Soleimani, 2011). In addition, alterations in epigenetic mechanisms can lead to altered gene function and the onset of malignancy (Sharma et al., 2010).

The most critical point that defines whether these genetic alterations are successful is the ability to proliferate and generate multiple clones bearing the same mutations. These accumulated mutations may confer cancer cell clones a selective advantage over other clones. The reproductive success of a malignant cell generates clone cells that no longer obey the strict homeostatic protocol of normal cells, ignoring the cooperative agenda of their regular counterparts, focusing instead on unrestrained multiplication and demonstrating phenotypic alterations that have been defined as the hallmarks of cancer (Greaves & Maley, 2012). Hanahan and Weinberg (2000) proposed the groundbreaking six hallmarks of cancer, which have more recently been updated to ten (Hanahan & Weinberg, 2000). These hallmarks represent distinctive features that are common to all types of cancer and are those responsible for tumour growth and dissemination of metastasis. The ten hallmarks are: sustained proliferative signaling, inducing angiogenesis, enabling replicative immortality, resisting cell death, evading growth suppressors, activating invasion and metastasis, tumour-promoting inflammation, avoiding immune destruction, deregulating cellular energetics and genome instability and mutation (Hanahan & Weinberg, 2011).

The proliferation of cancer cells leads to a neoplasm, also known as a tumour. Not all tumours are malignant, benign tumours (e.g., melanocytic nevi) are not invasive or metastatic and do not cause significant health threats, thus they are not cancerous in nature. Malignant tumours display high heterogeneity in terms of phenotypic expression, which result of the influence of both genetic and non-genetic factors. This heterogeneity may complicate therapeutic approach (Marusyk et al., 2012).

The development of metastasis is in fact the main cause of death in cancer patients. Metastasis are formed when a cancer cell originated from a primary tumour develops an invasive phenotype and invades surrounding tissue, eventually penetrating the microvasculature of either blood or lymphatic systems, i.e. intravasation. These transitioning cancer cells (CTC) circulate through the bloodstream until reaching small vessels in a distant tissue, where they then exit, i.e. extravasion. These cells must then evade the innate immune response in order to survive, and when successful, they must adapt to the new microenvironment and thus proliferate, forming a secondary tumour, i.e. colonization (Chaffer & Weinberg, 2011). Metastatic sources for common cancers are exemplified in Figure 1.

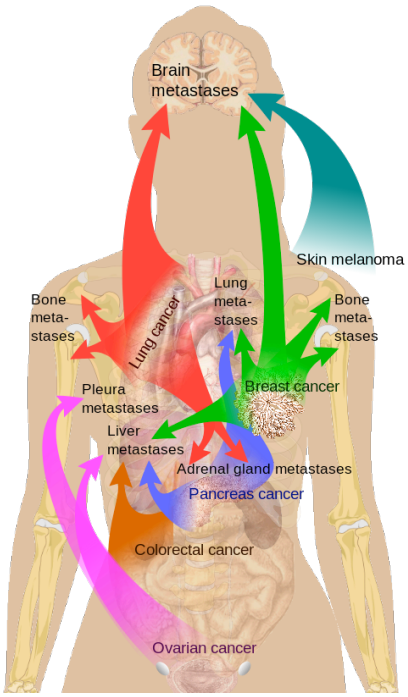

Figure 1 – Sources of metastasis for common cancers. *Source*: Mikael Häggström, via Wikimedia Commons, Creative Commons CC0 1.0.

It is widely accepted that tumours possess cancer cells that are heterogenous in terms of phenotype and function. Recent studies have shown that cancer cells in tumours obey to a hierarchy in terms of cells with tumorigenic and invasive potential. This hierarchy is consisted by stem cells, progenitor cells and differentiated cells. Cancer stem cells (CSC) lie on the top of this hierarchy, and they have been labeled as self-renewing and as the source of the heterogenous lineages of cancer cells contained in the tumour, as they are able to differentiate, much like normal stem cells (Sugihara & Saya, 2013). CSCs possess a high clonogenic potential, and are thought to be responsible for the enhancement tumour growth, local tissue invasion and the formation of distant metastasis (Sampieri &

Fodde, 2012). Recent research has tried to solidify the hypothesis of CSCs being the main culprits in the phenomenon of migration and metastasis. In fact, the latest studies suggest that CSCs may very well be the root of cancer, and highly responsible not only for the invasion by metastasis, but also for relapses in patients who have been in remission for several years, for CSCs can survive in dormancy for extended periods of time (Tirino et al., 2013). Considering that CSCs have also been found to be highly resistant to both chemotherapy and radiotherapy, efforts are being made to produce targeted therapy towards CSCs in hopes of limiting the aggressiveness of the cancer and its invasive potential, as well as eliminating residual cancer stem cells and mitigating relapses of the disease (Shiozawa et al., 2013).

1.1.2.2. Programmed cell death and cancer

Programmed cell death (PCD) is an essential mechanism involved in cell death and survival. There are three known types of PCD, namely apoptosis, autophagy and programmed necrosis. All these types of PCD are involved to some extent in the pathological process of cell death, and more specifically in this approach, in the development of cancer (Ouyang et al., 2012).

Apoptosis is mediated by a strict intracellular program, which ultimately leads a cell exposed to a certain stimulus to pursue a path that leads to its own death (Kerr et al., 1972). It is involved in several physiological processes, such as lymphocyte development and homeostasis, sculpting of tissue during embryonic development, destruction of cells with consequent proliferative replacement and physiologic involution (Taylor et al., 2008; Zhang et al., 2005). Disorders in apoptosis play a major role in pathogenesis, namely in cancer, neurological and cardiovascular disorders and autoimmune diseases (Favaloro et al., 2012).

Autophagy is a highly regulated process, which plays a role in the elimination of dysfunctional or unnecessary cellular components. Autophagy is not only involved in normal cell homeostasis, development, fight against infection and disease, but also in the response to metabolic stress (Mizushima et al., 2008). Its course of action is followed when the unnecessary cellular components are trapped by autophagic vacuoles and their degradation is enforced by lysosomes. In fact, autophagy may play a pro-survival role or a pro-death role. This pro-death role has been suggested as being triggered in situations where apoptosis is inhibited or impeded, acting as a last resort. Autophagic cell death is proposed to occur when the autophagic process eliminates such a large portion of cellular

components that the cells' function and viability are mortally compromised (Levine & Yuan, 2005). Nonetheless, autophagic induced cell death is still a controversial subject, as there is still no consensual evidence that autophagy can effectively kill a cell (Shen et al., 2012). Moreover, autophagy may also trigger apoptosis and *vice-versa*. The exact mechanisms involved in the cross-talk between apoptosis and autophagy remain largely unclear, however, both seem to be regulated by Bcl-2 family proteins, and autophagic proteins (Atg) have been proposed as pro-apoptotic effectors and regulators of caspase induction (Ryter et al., 2014). Autophagy plays a dual role in cancer, as it can be both tumour-suppressing by removing damaged organelles, toxic unfolded proteins and oncogenic proteins, or be tumour-promoting, by providing the cancer cells with substrates for metabolism, maintaining mitochondria and thus inducing stress-tolerance (White, 2012).

Necrosis is most commonly referred as uncontrolled cell death. In contrast, new forms of necrotic cell death have been described, such as necroptosis, are considered to have regulated signaling pathways, which in turn may be linked to cross-talk with apoptosis and autophagy (Feoktistova & Leverkus, 2014). Necrosis occurs when a cell is exposed to trauma, infection and toxins that cause damage beyond a threshold of feasible repair, and ultimately leads to cell and organelle swelling, loss of membrane integrity and the leakage of intracellular components to the extracellular medium, which in turn causes an aggravated inflammatory response by the immune system. This type of immune response is not only costly to the organism, but can also lead to the development of further damage to surrounding cells and tissues. This localized induction of inflammation may promote tumour growth (Vakkila & Lotze, 2004). Nonetheless, the exploitation of necrotic cell death in cancer therapy may be of interest (Ouyang et al., 2012).

1.1.2.2.1. Apoptosis in cancer

Apoptosis is characterized by a series of morphological and biochemical hallmarks, such as membrane blebbing, cell shrinkage, nuclear condensation and fragmentation, phosphatidylserine externalization, detachment from the cellular matrix, mitochondrial fragmentation amongst others (Elmore, 2007). In contrast with necrosis, where the leaking of cell content stimulates inflammation, apoptotic cells trigger direct chemotactic signaling to phagocytes, thus avoiding inflammation and damage to neighboring cells (Taylor et al., 2008).

Apoptosis is triggered when an exogenous or endogenous stressor stimulates the cell to produce apoptotic signals, which cause regulatory proteins to initiate the apoptotic pathway. This process usually occurs either by the extrinsic or death receptor pathway or the intrinsic or mitochondrial pathway, which ultimately activate executioner caspases and induce cell death. Briefly, the extrinsic pathway initiates apoptosis by involving transmembrane receptor-mediated pathways, where cytokine ligands (e.g., TNF) bind to the death receptors (Fas), which will aggregate to the cell surface and form a Death Inducing Signaling Complex (DISC) that will then activate the caspase cascade (Elmore, 2007). The intrinsic pathway occurs with the involvement of mitochondria, the Bcl-2 family proteins, which regulate mitochondrial permeability, and the p53 tumour suppressor protein, which regulates the Bcl-2 proteins. The initial response is based on a response to a stress stimulus, which is then sensed by cytosolic or intra-membrane molecules, that consequently send the signal to the mitochondria. This results in changes in the mitochondrial membrane, such as loss of mitochondrial membrane potential that increases protein permeability, the SMAC proteins (small mitochondria-derived activator of caspases) then exit the mitochondria and diffuse in the cytosol. SMAC proteins will bind to and deactivate inhibitor of apoptosis proteins (IAP), thus avoiding the arrest of the apoptotic pathway. The mitochondrial cytochrome *c* will bind to the apoptosis protease activating factor-1 (Apaf-1), thus a series of subsequent processes will create the apoptosome and activate the caspase cascade (Elmore, 2007; Khosravi-Far & Esposti, 2004).

Cancer cells manage to avoid the induction of apoptosis due to three main factors: reduced caspase function, imbalance of anti-apoptotic and pro-apoptotic proteins and impaired death receptor signaling. The reduction of apoptotic caspase activity, which comprises initiator caspases (e.g., caspase-2) and effector caspases (e.g., caspase-3), may lead to impaired apoptosis and carcinogenesis. Abnormalities in pathways involved in the signaling of death, such as the downregulation or impairment of receptors, lead to deficient signaling, thus the stimulus of apoptosis is reduced and may lead to carcinogenesis (Wong, 2011). Several proteins are involved in promoting or inhibiting apoptotic activity. The main proteins of interest involved in this process are proteins of the Bcl-2 family, p53 and IAPs.

The Bcl-2 family is comprised of pro-apoptotic proteins (e.g. Bax, Bak, Bad, Bcl-XS, Bid, Bik ad Bim) and anti-apoptotic proteins (e.g., Bcl-2, Bcl-xL and Mcl-1), which are located in the outer mitochondrial membrane (Ouyang et al., 2012). An imbalance of expression of these proteins can cause a dysregulation of apoptosis, in particular when overexpressing anti-apoptotic proteins and underexpressing pro-apoptotic proteins.

Mutations in genes encoding these proteins are common to many cancers and may even lead to multi-drug resistance (Kelly & Strasser, 2011; Wong, 2011).

The protein p53 is the most notorious tumor suppressor protein, encoded by the gene *TP53*. It is regarded as the guardian of the genome, being responsible for apoptosis induction, autophagy modulation, cell cycle regulation, DNA repair, differentiation and development, DNA recombination, cell senescence, chromosomal segregation and gene amplification (Maiuri et al., 2010; Wong, 2011). Studies have shown that p53 is mutated in at least 50% of cancers (Bai & Zhu, 2006). The p53 tumour suppressor gene reacts when the cell suffers DNA damage, by arresting the cell cycle until the DNA is repaired. If p53 is mutated, cells will continue to divide disregarding the DNA damage, which may lead to the appearance of malignancy (Bai & Zhu, 2006).

The inhibitor of apoptosis proteins (IAP) are involved in apoptosis, signal transduction, cytokinesis immunity, and are also endogenous caspase inhibitors. Overexpression of these proteins may lead to inhibition of apoptosis and promotion of pro-survival signals that contribute towards tumour proliferation (De Almagro & Vucic, 2012).

1.1.3. Therapeutic approaches to cancer

Early cancer treatments were mostly based on exploratory surgery resulting in the resection of cancerous lesions, even so, many lesions were deemed unresectable and afflicted patients were treated only with palliative medication. At the present time, the surgical approach to cancer treatment has been much improved due to the diagnostic imaging techniques which are now available, such as computed tomography (CT), ultrasound sonography, positron emission tomography (PET) and magnetic resonance imaging (MRI). The development of less invasive surgical techniques with the aid of scopes, video cameras, lasers and other technological tools has greatly aided patient survival and quality of life (Fisher, 2008). However, the effectiveness of surgical treatment is undermined when the cancer has spread to other organs by metastasis.

The use of ionizing radiation for cancer treatment began in the 19th century, soon after the discovery of X-rays and radium, however, to a limited success, until the pioneering of fractioned radiotherapy in the early 20th century. Radiation therapy continued to evolve, in the light of technological advances in X-ray therapy, the development of intensity-modulated radiation therapy and extensive studies on the response of tumours and cells to radiation. It was later found that ionizing radiation acts through several pathways, such

as the activation of cell surface receptors, induction of double strand DNA breaks, production of ceramide (a pro-apoptotic molecule) by cell-membrane sphyngomyelin, activation of intracellular signaling pathways and by bystander effect to other neighboring cells (Connell & Hellman, 2009). Currently, radiation therapy remains a standard treatment for several cancers, and is also frequently used in combination with surgery or chemotherapy to potentiate results (Siegel et al., 2012).

In parallel, in beginning of the 20th century, efforts were made to develop chemotherapeutic agents with specificity towards cancer, by screening chemicals using transplantable tumour systems in rodents as models. The interest in screening for chemotherapeutical agents continued to rise, and major breakthroughs were achieved both during and in the aftermath of World War II, which lead to the use of many of the discovered chemotherapeutic agents in hematologic cancers. The discovery of truly successful chemotherapeutic agents only came to be in the 1960s, where the first cases of chemotherapy-induced remissions were observed. From hereon, the acceptance of this approach gave rise to the progress of more agents, as well as the development of adjuvant and combination chemotherapy (DeVita & Chu, 2008). Several types of chemotherapeutic drugs have been developed, such as alkylating agents, which damage the DNA (e.g., mechlorethamine and dacarbazine); antimetabolite agents, which induce cell death at S phase and inhibit enzymes responsible for RNA and DNA production (e.g., 5-fluorouracil and cytarabine); compounds that interfere with enzymes involved in DNA replication, for example, by inhibiting topoisomerase enzymes responsible for DNA strand separation (e.g., doxorubicin, etoposide and topotecan); mitotic inhibitors, which inhibit the progression of mitosis and associated enzymes (e.g., taxol and vinblastine) and corticosteroids with cytotoxic and cytostatic activity (e.g., prednisone and dexamethasone) (Skeel & Khleif, 2011). Nonetheless, the use of chemotherapy faces several setbacks, as for example, the non-specific cytotoxic effect with high toxicity and giving rise to resistance (Gordon & Nelson, 2012; Hedigan, 2010).

Common side effects related to current cancer therapies include cardiomyopathy, nausea, cognitive deficits, peripheral neuropathy, fatigue, infertility, osteopenia, osteoporosis and pulmonary dysfunction (Farrell et al., 2013; Saad et al., 2014; Siegel et al., 2012; Yahalom & Portlock, 2011).

More recently, efforts have been made in developing targeted therapy, which is a more selective and mechanism-based treatment. Targeted cancer therapy is based on molecules that specifically block vital biochemical pathways or abnormal proteins that are essential for the survival and proliferation of tumours (Vanneman & Dranoff, 2012). Such

examples are hormone therapies, apoptosis inducers, immunotherapies, gene expression modulators, angiogenesis inhibitors, signal transduction inhibitors, and delivery molecules (e.g. nanoparticles) (Brannon-Peppas & Blanchette, 2012; Garzon et al., 2010; Jordan, 2014; Vanneman & Dranoff, 2012; Wiezorek et al., 2010).

1.2. MARINE SYSTEMS AS A SOURCE OF NATURAL BIOACTIVE COMPOUNDS

The marine environment is home to an immensely vast and complex array of species and ecosystems, most of which remain undiscovered. This does not come as a surprise, considering that the ocean covers approximately 70% of the planet's surface. This massive body of water encompasses different ecological niches, some of which are highly productive and prosperous in biodiversity, such as the sea-land interface and deep ocean thermal vent communities, others, such as the vast open ocean waters, possess limited production and are poor in biomass and diversity. Appeltans et al. (2012) enumerate a total of approximately 226.000 described marine eukaryotic species, and estimate that one-third to two-thirds of marine species are yet to describe (Appeltans et al., 2012).

The quest for novel compounds of natural origin has been a persistent ambition for pharmaceutical research. Natural compounds are the main source of active ingredients in medicines, and in spite of modern pharmaceutical synthesis techniques, natural products are still the basis of almost half of all approved drugs. This success is in part due to the fact that natural products usually display high bioavailability, high affinity to target, as well as a minor loss of entropy when binding to proteins (Harvey, 2008). Indeed, the discovery of natural products in terrestrial fauna and flora has produced a grand diversity of bioactive compounds with the most varied chemistry and effects, as diverse as anticancer, anti-inflammatory, anti-parasitic, antiviral, analgesic, immunomodulator, anti-diabetic activity, amongst many others (Newman & Cragg, 2012).

The focus is now turning also towards to the marine environment, where the broad and yet to explore biodiversity make promise of new chemical structures. In spite of this interest, the quest for marine natural products poses several challenges which limit its expansion; large and complex molecules, enhanced costs to collect and manipulate species, difficult culturability in laboratory conditions, the lack of technological tools and innovations and also environmental concerns (Bhatnagar & Kim, 2010). Approaches to

solving the problem of product availability may include the optimization of cultivation of target organisms with the goal of achieving mass-cultivation, either by adapting cultivation conditions (e.g., development of appropriate medium, adapting of growth conditions, novel technological tools) or by genetic engineering (Lang et al., 2005; C. Raghukumar, 2008). However, this is particularly hard to achieve with more complex organisms, such as many invertebrates, nonetheless, it is possibly more approachable when considering marine microorganisms. Marine microorganisms are solid candidates for the isolation of bioactive secondary metabolites, and as microbial cultivation and fermentation technologies advance in the future, there may be a substantial improvement in the availability of compounds from microbial origin and a lowering of the associated cost (Xiong et al.,, 2013).

The screening for novel bioactive compounds from marine sources can be undertaken by an array of procedures. These procedures must have as initial intent the choice of the target organism. Current screening strategies encompass conventional bioactivity guided screening, metagenomics, genomics, synthetic biology and combinatorial biosynthesis (Xiong et al., 2013).

As a result of several marine compound screening initiatives, there are already a few drugs originated from marine compounds that have been approved for pharmaceutical use in humans or are currently under clinical trials (Martins et al., 2014). ET743 (also known as Trabectedin or Yondelis®) is a drug originated from the sponge *Ecteinascidia turbinata* and is currently approved in the EU for the treatment of advanced tissue sarcoma, as well as for the treatment of platinum-sensitive ovarian cancer in other countries (Newman & Cragg, 2014). Ziconotide (Prialt®) was approved by the FDA and EMA for use in chronic pain management, and is derived from the peptide ω-conotoxin isolated from the venom of a cone snail (Schmidtko et al., 2010). Another example of a marine drug currently in clinical use is cytarabine (Cytosar-U®, also known as arabinosyl cytosine), a synthetic pyramidine nucleoside based on spongothymidine, a nucleoside origined from the sponge *Tethya crypta*, is an FDA and EMA approved cytotoxic drug used in the treatment of several types of leukemia (Löwenberg et al., 2011; Mayer et al., 2010).

1.2.1. Marine fungi

In the last few years, marine fungi have gained a growing interest from the scientific community as sources of bioactive compounds of biotechnological interest. This interest

rises from the fact that fungi produce secondary metabolites with potential concern in pharmacological and biological studies (Rateb & Ebel, 2011).

Marine fungi differ from their terrestrial and freshwater counterparts in a critical factor. Fungi inhabiting the marine medium suffer a great influence from the seawater salinity, which is on average of 33 to 35 ppt. This poses several challenges for marine organisms, for they must adapt to several factors such as an increased pH, exposure to high sodium levels and alterations in internal water potential, and also low temperature, high hydrostatic pressure, oligotrophic nutrient conditions in deep-sea environments (Jennings, 1983; Raghukumar, 2008). The latter is of critical importance, for marine fungi must present efficient osmoregulatory mechanisms to counter high salinity environments, as is the case of the observation of greater production of osmolytes such as glycerol when exposed to such conditions (Blomberg & Adler, 1992). The tolerance of salinity is also highly dependent on temperature (Lorenz & Molitoris, 1992). In actual fact, these factors are of such vital consequence that marine fungi geographic distribution is mostly influenced by sea temperature and salinity (Jones, 2000).

Marine fungi can be effectively divided into two groups: obligate and facultative marine fungi. Those classified as obligate must by definition grow and sporulate exclusively in the marine environment, while those classified as facultative are commonly found in terrestrial or freshwater environments, however, are able to grow and sporulate in the marine medium due to a series of physiological adaptations (Kohlmeyer, 1974). However, the distinction between marine obligate and facultative is not always clear. Current studies have listed 530 obligate filamentous marine fungus species, inserted into 321 genera. A great majority of these species belong to the orders Ascomycota (424 species in 251 genera), Halosphaeriales (126 species in 53 genera) and Deuteromycota (94 species in 61 genera) and the remaining 12 species belonging to Basidiomycota (in 9 genera) (Jones et al., 2009). These numbers may however manifest a significant rise as research in this field increases and more recent molecular tools are applied. In spite of a potential escalation of the number of species, the actual number of marine fungi species culturable in laboratorial conditions is still extremely low (<1% of all estimated fungi biodiversity). This low culturability may be due to the artificial nature of the regularly used culture media for marine organisms, which may be lacking in essential nutrients (Alain & Querellou, 2009).

In an ecological perspective, marine fungi may be separated into various groups according to their habitat within the marine environment, such as oceanic, coastal, estuarine, deep-water, areniculous, or manglicolous marine fungi – different habitats in the

marine environment exert great influence on overall fungi diversity and adaptations (E. Jones, 2000). These fungi also happen to be associated to a large diversity of hosts, comprising several types of marine vertebrates and invertebrates, marine plants, algae, and marine microbial communities (Debbab et al., 2012; König et al., 2006; Raghukumar, 2006; Rateb & Ebel, 2011). The sources of marine-derived fungi from which compounds have been extracted are portrayed in Figure 2.

Fungi are known to produce secondary metabolites, which are small molecules that generally do not interfere in fungus' normal development and growth (Fox & Howlett, 2008). Marine-derived fungi strains are known to mostly produce polyketides, peptides, terpenoids, prenylated polyketides, alkaloids, shikimates, lipids and mixed biosynthesis compounds, which are in agreement with the secondary metabolites commonly produced by fungi (Rateb & Ebel, 2011; Swathi et al., 2013).

There is currently one anticancer compound based on marine-derived fungi metabolites that has entered clinical trials. This alkaloid compound, plinabulin, is a modified structure based on (-)-phenylahistin, a metabolite of both terrestrial and marine fungi *Aspergillus ustus* (Hayashi et al., 2013; Kanoh et al., 1997).

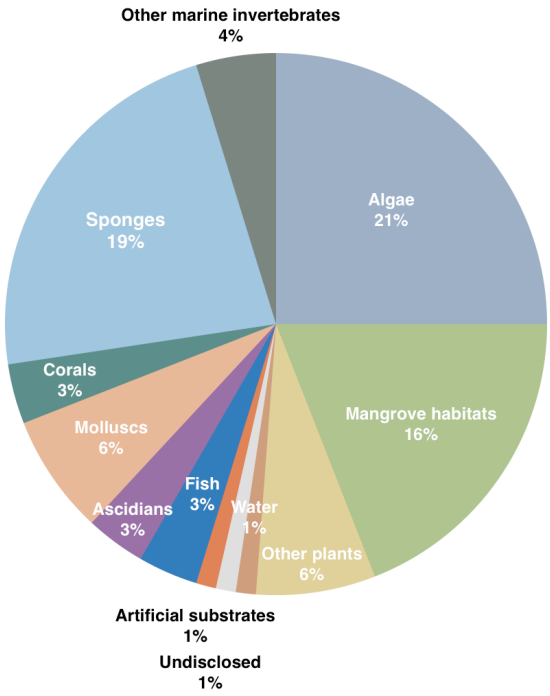

Figure 2 – Sources of fungi from which bioactive compounds have been isolated. Adapted from Rateb & Ebel (2011).

1.2.2. Fungi associate relationships as a source of bioactive compounds

The total scale of marine fungi species is most probably greatly underrated. Current research has aimed at understanding marine fungi's associations with other species, which has resulted in the collection of several novel species and strains (Passarini et al., 2013; Wang et al., 2012). As previously mentioned, marine fungi are associated to several organisms, most importantly invertebrates, algae and plants. While in the case of invertebrates (e.g. corals, ascidians, holothurians, gorgonians and sponges) and algae, fungi usually interact by association, when concerning marine plants, marine fungi habitually act as endophytes (Debbab et al., 2012). An endophyte is a fungal or bacterial microorganism that colonizes a plant or algae either at an intra-cellular or inter-cellular level, this colonization takes place without an apparent pathogenicity towards the host and may occur during a part or the whole of the host's life cycle (Tan & Zou, 2001).

In the case of endophyte-host plant interaction, fungi secondary metabolites have been proven to contribute to the host's survival and performance, by affecting factors such as chemical defense against predation, competitors and pathogens, growth rate, salt tolerance, photosynthesis and overall fitness, amongst others (Debbab et al., 2012). Whereas in fungal association to marine invertebrates, in particular sessile invertebrates, the role of fungi remains essentially unknown, however, fungi have been proposed as being able to improve the stability of the host skeleton, enable chemical defense against predators and competitors, parasitize pathogens, enhance nutrition and stimulate the host's immune system (Selvin et al., 2010). This intricate relationship provided by association or endophytic behavior proves itself interesting for bioactive compound research since the colonization process and fungal-host interaction relies on chemical communication

Sponges (phylum Porifera) represent some of oldest metazoans in existence. They are simply structured sessile invertebrates, which inhabit mostly marine and seldom freshwater systems. They are habitually attached to benthic substrates of the intertidal or deeper zones of tropical, temperate and polar waters (Thomas et al., 2010). Considering their relatively simple body structure and function, they heavily rely on chemical defense mechanisms to avoid predation and combat pathogens and competitors. Intense competition is in fact one of the main drivers for the production of toxic compounds, as the concentration of biomass is often overwhelming and space is limited in many ecological niches. Consequently, these compounds must reveal a surprising efficiency to exert their

effect as they are rapidly diluted in the seawater (Haefner, 2003). As a result, a great deal of attention was guided towards sponge secondary metabolites, and an extensive number of compounds have been extracted from these organisms. Several studies have attributed numerous pharmacological attributes to sponge metabolites, such as neuroprotective, antifungal, antibacterial, cyotoxic and antiparasitic activity (Kossuga et al., 2008; Qaralleh et al., 2010; Sasaki et al., 2011).

Sponges are home to a massive microenvironment of associate microorganisms. These associated microorganisms are essentially of bacterial nature, however, recent research has shed some light into the actual diversity of symbiotic marine fungi attached to sponges. Höller et al. (2000) collected 16 sponge species from 6 different locations, from which they isolated 681 fungal strains. These fungal strains were predominantly representatives of ubiquitous genera, but marine genera were also observed (Holler et al., 2000). In parallel, Morrison-Gardiner (2002) described the isolation of 208 fungal strains from marine sponges of tropical waters of Australia (Morrison-Gardiner, 2002). In similarity to the previous case, both ubiquitous and marine fungi genera were observed. The persistent observation of ubiquitous genera suggests that these fungi may be highly similar to their terrestrial counterparts, and may be in fact contaminants to the sponge. In fact, the exact role of marine fungi in their relationship with sponges remains unclear. However, the adaptations needed to endure marine conditions, vast marine fungal biodiversity and a prospective bioactive potential comparable to that of terrestrial fungi, make these fungi an undoubtedly interesting focus for the search of bioactive compounds (Taylor et al., 2007). Table 1 shows examples of the bioactivity of several compounds isolated from sponge-derived fungi.

Gorgonian corals (phylum Cnidaria), also known as sea fans, are sessile colonial cnidarians which are mainly distributed in the tropics and sub-tropics. Much like sponges, due to their bodies and sessile nature, they are also exposed and vulnerable to predation and competition. While the need of sea fan for chemical defense and interaction makes these organisms themselves a prolific source of bioactive compounds (Rocha, Peixe, Gomes, & Calado, 2011), the focus on their microbial associates is beginning to arise. The interaction of fungi with sea fans is far less studied than that of other corals, in exception of the pathogenic relationship often observed, since sea fan communities are frequently attacked by violent fungal infections (Toledo-Hernández et al., 2013). However, there are a series of studies that have examined healthy sea fan populations and observed that healthy populations possess a more diverse array of fungi, which are considered residential (Toledo-Hernández et al., 2007). The association between non-

pathogenic fungi and sea fans has been proposed to be of saprophytic nature, where the fungus consumes dead tissue from the coral (Kanoh et al., 1997). Meanwhile, reports of novel compounds derived from these sea fan associate fungi are rapidly increasing, and descriptions of antifouling, antibacterial, cytotoxic and other activities are already available (Bao et al., 2012; C. Li et al., 2011).

Table 1 - Examples of compounds isolated from marine sponge-associated fungi and their bioactivity.

Bioactivity	Fungus	Sponge	Metabolite	Reference
Antibacterial	*Aspergillus* sp.	*Xestospongia testudinaria*	(−)-sydonic acid	(D. Li et al., 2012)
	Aspergillus sp.	*Tethya aurantium*	Austalide R	(Zhou et al., 2014)
	Exophiala sp.	*Halichondria panacea*	Chlorohydroaspyrones A and B	(D. Zhang, Yang, Kang, Choi, & Son, 2008)
Antifungal	*Penicillium* spp.	*Tethya aurantium*	Nortryptoquivalin	(Wiese, Ohlendorf, Blümel, Schmaljohann, & Imhoff, 2011)
	Aspergillus insuetus	*Psammocinia* sp.	Insuetolides A, B and C	(E. Cohen et al., 2011)
Cytotoxic	*Clonostachys* sp.	Unidentified sponge	IB-01212	(Cruz et al., 2006)
	Acremonium sp.	*Axinella* sp.	Efrapeptin E, F, Eα, G, H	(Claudia M Boot et al., 2007; C. M. Boot, Tenney, Valeriote, & Crews, 2006)
	Penicillium auratiogriseum	*Mycale plumose*	(S)-2,4-dihydroxy-1-butyl(4-hydroxy)-benzoate	(Xin et al., 2005)
	Metarrhizium sp.	*Pseudoceratina purpurea*	Destruxin A and B2	(Claudia M Boot et al., 2007)

1.2.3. Anticancer compounds of sponge and sea fan derived fungi origin

Marine invertebrates have been on the spotlight of marine anticancer compound screening for a few decades. Sponges, gorgonians and soft corals have been the main targets for compound isolation (Bhatnagar & Kim, 2010), however, more recently, research has centered on discovering and isolating microorganism associates of these species and assessing compounds and extracts for anticancer activity.

The screening of extracts is often the first route to examine the potential in investing in the isolation of compounds. As an example of a sponge-associated fungus extract possessing anti-proliferative activity, the ethyl acetate extract of the fungus *Eurotium cristatum* obtained from the sponge *Mycale* sp. was shown to possess activity against three cancer cell lines: MCF-7 (breast adenocarcinoma), NCI-H460 (lung cancer), and A375-C5 (melanoma). The IC_{50} values were, respectively, 44.3, 45.5 and 71.3 µg/ml. Three compounds were isolated from this extract, of which 2-(2', 3-epoxy-1',3'-heptadienyl)-6-hydroxy-5-(3-methyl-2-butenyl) benzaldehyde exhibited the most interesting cytotoxic activity in all three cell lines, however, the overall cytotoxic effect displayed by the crude extract quite possibly is also due to other unidentified compounds (Almeida et al., 2010).

There are several examples of compounds isolated from sponge-associated fungi, as is the case of dankastatin C, a polyketide tyrosine derivative, isolated from the marine sponge-derived (*Homaxinella* sp.) fungus *Gymnascella dankaliensis*. This compound was a potent inhibitor of the P388 lymphocytic leukemia cell line, with an ED_{50} (half maximal effective concentration) of 57 ng/ml, having obtained an equivalent potency to that of the common chemotherapeutic drug 5-fluorouracil (Amagata et al., 2013). Another example is of epoxyphomalin A, isolated from the sponge-derived (*Ectyplasia perox*) fungus *Phoma* sp., which revealed a cytotoxicity at nanomolar concentrations against 12 human tumour cell lines, with IC_{50} values ranging from 0.010 µg/ml to 0.038 µg/ml (Mohamed et al., 2009).

While gorgonians or sea fans have been widely screened for cytotoxic compounds, their associate fungi counterparts have only very recently been approached in this matter. The following examples illustrate a few examples of compounds with different chemical composition and cytotoxic activity, originating from sea fan associate fungi. Nigrosporanene A, a cylohexene derivative isolated from the fungus *Nigrospora* sp. PSU-F11 derived from the gorgonian sea fan *Annella* sp. presented an IC_{50} of 9.37 µg/ml in MCF-7 breast adenocarcinoma cells (Rukachaisirikul et al., 2010). Aspergillone A, a benzylazaphilone derivative isolated from the fungus *Aspergillus* sp. associated to the sea fan *Dichotella gemmacea* was found to demonstrate a potent cytotoxic effect against MCF-7 breast adenocarcinoma, A549 lung cancer and HL-60 human promyelocytic leukemia with the respective IC_{50} values of 25.0, 37.0 and 3.2 µg/ml (Shao et al., 2011). Asperterrestide A, a cyclic tetrapeptide, was isolated from the fungus *Aspergillus terreus* that was isolated from the tissue of the sea fan *Echinogorgia aurantiaca*, exhibited a cytotoxic effect against two human leukemia cell lines, U937 leukemic monocyte

lymphoma and MOLT4 acute lymphoblastic leukemia (IC_{50} of 6.4 and 6.2 μM, respectively) (He et al., 2013). Oxalicumones D and E, two dihydrothiophene-condensed chromones, were isolated from the fungus *Penicillium oxalicum* SCSGAF 0023 associated to the sea fan *Muricella flexuosa*. Oxalicumone E demonstrated a strong cytotoxic activity against a panel of eight cancer cell lines (H1975, U937, K562, BGC823, MOLT4, MCF-7, HL60 and Huh-7) with IC_{50} values ranging from 1.36 to 8.80 μM. Oxalicumone D, however, exhibited a cytotoxic effect against BGC823 gastric cancer and MOLT4 acute lymphoblastic leukemia cell lines with an IC_{50} of 10.10 and 5.74 μM, respectively (Bao et al., 2014).

1.2.4. *Neosartorya* genus

Neosartorya is a genus of fungi that is common in the terrestrial environment, where it has been described as occurring in the soil, organic materials, air, food and human habitations but also in the marine environment (Yaguchi et al., 2010). The *Neosartorya* genus belongs to the phylum Ascomycota and species of this genus are a telemorphic (i.e., sexual reproductive) stage of the *Aspergillus* section *Fumigati. Neosartorya* species are known to produce an asexual state with conidiospores and a sexual state with ascospores (Frisvad et al., 2008). Although some *Neosartorya* species have been identified as the cause of several human pathologies, such as invasive aspergillosis, endocarditis, and osteomyelitis (Peláez et al., 2013; Summerbell et al., 1992), recent studies have approached these species as a source of interesting bioactive compounds with potential application in human health (Eamvijarn et al., 2013; Gomes et al., 2014; Padhye et al., 1994).

Several terrestrial strains of *Neosartorya* have yielded interesting cytotoxic compounds, for instance, the pyrroloindole sesquiterpenoid fischerindoline, isolated from *Neosartorya pseudofischeri* CBS 404.67. This compound presented moderate cytotoxic activity in five human cancer cell lines (A549, Hs683, MCF-7, SKMEL-28 and U373) and one murine melanoma cell line (B16F10), with IC_{50} values ranging from 25 to 37 μM (Masi et al., 2013). Another example is the indoloazepinone deriviative sartorymensin, isolated from *Neosartorya siamensis* KUFC 6349, which exhibited cytotoxic activity against five human cancer cell lines (U373, Hs683, A549, MCF-7 and SKMEL-28) with IC_{50} values that ranged from 39 to 73 μM (Buttachon et al., 2012).

More recently, marine strains of *Neosartorya* have been subjected to isolation of compounds and subsequent screening of cytotoxic activity. In a study by Eamvijarn et al. (2013) several compounds were isolated from *Neosartorya tsunodae* (KUFC 9213), *Neosartorya fischeri* (KUFC 6344) and *Neosartorya laciniosa* (KUFC 7896). The isolated compounds, 13-oxofumitremorgin B, sartorypyrone A, aszonapyrone A and sartorypyrone B, presented low to high cytotoxic activity (GI_{50} values ranging from 123.3 to 10.2 μM) against MCF-7, NCI-H460 and A375-C5 (Eamvijarn et al., 2013).

References

Alain, K., & Querellou, J. (2009). Cultivating the uncultured: limits, advances and future challenges. *Extremophiles, 13*(4), 583-594.

Almeida, A. P., Dethoup, T., Singburaudom, N., Lima, R., Vasconcelos, M. H., Pinto, M., & Kijjoa, A. (2010). The in vitro anticancer activity of the crude extract of the sponge-associated fungus *Eurotium cristatum* and its secondary metabolites. *J Nat Pharm, 1*(1), 25-29.

Amagata, T., Tanaka, M., Yamada, T., Chen, Y.-P., Minoura, K., & Numata, A. (2013). Additional cytotoxic substances isolated from the sponge-derived *Gymnascella dankaliensis*. *Tetrahedron Lett, 54*(45), 5960-5962.

Anand, P., Kunnumakara, A. B., Sundaram, C., Harikumar, K. B., Tharakan, S. T., Lai, O. S, Sung, B., & Aggarwal, B. B. (2008). Cancer is a preventable disease that requires major lifestyle changes. *Pharm Res, 25*(9), 2097-2116.

Appeltans, W., Ahyong, S. T., Anderson, G., Angel, M. V., Artois, T., Bailly, N., et al. (2012). The Magnitude of Global Marine Species Diversity. *Curr Biol, 22*(23), 2189-2202.

Babashah, S., & Soleimani, M. (2011). The oncogenic and tumour suppressive roles of microRNAs in cancer and apoptosis. *Eur J Cancer, 47*(8), 1127-1137.

Bai, L., & Zhu, W.-G. (2006). p53: structure, function and therapeutic applications. *J Cancer Mol, 2*(4), 141-153.

Bao, J., Luo, J.-F., Qin, X.-C., Xu, X.-Y., Zhang, X.-Y., Tu, Z.-C., & Qi, S.-H. (2014). Dihydrothiophene-condensed chromones from a marine-derived fungus *Penicillium oxalicum* and their structure–bioactivity relationship. *Bioorg Med Chem Lett, 24*(11), 2433-2436.

Bao, J., Sun, Y.-L., Zhang, X.-Y., Han, Z., Gao, H.-C., He, F., Qian, P.-Y., & Qi, S.-H. (2012). Antifouling and antibacterial polyketides from marine gorgonian coral-associated fungus *Penicillium* sp. SCSGAF 0023. *J Antibiot , 66*(4), 219-223.

Bertram, J. S. (2000). The molecular biology of cancer. *Mol Aspects Med, 21*(6), 167-223.

Bhatnagar, I., & Kim, S.-K. (2010). Marine antitumor drugs: Status, shortfalls and strategies. *Mar Drugs, 8*(10), 2702-2720.

Blachier, M., Leleu, H., Peck-Radosavljevic, M., Valla, D. C., & Roudot-Thoraval, F. (2013). The burden of liver disease in Europe: a review of available epidemiological data. *J Hepatol, 58*(3), 593-608

Blomberg, A., & Adler, L. (1992). Physiology of osmotolerance in fungi. *Adv Microb Physiol, 33*, 145-212.

Boot, C. M., Amagata, T., Tenney, K., Compton, J. E., Pietraszkiewicz, H., Valeriote, F. A., & Crews, P. (2007). Four classes of structurally unusual peptides from two marine-derived fungi: structures and bioactivities. *Tetrahedron, 63*(39), 9903-9914.

Boot, C. M., Tenney, K., Valeriote, F. A., & Crews, P. (2006). Highly N-methylated linear peptides produced by an atypical sponge-derived *Acremonium* sp. *J Nat Prod, 69*(1), 83-92.

Brannon-Peppas, L., & Blanchette, J. O. (2012). Nanoparticle and targeted systems for cancer therapy. *Adv Drug Deliv Rev, 64*, 206-212.

Buttachon, S., Chandrapatya, A., Manoch, L., Silva, A., Gales, L., Bruyère, C., Kiss, R., & Kijjoa, A. (2012). Sartorymensin, a new indole alkaloid, and new analogues of tryptoquivaline and fiscalins produced by *Neosartorya siamensis* (KUFC 6349). *Tetrahedron, 68*(15), 3253-3262.

Chaffer, C. L., & Weinberg, R. A. (2011). A perspective on cancer cell metastasis. *Science, 331*(6024), 1559-1564.

Choi, Y. S., & Fisher, D. E. (2014). UV and melanoma: mechanistic contributions, complexities and opportunities. *Cell Res*.

Cohen, E., Koch, L., Thu, K. M., Rahamim, Y., Aluma, Y., Ilan, M., Yarden, O., & Carmeli, S. (2011). Novel terpenoids of the fungus *Aspergillus insuetus* isolated from the Mediterranean sponge *Psammocinia* sp. collected along the coast of Israel. *Bioorg Med Chem, 19*(22), 6587-6593.

Cohen, S. M., & Arnold, L. L. (2008). Cell proliferation and carcinogenesis. *J Toxicol Pathol, 21*(1), 1-7.

Connell, P. P., & Hellman, S. (2009). Advances in Radiotherapy and Implications for the Next Century: A Historical Perspective. *Cancer Res, 69*(2), 383-392.

Cruz, L. J., Insua, M. M., Baz, J. P., Trujillo, M., Rodriguez-Mias, R. A., Oliveira, E., Giralt, E., Albericio, F., & Canedo, L. M. (2006). IB-01212, a new cytotoxic cyclodepsipeptide isolated from the marine fungus *Clonostachys* sp. ESNA-A009. *J Org Chem, 71*(9), 3335-3338.

De Almagro, M., & Vucic, D. (2012). The inhibitor of apoptosis (IAP) proteins are critical regulators of signaling pathways and targets for anti-cancer therapy. *Exp Oncol, 34*(3), 200-211.

Debbab, A., Aly, A. H., & Proksch, P. (2012). Endophytes and associated marine derived fungi—ecological and chemical perspectives. *Fungal Divers, 57*(1), 45-83.

DeVita, V. T., & Chu, E. (2008). A History of Cancer Chemotherapy. *Cancer Res, 68*(21), 8643-8653.

Eamvijarn, A., Gomes, N. M., Dethoup, T., Buaruang, J., Manoch, L., Silva, A., Pedro, M., Marini, I., Roussis, V., & Kijjoa, A. (2013). Bioactive meroditerpenes and indole alkaloids from the soil fungus *Neosartorya fischeri* (KUFC 6344), and the marine-derived fungi *Neosartorya laciniosa* (KUFC 7896) and *Neosartorya tsunodae* (KUFC 9213). *Tetrahedron, 69*(40), 8583-8591.

El-Serag, H. B., & Rudolph, K. L. (2007). Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology, 132*(7), 2557-2576.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicol Pathol, 35*(4), 495-516.

Farrell, C., Brearley, S. G., Pilling, M., & Molassiotis, A. (2013). The impact of chemotherapy-related nausea on patients' nutritional status, psychological distress and quality of life. *Support Care Cancer, 21*(1), 59-66.

Favaloro, B., Allocati, N., Graziano, V., Di Ilio, C., & De Laurenzi, V. (2012). Role of Apoptosis in disease. *Aging-Us, 4*(5), 330-349.

Feoktistova, M., & Leverkus, M. (2014). Programmed necrosis and necroptosis signalling. *FEBS J*. doi: 10.1111/febs.13120

Ferlay, J., Shin, H. R., Bray, F., Forman, D., Mathers, C., & Parkin, D. M. (2010). Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer, 127*(12), 2893-2917.

Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F. (2013) GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; 2013. Available from: http://globocan.iarc.fr, accessed on 9/09/2014.

Fisher, B. (2008). Biological research in the evolution of cancer surgery: a personal perspective. *Cancer Res, 68*(24), 10007-10020.

Fox, E. M., & Howlett, B. J. (2008). Secondary metabolism: regulation and role in fungal biology. *Curr Opin Microbiol, 11*(6), 481-487.

Frisvad, J. C., Rank, C., Nielsen, K. F., & Larsen, T. O. (2008). Metabolomics of *Aspergillus fumigatus*. *Medical Mycol, 47*(S1), S53-S71.

Garraway, L. A., & Chin, L. (2011). Molecular Biology of Cutaneous Melanoma. *Cancer Prin Pract Oncol, 9*.

Garzon, R., Marcucci, G., & Croce, C. M. (2010). Targeting microRNAs in cancer: rationale, strategies and challenges. *Nat Rev Drug Discov, 9*(10), 775-789.

Gomes, N. M., Bessa, L. J., Buttachon, S., Costa, P. M., Buaruang, J., Dethoup, T., Silva, A., & Kijjoa, A. (2014). Antibacterial and Antibiofilm Activities of Tryptoquivalines and Meroditerpenes Isolated from the Marine-Derived Fungi *Neosartorya paulistensis*, *N. laciniosa*, *N. tsunodae*, and the Soil Fungi *N. fischeri* and *N. siamensis*. *Mar Drugs, 12*(2), 822-839.

Gordon, R. R., & Nelson, P. S. (2012). Cellular senescence and cancer chemotherapy resistance. *Drug Resist Updat, 15*(1), 123-131.

Greaves, M., & Maley, C. C. (2012). Clonal evolution in cancer. *Nature, 481*(7381), 306-313.

Haefner, B. (2003). Drugs from the deep: marine natural products as drug candidates. *Drug Discov Today, 8*(12), 536-544.

Hajdu, S. I. (2011). A note from history: landmarks in history of cancer, part 1. *Cancer, 117*(5), 1097-1102.

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell, 100*(1), 57-70.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell, 144*(5), 646-674.

Harvey, A. L. (2008). Natural products in drug discovery. *Drug Discov Today, 13*(19), 894-901.

Hayashi, Y., Yamazaki-Nakamura, Y., & Yakushiji, F. (2013). Medicinal Chemistry and Chemical Biology of Diketopiperazine-Type Antimicrotubule and Vascular-Disrupting Agents. *Chem Pharm Bull (Tokyo), 61*(9), 889-901.

He, F., Bao, J., Zhang, X. Y., Tu, Z. C., Shi, Y. M., & Qi, S. H. (2013). Asperterrestide A, a cytotoxic cyclic tetrapeptide from the marine-derived fungus *Aspergillus terreus* SCSGAF0162. *J Nat Prod, 76*(6), 1182-1186.

Hedigan, K. (2010). Cancer: Herbal medicine reduces chemotherapy toxicity. *Nat Rev Drug Discov, 9*(10), 765-765.

Holler, U., Wright, A. D., Matthee, G. F., Konig, G. M., Draeger, S., Aust, H. J., & Schulz, B. (2000). Fungi from marine sponges: diversity, biological activity and secondary metabolites. *Mycol Res, 104*, 1354-1365.

Jasperson, K. W., Tuohy, T. M., Neklason, D. W., & Burt, R. W. (2010). Hereditary and familial colon cancer. *Gastroenterology, 138*(6), 2044-2058.

Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E., & Forman, D. (2011). Global cancer statistics. *CA Cancer J Clin, 61*(2), 69-90.

Jennings, D. (1983). Some aspects of the physiology and biochemistry of marine fungi. *Biol Rev, 58*(3), 423-459.

Jones, E. (2000). Marine fungi: some factors influencing biodiversity. *Fungal Divers, 4*, 53-73.

Jones, E. B. G., Sakayaroj, J., Suetrong, S., Somrithipol, S., & Pang, K. L. (2009). Classification of marine *Ascomycota,* anamorphic taxa and Basidiomycota. *Fungal Divers, 35*, 1-187.

Jordan, V. C. (2014). Tamoxifen as the first successful targeted therapy in cancer: the gift that kept on giving. *Breast Cancer Manage, 3*(4), 321-326.

Kanoh, K., Kohno, S., Asari, T., Harada, T., Katada, J., Muramatsu, M., Kawashima, H., Sekiya, H., & Uno, I. (1997). (−)-Phenylahistin: A new mammalian cell cycle inhibitor produced by *Aspergillus ustus*. *Bioorg Med Chem Lett, 7*(22), 2847-2852.

Kelly, P. N., & Strasser, A. (2011). The role of Bcl-2 and its pro-survival relatives in tumourigenesis and cancer therapy. *Cell Death Differ, 18*(9), 1414-1424.

Kerr, J. F., Wyllie, A. H., & Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer, 26*(4), 239-257.

Khosravi-Far, R., & Degli Esposti, M. (2004). Review Death Receptor Signals to Mitochondria. *Cancer Biol Ther, 3*(11), 1051-1057.

Kohlmeyer, J. (1974). On the definition and taxonomy of higher marine fungi. *Vero ffentlichungen des Instituts fur Meeresforschung in Bremerhaven, 5*, 263-286.

König, G. M., Kehraus, S., Seibert, S. F., Abdel-Lateff, A., & Müller, D. (2006). Natural products from marine organisms and their associated microbes. *Chem Bio Chem, 7*(2), 229-238.

Kossuga, M. H., Nascimento, A. M., Reimão, J. Q., Tempone, A. G., Taniwaki, N. N., Veloso, K., Ferreira, A. G., Cavalcanti, B. C., Pessoa, C., & Moraes, M. O. (2008). Antiparasitic, Antineuroinflammatory, and Cytotoxic Polyketides from the Marine Sponge *Plakortis angulospiculatus* Collected in Brazil. *J Nat Prod, 71*(3), 334-339.

Kryston, T. B., Georgiev, A. B., Pissis, P., & Georgakilas, A. G. (2011). Role of oxidative stress and DNA damage in human carcinogenesis. *Mutat Res, 711*(1), 193-201.

Lang, S., Hüners, M., & Lurtz, V. (2005). Bioprocess engineering data on the cultivation of marine prokaryotes and fungi. *Mar Biotechnol II* (pp. 29-62): Springer.

LeMarchand, L., Wilkens, L. R., Kolonel, L. N., Hankin, J. H., & Lyu, L. C. (1997). Associations of sedentary lifestyle, obesity, smoking, alcohol use, and diabetes with the risk of colorectal cancer. *Cancer Res, 57*(21), 4787-4794.

Levine, B., & Yuan, J. (2005). Autophagy in cell death: an innocent convict? *J Clin Invest, 115*(10), 2679-2688.

Li, C., La, M. P., Sun, P., Kurtan, T., Mandi, A., Tang, H., . . . Zhang, W. (2011). Bioactive (3Z,5E)-11,20-Epoxybriara-3,5-dien-7,18-olide Diterpenoids from the South China Sea Gorgonian *Dichotella gemmacea*. *Mar Drugs, 9*(8), 1403-1418.

Li, D., Xu, Y., Shao, C.-L., Yang, R.-Y., Zheng, C.-J., Chen, Y.-Y., Fu, X.-M., Qian, P.-Y., She, Z.-G., & Voogd, N. J. D (2012). Antibacterial bisabolane-type sesquiterpenoids from the sponge-derived fungus *Aspergillus* sp. *Mar Drugs, 10*(1), 234-241.

Lorenz, R., & Molitoris, H. P. (1992). Combined Influence of Salinity and Temperature (Phoma-Pattern) on Growth of Marine Fungi. *Can J Bot, 70*(10), 2111-2115.

Löwenberg, B., Pabst, T., Vellenga, E., van Putten, W., Schouten, H. C., Graux, C., Ferrant, A., Sonneveld, P., Biemond, B. J., & Gratwohl, A. (2011). Cytarabine dose for acute myeloid leukemia. *N Engl J Med, 364*(11), 1027-1036.

Maiuri, M. C., Galluzzi, L., Morselli, E., Kepp, O., Malik, S. A., & Kroemer, G. (2010). Autophagy regulation by p53. *Curr Opin Cell Biol, 22*(2), 181-185.

Martins, A., Vieira, H., Gaspar, H., & Santos, S. (2014). Marketed Marine Natural Products in the Pharmaceutical and Cosmeceutical Industries: Tips for Success. *Mar Drugs, 12*(2), 1066-1101.

Marusyk, A., Almendro, V., & Polyak, K. (2012). Intra-tumour heterogeneity: a looking glass for cancer? *Nat Rev Cancer, 12*(5), 323-334.

Masi, M., Andolfi, A., Mathieu, V., Boari, A., Cimmino, A., Banuls, L. M. Y., Vurro, M., Kornienko, A., Kiss, R., & Evidente, A. (2013). Fischerindoline, a pyrroloindole sesquiterpenoid isolated from *Neosartorya pseudofischeri*, with in vitro growth inhibitory activity in human cancer cell lines. *Tetrahedron, 69*(35), 7466-7470.

Mayer, A., Glaser, K. B., Cuevas, C., Jacobs, R. S., Kem, W., Little, R. D., McIntosh, J. M., Newman, D. J., Potts, B. C., & Shuster, D. E. (2010). The odyssey of marine pharmaceuticals: a current pipeline perspective. *Trends Pharmacol Sci, 31*(6), 255-265.

Mizushima, N., Levine, B., Cuervo, A. M., & Klionsky, D. J. (2008). Autophagy fights disease through cellular self-digestion. *Nature, 451*(7182), 1069-1075.

Mohamed, I. E., Gross, H., Pontius, A., Kehraus, S., Krick, A., Kelter, G., Maier, A., Fiebig, H.-H., & König, G. M. (2009). Epoxyphomalin A and B, prenylated polyketides with potent cytotoxicity from the marine-derived fungus *Phoma* sp. *Org Lett, 11*(21), 5014-5017.

Morrison-Gardiner, S. (2002). Dominant fungi from Australian coral reefs. *Fungal Divers, 9*, 105-121.

Newman, D. J., & Cragg, G. M. (2012). Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J Nat Prod, 75*(3), 311-335.

Newman, D. J., & Cragg, G. M. (2014). Marine-Sourced Anti-Cancer and Cancer Pain Control Agents in Clinical and Late Preclinical Development. *Mar Drugs, 12*(1), 255-278.

Nordenstedt, H., White, D. L., & El-Serag, H. B. (2010). The changing pattern of epidemiology in hepatocellular carcinoma. *Dig Liver Dis, 42 Suppl 3*, S206-214.

Ouyang, L., Shi, Z., Zhao, S., Wang, F. T., Zhou, T. T., Liu, B., & Bao, J. K. (2012). Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. *Cell Prolif, 45*(6), 487-498.

Padhye, A. A., Godfrey, J. H., Chandler, F. W., & Peterson, S. W. (1994). Osteomyelitis caused by *Neosartorya pseudofischeri*. *J Clin Microbiol, 32*(11), 2832-2836.

Passarini, M. R. Z., Santos, C., Lima, N., Berlinck, R. G. S., & Sette, L. D. (2013). Filamentous fungi from the Atlantic marine sponge *Dragmacidon reticulatum*. *Arch Microbiol, 195*(2), 99-111.

Peláez, T., Álvarez-Pérez, S., Mellado, E., Serrano, D., Valerio, M., Blanco, J. L., Garcia, M. E., Muñoz, P., Cuenca-Estrella, M., & Bouza, E. (2013). Invasive aspergillosis caused by cryptic *Aspergillus* species: a report of two consecutive episodes in a patient with leukaemia. *J Med Microbiol, 62*(Pt 3), 474-478.

Qaralleh, H., Idid, S., Saad, S., Susanti, D., Taher, M., & Khleifat, K. (2010). Antifungal and antibacterial activities of four Malaysian sponge species (Petrosiidae). *J Med Mycol, 20*(4), 315-320.

Raghukumar, C. (2006). Algal-fungal interactions in the marine ecosystem: symbiosis to parasitism.

Raghukumar, C. (2008). Marine fungal biotechnology: an ecological perspective. *Fungal Divers, 31*, 19-35.

Rateb, M. E., & Ebel, R. (2011). Secondary metabolites of fungi from marine habitats. *Nat Prod Rep, 28*(2), 290-344. doi: Doi 10.1039/C0np00061b

Rocha, J., Peixe, L., Gomes, N., & Calado, R. (2011). Cnidarians as a source of new marine bioactive compounds—An overview of the last decade and future steps for bioprospecting. *Mar Drugs, 9*(10), 1860-1886.

Rukachaisirikul, V., Khamthong, N., Sukpondma, Y., Phongpaichit, S., Hutadilok-Towatana, N., Graidist, P., . . . Kirtikara, K. (2010). Cyclohexene, diketopiperazine, lactone and phenol derivatives from the sea fan-derived fungi *Nigrospora* sp. PSU-F11 and PSU-F12. *Arch Pharm Res, 33*(3), 375-380.

Ryter, S. W., Mizumura, K., & Choi, A. M. (2014). The impact of autophagy on cell death modalities. *Int J Cell Biol, 2014*, 502676.

Saad, M., Tafani, C., Psimaras, D., & Ricard, D. (2014). Chemotherapy-induced peripheral neuropathy in the adult. *Curr Opin Oncol, 26*(6), 634-641.

Sampieri, K., & Fodde, R. (2012). *Cancer Stem Cells And Metastasis.* Paper presented at the Seminars in cancer biology.

Sasaki, S., Tozawa, T., Van Wagoner, R. M., Ireland, C. M., Harper, M. K., & Satoh, T. (2011). Strongylophorine-8, a pro-electrophilic compound from the marine sponge *Petrosia* (*Strongylophora*) *corticata*, provides neuroprotection through Nrf2/ARE pathway. *Biochem Biophys Res Commun, 415*(1), 6-10.

Schmidtko, A., Lotsch, J., Freynhagen, R., & Geisslinger, G. (2010). Ziconotide for treatment of severe chronic pain. *Lancet, 375*(9725), 1569-1577.

Selvin, J., Ninawe, A., Seghal Kiran, G., & Lipton, A. (2010). Sponge-microbial interactions: Ecological implications and bioprospecting avenues. *Crit Rev Microbiol, 36*(1), 82-90.

Shao, C. L., Wang, C. Y., Wei, M. Y., Gu, Y. C., She, Z. G., Qian, P. Y., & Lin, Y. C. (2011). Aspergilones A and B, two benzylazaphilones with an unprecedented carbon skeleton from the gorgonian-derived fungus *Aspergillus* sp. *Bioorg Med Chem Lett, 21*(2), 690-693.

Sharma, S., Kelly, T. K., & Jones, P. A. (2010). Epigenetics in cancer. *Carcinogenesis, 31*(1), 27-36.

Shen, S., Kepp, O., & Kroemer, G. (2012). The end of autophagic cell death? *Autophagy, 8*(1), 1-3.

Shi, J., Yang, X. R., Ballew, B., Rotunno, M., Calista, D., Fargnoli, M. C., Ghiorzo, P., Bressac-de Paillerets, B., Nagore, E., & Avril, M. F. (2014). Rare missense variants in POT1 predispose to familial cutaneous malignant melanoma. *Nat Genet*.

Shiozawa, Y., Nie, B., Pienta, K. J., Morgan, T. M., & Taichman, R. S. (2013). Cancer stem cells and their role in metastasis. *Pharmacol Ther, 138*(2), 285-293.

Siegel, R., DeSantis, C., Virgo, K., Stein, K., Mariotto, A., Smith, T., Cooper, D., Gansler, T., Lerro, C., & Fedewa, S. (2012). Cancer treatment and survivorship statistics, 2012. *CA Cancer J Clin, 62*(4), 220-241.

Skeel, R. T., & Khleif, S. N. (2011). *Handbook of Cancer Chemotherapy*: Lippincott Williams & Wilkins.

Sugihara, E., & Saya, H. (2013). Complexity of cancer stem cells. *Int J Cancer, 132*(6), 1249-1259.

Summerbell, R. C., de Repentigny, L., Chartrand, C., & St Germain, G. (1992). Graft-related endocarditis caused by *Neosartorya fischeri* var. *spinosa*. *J Clin Microbiol, 30*(6), 1580-1582.

Swathi, J., Narendra, K., Sowjanya, K., & Satya, A. K. (2013). Marine fungal metabolites as a rich source of bioactive compounds. *Afr J Biochem Res, 7*(10), 184-196.

Tan, R. X., & Zou, W. X. (2001). Endophytes: a rich source of functional metabolites. *Nat Prod Rep, 18*(4), 448-459.

Taylor, M. W., Radax, R., Steger, D., & Wagner, M. (2007). Sponge-associated microorganisms: Evolution, ecology, and biotechnological potential. *Microbiol Mol Biol Rev, 71*(2), 295-+.

Taylor, R. C., Cullen, S. P., & Martin, S. J. (2008). Apoptosis: controlled demolition at the cellular level. *Nat Rev Mol Cell Biol, 9*(3), 231-241.

Thomas, T. R. A., Kavlekar, D. P., & LokaBharathi, P. A. (2010). Marine Drugs from Sponge-Microbe Association-A Review. *Mar Drugs, 8*(4), 1417-1468.

Tirino, V., Desiderio, V., Paino, F., De Rosa, A., Papaccio, F., La Noce, M., Laino, L., De Francesco, F., & Papaccio, G. (2013). Cancer stem cells in solid tumors: an overview and new approaches for their isolation and characterization. *FASEB J, 27*(1), 13-24.

Toledo-Hernández, C., Bones-González, A., Ortiz-Vázquez, O., Sabat, A., & Bayman, P. (2007). Fungi in the sea fan Gorgonia ventalina: diversity and sampling strategies. *Coral Reefs, 26*(3), 725-730.

Toledo-Hernández, C., Gulis, V., Ruiz-Diaz, C. P., Sabat, A. M., & Bayman, P. (2013). When aspergillosis hits the fan: Disease transmission and fungal biomass in diseased versus healthy sea fans (*Gorgonia ventalina*). *Fungal Ecol, 6*(2), 161-167.

Vakkila, J., & Lotze, M. T. (2004). Inflammation and necrosis promote tumour growth. *Nat Rev Immunol, 4*(8), 641-648.

Vanneman, M., & Dranoff, G. (2012). Combining immunotherapy and targeted therapies in cancer treatment. *Nat Rev Cancer, 12*(4), 237-251.

Wang, H., Chen, J., & Xie, Y. Patent Request CN102952756-A. 23 August 2012.

White, E. (2012). Deconvoluting the context-dependent role for autophagy in cancer. *Nat Rev Cancer, 12*(6), 401-410.

Wiese, J., Ohlendorf, B., Blümel, M., Schmaljohann, R., & Imhoff, J. F. (2011). Phylogenetic identification of fungi isolated from the marine sponge *Tethya aurantium* and identification of their secondary metabolites. *Mar Drugs, 9*(4), 561-585.

Wiezorek, J., Holland, P., & Graves, J. (2010). Death receptor agonists as a targeted therapy for cancer. *Clin Cancer Res, 16*(6), 1701-1708.

Wong, R. S. Y. (2011). Apoptosis in cancer: from pathogenesis to treatment. *J Exp Clin Cancer Res, 30*.

Xin, Z. H., Zhu, W. M., Gu, Q. Q., Fang, Y. C., Duan, L., & Cui, C. B. (2005). A new cytotoxic compound from Penicillium auratiogriseum, symbiotic or epiphytic fungus of sponge *Mycale plumose*. *Chin Chem Lett, 16*(9), 1227-1229.

Xiong, Z. Q., Wang, J. F., Hao, Y. Y., & Wang, Y. (2013). Recent Advances in the Discovery and Development of Marine Microbial Natural Products. *Mar Drugs, 11*(3), 700-717.

Yaguchi, T., Matsuzawa, T., Tanaka, R., Abliz, P., Hui, Y., & Horie, Y. (2010). Two new species of *Neosartorya* isolated from soil in Xinjiang, China. *Mycoscience, 51*(4), 253-262.

Yahalom, J., & Portlock, C. S. (2011). Long-term cardiac and pulmonary complications of cancer therapy. *Heart Fail Clin, 7*(3), 403-411.

Zhang, D., Yang, X., Kang, J. S., Choi, H. D., & Son, B. W. (2008). Chlorohydroaspyrones A and B, antibacterial aspyrone derivatives from the marine-derived fungus Exophiala sp. *J Nat Prod, 71*(8), 1458-1460.

Zhang, N., Hartig, H., Dzhagalov, I., Draper, D., & He, Y. W. (2005). The role of apoptosis in the development and function of T lymphocytes. *Cell Res, 15*(10), 749-769.

Zhang, X. Y., Bao, J., Wang, G. H., He, F., Xu, X. Y., & Qi, S. H. (2012). Diversity and Antimicrobial Activity of Culturable Fungi Isolated from Six Species of the South China Sea Gorgonians. *Microb Ecol, 64*(3), 617-627.

Zhou, Y., Debbab, A., Wray, V., Lin, W., Schulz, B., Trepos, R., Pile, C., Hellio, C., Proksch, P., & Aly, A. H. (2014). Marine bacterial inhibitors from the sponge-derived fungus *Aspergillus* sp. *Tetrahedron Lett, 55*(17), 2789-279.

CHAPTER II

TESTING THE POTENTIAL OF FOUR
MARINE-DERIVED FUNGI EXTRACTS
AS ANTI-PROLIFERATIVE
AND CELL DEATH-INDUCING AGENTS
IN SEVEN HUMAN CANCER CELL LINES

This chapter comprises data included in one original manuscript submitted for publication, and it was formatted according to requirements of the *Journal of Applied Microbiology*:

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2.1. Manuscript

Abstract

Aim: To evaluate the *in vitro* anticancer activity of crude ethyl acetate extracts of the culture of four marine-derived fungi *Aspergillus similanensis* KUFA 0013 (E1), *Neosartorya paulistensis* KUFC 7897 (E2), *Neosartorya siamensis* KUFA 0017 (E4) and *Talaromyces trachyspermus* KUFC 0021 (E3) on a panel of seven human cancer cell lines.

Methods and results: Effects on cell proliferation, induction of DNA damage and cell death were assessed by MTT and clonogenic assay, comet assay and nuclear condensation assay, respectively. The proliferation of HepG2, HCT116 and A375 cells decreased after 48 h incubation with the extracts from *N. paulistensis* and *N. siamensis*. The anti-proliferative effect was confirmed by MTT assay, morphologic alterations and by clonogenic assay. Both extracts also induced cell death in HepG2 and HCT116 cells. Doxorubicin was used as a positive control and showed *in vitro* anticancer activity.

Conclusions: This study demonstrated for the first time that extracts from *N. paulistensis* and *N. siamensis* have selective anti-proliferative and cell death activity in HepG2, HCT16 and A375 cells.

Significance and Impact of the Study: The bioactivity of these extracts suggests a potential for biotechnological applications, and substantiates that both should be further considered for identification of compounds with anticancer activity and elucidation about the molecular targets and signal transduction pathways involved.

Keywords:

Marine-derived fungi; anti-proliferative; cell death; cytotoxicity; anticancer activity; human cancer cell lines.

Abbreviations:

Doxorubicin (Dox); Strand breaks (SB); Dimethylsulphoxide (DMSO); *Aspergillus similanensis* extract (E1); *Neosartorya paulistensis* extract (E2); *Talaromyces trachyspermus* extract (E3); *Neosartorya siamensis* extract (E4).

Introduction

Cancer is one of the main causes of death worldwide. In the following decades, the number of people with cancer will continue to increase, largely due to lifestyle, nutrition and environmental conditions in developed countries (Marmot et al. 2007; Veer and Kampman 2007; Jemal et al. 2011). During cancer development, cells acquire several genetic and epigenetic changes. These changes result in the progressive acquisition of biological characteristics such as sustained proliferative signaling, insensitivity to growth suppressors, evading apoptosis, increasing genomic instability, activating mobility, invasion, metastasis and angiogenesis that may thus evolve into a malignant phenotype (Hanahan and Weinberg 2011).

Apoptosis is a mechanism of programmed cell death essential to maintain tissue homeostasis since it is a genetically determined elimination of abnormal or damaged cells. Apoptosis occurs by a programmed pathway and does not involve inflammation (Portt et al. 2011). Its dysfunction is associated with cancer development, and several strategies have been used to reactivate apoptosis in cancer cells for eliminating them. Apoptotic cells develop typical morphological changes such as shrinkage, membrane blebbing, chromatin condensation, nuclear fragmentation and the formation of apoptotic bodies, all of which are features that allow its identification (Nguyen et al. 2013).

Advances in cancer biology knowledge have allowed the development of new treatment strategies, including new anticancer drugs that may act in one or more of the hallmarks described above. In fact, compounds that reactivate cell death and/or decrease proliferative ability in cancer cells show a potential anticancer activity. However, most of the anticancer drugs currently used give rise to undesirable side effects. One such example is doxorubicin (Dox), an anticancer drug used in the treatment of a wide range of cancers, and known to induce severe side effects such as cardiotoxicity and tumor drug-resistance (Carvalho et al. 2009). Therefore, new anticancer drugs with more efficiency and ability to mitigate side effects are in need.

It is interesting to note that more than 50% of the drugs used in cancer treatment are from natural origin, mainly from plant sources (Sithranga Boopathy and Kathiresan 2010). Nonetheless, the marine environment represents about 95% of the world's biosphere and is an important source of bioactive compounds to be explored (Munro et al. 1999; Jimeno et al. 2004). Marine organisms have been used as sources of folk medicine since ancient times, for instance, for the treatment of gout, cough, wounds, goiter and other illnesses (Sithranga Boopathy and Kathiresan 2010). On the other hand the discovery of penicillin in 1928 by Alexander Fleming greatly increased the interest in the use of microorganisms as a source

of bioactive compounds, in particular of fungi (Debbab et al. 2010). Marine fungi remained until recently much less studied than terrestrial fungi, nevertheless, novel metabolites have been found in marine fungi that greatly differ from those found in terrestrial counterparts (Sithranga Boopathy and Kathiresan 2010). The production of secondary metabolites by marine fungi can be influenced by the combination of the marine environment's unique conditions such as variations in temperature, light, water current, salinity, nutrient availability, all of which create a highly competitive environment and thus force marine organisms to evolve complex chemical adaptations, many of which developed under a symbiotic relationship with other species (Simmons et al. 2005; Tohme et al. 2011). In fact, recent research has exploited these symbiotic relationships in marine ecosystems as a source for bioactive compounds. This is particularly pertinent when analyzing the case of microbe-sponge relationships, and whereas sponges are known to be notable sources for bioactive compounds, where origin has also been attributed to the sponge's microbial associates, namely fungi and bacteria (Thomas et al. 2010). Furthermore, compounds are frequently produced as a chemical manner of defense by many marine organisms, and are released into the water and thus diluted. Consequently, these compounds must be extremely efficient in order to produce their effect in spite of their dilution in the water. Hence, these metabolites seem to have interest as novel lead structures for the synthesis of new bioactive compounds (Haefner 2003; Debbab et al. 2010; Tohme et al. 2011).

In regard to biological activity, several metabolites produced by marine-derived fungi have been reported as antibacterial, antiviral, antifungal, antioxidant and anticancer agents (Shen et al. 2009; Tsukada et al. 2011; Arasu et al. 2013; Pejin et al. 2013; Qi et al. 2013; Gomes et al. 2014; Henriquez et al. 2014). Notwithstanding the increasing interest in these bioactive compounds, there is frequently an effective difficulty in extracting these compounds from nature since their source organisms are often hard to reproduce and manipulate in laboratorial conditions, limiting their availability and use. Interestingly, some marine fungi may grow efficiently under laboratory conditions, which may therefore enable the use of biotechnological tools for a massive production of the compounds of interest (Kjer et al. 2010; Cai et al. 2011). In summary, marine-derived fungi seem to be good candidates as a source of new bioactive compounds, thus making them a pivotal part of the emergent marine biotechnology applications.

Our present purpose was to assess the *in vitro* anticancer activity of crude ethyl acetate extracts of the sponge-derived fungi *A. similanensis* KUFA 0013 (E1), *N. paulistensis* KUFC 7897 (E2) and *T. trachyspermus* KUFC 0021 (E3), and the sea fan-derived fungi *N. siamensis* KUFA 0017 (E4), on a panel of seven human cancer cell lines, namely, colorectal carcinoma (HT29, HCT116), hepatocellular carcinoma (HepG2), breast adenocarcinoma

(MCF-7), malignant melanoma (A375), non-small cell lung carcinoma (A549) and glioblastoma (U251) cells.

Materials and methods

Chemicals

Doxorubicin, DMEM, MEM, RPMI-1640, sodium pyruvate, sodium bicarbonate, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), penicillin/streptomycin, trypsin solution, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Biochrom KG (Berlin, Germany). All other reagents and chemicals used were of analytical grade.

Fungal material

Aspergillus similanensis (KUFA 0013) was isolated from the marine sponge *Rhabdermia* sp., which was collected from the coral reef of the Similan Island in the Andaman Sea, off the coast of Phanga Province, Thailand, by scuba diving at 10 m depth, in April 2010. The fungus was identified by one of us (T. Dethoup) by morphological features, including characteristic of ascospores, conidiogenesis and colonies and by sequence analysis of the calmodulin gene (Glass and Donaldson 1995). The pure cultures were deposited as KUFA 0013 at the Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand (Prompanya et al. 2014).

N. paulistensis (KUFC 7897) was isolated from the marine sponge *Chondrilla australiensis* which was collected from Mu Kho Lan Beach, Chonburi Province, Thailand in May 2010. The fungus was identified by Prof. Leka Manoch (Department of Plant Pathology, Kasetsart University, Bangkok, Thailand), and the identification was supported by sequence analysis of the β-tubulin gene (Glass and Donaldson 1995) and the pure cultures were deposited as KUFC 7897 at the Kasetsart University Fungal Collection, Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand, and as MMERU 02 at the Microbes Marine Environment Research Unit, Division of Environmental Science, Faculty of Science, Ramkhamhaeng University, Bangkok, Thailand (Gomes et al. 2014).

T. trachyspermus (KUFC 0021) was isolated from the marine sponge *Clathria reianwardii*, which was collected from the coral reef at Kram Island, Chonburi Province, Thailand, by scuba diving at 10 m depth, in September 2011. The fungus was identified as *Talaromyces*

trachyspermus and the pure cultures were deposited as KUFC 0021 at the Kasetsart University Fungal Collection, Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok,Thailand (Kuml et al. 2014).

N. siamensis (KUFA 0017) was isolated from sea fan (*Rumphella* sp.), collected from the coral reef at Similan island, PhangNga province, Southern Thailand, by scuba diving at 10 m depth, in April 2010. Briefly, the sea fan tissue was cut into a piece of 0.5 cm x 0.5 cm, placed on the malt extract agar (MEA) with 70% sea water and incubated for 28ºC for 7 days. The fungus was identified by one of us (T. Dethoup) by morphological features, including the characteristic of ascospores and colonies, and by sequence analysis of the β-tubulin gene (Glass and Donaldson 1995). The pure cultures were deposited as KUFA0017 at Kasetsart University Fungal Collection, Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand.

Preparation of crude ethyl acetate extracts from marine-derived fungi

A. similanensis (KUFA 0013), *N. paulistensis* (KUFC 7897) and *T. trachyspermus* (KUFA 0021) were cultured as described by Prompanya et al. (2014), Gomes et al. (2014) and Kumla et al. (2014), respectively. Briefly, the fungi were cultured for one or two week in 90 mm Petri dishes with 25 ml of malt extract agar (MEA) with 70% sea water. Erlenmeyer flasks of 1000 ml, containing rice and water, were inoculated with mycelia plugs of the fungi and incubated at 28ºC for 30 days after which the moldy rice was macerated in ethyl acetate for seven or ten days and filtered. The two layers were separated and the ethyl acetate solution was concentrated at a reduced pressure to yield 97, 51 and 102 g of the crude ethyl acetate extract, respectively (Kuml et al. 2014; Prompanya et al. 2014).

N. siamensis (KUFA 0017) was cultured for one week in five 90 mm Petri dishes with 25 ml of potato dextrose agar per dish. Thirty-five 1000 ml Erlenmeyer flasks containing 200 g of rice and 100 ml of water were autoclaved at 121ºC for 15 min, and then inoculated with ten mycelium plugs of the fungus. The culture was incubated at 28ºC for thirty days. To each flask with the mouldy rice was added 500 ml of ethyl acetate and the content was left to macerate for seven days. The content of the flasks was filtered by filter paper and the filtrate was evaporated under reduced pressure to give 1000 ml of the solution and then anhydrous sodium sulphate was added and filtered. The ethyl acetate solution was evaporated under reduced pressure to give 5 g of dark brown viscous mass of a crude ethyl acetate extract.

Cell culture

Seven human cancer cell lines were used to assess anti-proliferative activity. HT29 and HCT116 (colorectal carcinoma) were kindly provided by Prof. Carmen Jerónimo from IPO, Porto. HepG2 cells were kindly provided by Prof. Rosário Martins from CIIMAR, Porto. MCF-7, A375, A549 and U251 cells were obtained from European Collection of Cell Cultures (ECACC). Human cancer cell lines were maintained as monolayer cultures in DMEM (HT29, A375 and A549), MEM (HepG2, MCF-7 and U251) and RPMI (HCT116) supplemented with 10% FBS and 1% of antibiotic solution (100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin), 0.1 mM sodium pyruvate and 10 mM HEPES under an atmosphere of 5% CO_2 at 37ºC. Cells were trypsinised when nearly confluent.

Stock solutions of Dox and crude ethyl acetate extracts of marine fungi were prepared in DMSO and aliquots kept at -20ºC. The final concentration of DMSO in the medium was <0.5% (v/v). The controls received only DMSO. All the cells were incubated with extracts and Dox dissolved in RPMI just before use, in order to maintain the same conditions for all cell lines.

MTT reduction assay

To evaluate effects of the crude ethyl acetate extracts on cell viability/proliferation, cells were plated in 96-multiwell culture plates at a density of $0.8x10^4$ to $1x10^4$ cells/well. Twenty-four hours after plating, the medium was discarded and fresh medium containing extracts of marine-derived fungi at different concentrations (0.1 - 500 µg ml^{-1}) and Dox at (0.001 - 10 µM) as positive control, as well as 0.5% DMSO as negative control was added. After 48 h incubation with extracts, MTT was added at a final concentration of 0.5 mg ml^{-1} and incubated for 2 h. Then, the medium was removed, and the formazan crystals were dissolved in a DMSO:ethanol solution (1:1) (v/v). Absorbance was measured at 570 nm in a microplate reader (Multiskan EX, Labsystems, USA). The MTT colorimetric assay is based upon mitochondrial conversion of tetrazolium salt (MTT) into formazan crystals, and thus alterations in the number of viable cells can be detected by measuring formazan crystals optical density (Ramos et al. 2008; Xavier et al. 2009). IC_{50} corresponds to the concentration of extract or Dox that decreases the number of viable cells by 50%. In this case, the absorbance in the control at 48 h corresponds to 100% viability. The IC_{50} values were determined using GraphPad Prism v5.0 software (GraphPad Software, La Jolla, CA, USA). To evaluate the effect on cell proliferation, the absorbance at the beginning of incubation (t = 0 h) was subtracted from all the experimental conditions used, including the control at 48 h.

Therefore, negative values can be interpreted as direct cytotoxic effects of extracts while positive values (between 0 and 100%) can be interpreted as inhibition of cell proliferation. This variant of the MTT assay allows a rapid and simple discrimination between inhibition of cell proliferation and cell death (Kiesslich et al. 2013). The results were expressed as the percentage of cell viability/proliferation relative to control (untreated cells) of at least six independent experiments; each one was carried out in duplicate. For the following assays, only fungi extracts that presented an IC_{50} equal or less than the arbitrary cut-off threshold of 200 µg ml^{-1} and without direct cytotoxic effects were used.

Clonogenic assay

To study the effect of the extracts of marine-derived fungi on the proliferation of clonogenic cells, cells were plated ($0.1x10^6$ cells/well) in 24-multiwell culture plates and treated with extracts or Dox (IC_{50}). After a 48 h treatment, survival cells were trypsinized, counted and plated in 6-multiwell culture plates at 200 cells/well with fresh medium. After 10 days of culture, colonies were fixed, stained with crystal violet (0.05% w/v) for 30 min and washed with water. Colonies containing more than 50 individual cells were counted using a stereomicroscope (Leica, ZOOM 2000). Plating efficiencies (PE) were calculated from the ratio between the number of colonies counted and the number of cells plated. The surviving fraction (SF) was calculated using the formula: SF = 100 × [PE of treated cells ÷ PE of control] (Munshi et al. 2005; Zips et al. 2005; Franken et al. 2006; Haloom et al. 2011; Rafehi et al. 2011). Results were expressed as the surviving fraction relative to control (cells treated with 0.5% DMSO).

Comet assay

To investigate the genotoxic effect of the tested fungal, cells were plated ($0.1x10^6$ cells/well) in 24-multiwell culture plates. Twenty-four hours after plating, cells were incubated with the extracts of marine-derived fungi (IC_{50}) for 4, 24 and 48 h and DNA damage (strand breaks and alkali-labile sites) was assessed by the alkaline version of the single cell gel electrophoresis (comet) assay (Collins et al. 2008; Ramos et al. 2010). In short, after treatment cells were trypsinized, washed, centrifuged, and the pellet suspended in low melting point agarose and about $2×10^4$ cells/gel were placed on a slide pre-coated with 1% normal melting point agarose. Slides were placed in lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris Base, pH 10 plus 1% Triton X-100) for 1 h at 4ºC, and then placed in a horizontal electrophoresis chamber with a buffered solution (300 mM NaOH, 1 mM

Na_2EDTA, pH >13), for 40 min at 4ºC, for the DNA to unwind. The electrophoresis was run for 20 min at 21V the slides were washed twice with PBS and dried at room temperature. For the analysis of the comet images, the slides were stained with DAPI solution (1 μg ml^{-1}) and visualized in a fluorescence microscope (Olympus IX71). Images were registered and studied with the image analysis software CometScore® (CometScore, TriTek Corp.), for quantifying the percentage of tail intensity. At least 100 simply randomly selected cells were analyzed per sample.

Nuclear condensation assay

To evaluate the ability of the extracts of marine-derived fungi to induce cell death, cells were plated ($0.1x10^6$ cells/well) in 24-multiwell culture plates. After cell adhesion, cells were treated with extracts or Dox (IC_{50}) for 48 h and nuclear condensation was assessed by observation in the mentioned fluorescence microscope. Briefly, both adherent and non-adherent cells were collected, washed, centrifuged, fixed with 4% (w/v) paraformaldehyde in PBS for 20 min at 37ºC, and then attached into a polylysine-treated slide using a Cytospin Cytocentrifuge (Thermo Scientific, USA). Once dried, slides were incubated with DAPI (1 μg ml^{-1}) for nuclei staining. The percentage of cells with condensed nuclei was calculated from the ratio between cells with nuclear condensation and total number of cells (nuclei staining with DAPI). More than 300 cells were counted per sample.

Statistical analysis

Results were expressed as mean ± SD from at least 3 independent experiments. The GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA) was used for inferential statistics. Eventual significant differences ($P \leq 0.05$) were evaluated by one-way ANOVA, followed by the post-hoc Newman-Keuls multiple comparison test or Dunnett's test as appropriate. In specific cases Student *t*-test was used as described. Data from the MTT assay were analysed for normal distribution using the D'Agostino-Pearson omnibus test. For the remaining data, normal distribution was not tested due to low "n", and was assumed to occur, which is consistent with the normal behaviour of cell culture responses as observed in similar published studies.

Results

Evaluation of the effect of the extracts of marine-derived fungi on cell viability and proliferation by MTT reduction assay in cancer cells

In order to test the effects of the extracts of four marine-derived fungi on cell viability and proliferation, seven human cancer cell lines, HT29, HCT116, HepG2, MCF-7, A375, A549 and U251 were used. The assay was performed on exponentially growing cells, as previously determined (data not shown). Each cell line was incubated for 48 h with extracts at different concentrations (0.1-500 μg ml^{-1}) and then cell viability and proliferation was assessed by MTT assay. Doxorubicin was tested in parallel over a concentration range (0.001-10 μM), corresponding to ($5.8x10^{-4}$ - 5.8 μg ml^{-1}), as a positive control. The IC_{50} (concentration of extract or Dox that reduces the number of viable cells by 50 %) for each cell line was calculated from the dose-response curves. The values of the IC_{50} are summarized in Table 1. Extracts E2 and E4 significantly decreased the number of viable cells in HepG2, HCT116 and A375 cells. Both extracts showed similar IC_{50} values for HepG2 cells. Regarding HCT116 and A375 cells, extract E4 showed a lower IC_{50} value (124 and 150 μg ml^{-1}, respectively) when compared to extract E2 (165 and 184 μg ml^{-1}). The extracts E1 and E3 had an IC_{50} higher than the cut-off level of 200 μg ml^{-1} in all tested cell lines, and therefore they were not used in the following experiments. Dox decreased cell viability in all tested cell lines and the related IC_{50} values ranged from 0.11 μM to 1.55 μM (corresponding to 0.06 – 0.9 μg ml^{-1}) (Table 1).

Table 1 – Determination of IC_{50} values (concentration that inhibits the number of viable cells in 50%) of four extracts in seven cells lines. Doxorubicin was used as a positive control. IC_{50} values are the mean at least 6 independent experiment each in duplicate.

			Extracts (µg ml^{-1})					
			KUFA 0013	KUFC 7897		KUFC 0021	KUFA 0017	
	Doxorubicin (µM)		(E1)	(E2)		(E3)	(E4)	
Cancer cell lines	**IC_{50}**	95% confidence interval	**IC_{50}**	**IC_{50}**	95% confidence interval	**IC_{50}**	**IC_{50}**	95% confidence interval
HepG2	0.11	(0.07-0.17)	>200	**198**	(179-208)	>200	**197**	(182-213)
HT29	0.87	(0.54-1.39)	>200	>200		>200	>200	
HCT116	0.13	(0.09-0.19)	>200	**165**	(136-201)	>200	**124**	(81-180)
U251	1.55	(0.70-2.50)	>200	>200		>200	>200	
A549	0.54	(0.30-0.94)	>200	>200		>200	>200	
A375	0.12	(0.09-0.16)	>200	**184**	(160-209)	>200	**150**	(131-173)
MCF7	0.37	(0.27-0.50)	>200	>200		>200	>200	

In order to evaluate the effects of the extracts of marine-derived fungi on cell proliferation, the number of viable cells at the beginning of experiment (when extracts were added, t = 0 h), assessed by MTT, were subtracted to the number of viable cells at the end of experiment (t = 48 h). The results were represented as percentage of cell proliferation relative to control (cells with 0.5% DMSO). Values between 0 and 100% can be interpreted as anti-proliferative effects, while negative values can be a direct cytotoxic effect. We observed that all cell lines, when treated only with 0.5% DMSO (negative control), grew significantly between the beginning (t = 0 h) and the end of the incubation (t = 48 h) (data not shown). The results showed that extracts E2 and E4 decreased cell proliferation in a concentration-dependent manner in HepG2, HCT116 and A375 cells (Figure 1). The anti-proliferative effect of both extracts was higher in HCT116 and A375 cells. The extract E2 at 100 µg ml^{-1} significantly decreased proliferation of HCT116 and A375 cells by 43% and 25%, respectively relative to control (Figure 1A). At the same concentration, extract E4 significantly decreased proliferation of HCT116 and A375 cells by 72% and 60%, respectively (Figure 1B). In the MTT assay, extract E4 was more active in the cells lines tested. For concentrations higher than 200 µg ml^{-1}, both extracts showed a direct cytotoxic effect in HepG2, HCT116 and A375 cells. Dox decreased cell proliferation in a concentration-dependent manner in all cell lines tested. At 1 and 10 µM, Dox exhibited a direct cytotoxic effect in HepG2, HCT116 and A375 cells (Figure 1C).

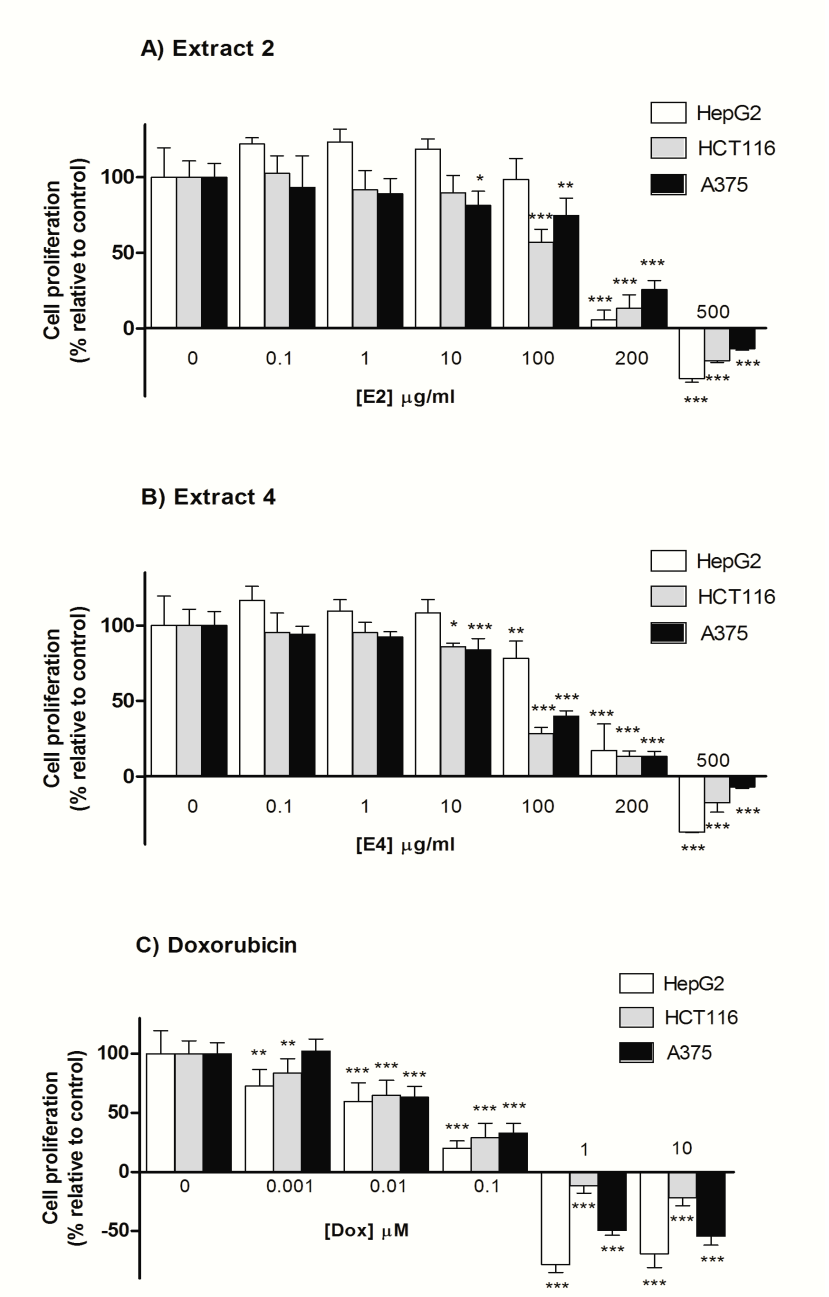

Figure 1. Dose-response effects of extracts E2 (A), E4 (B) and doxorubicin (C) on cell viability/proliferation in HepG2, HCT116 and A375 cells after 48 h, evaluated by MTT assay. Results are expressed as mean ± SD of at least six independent experiments, each made in duplicate. Significant differences (* P≤0.05; ** P≤0.01 and *** P≤0.001) among groups per situation of exposure were tested by one-way ANOVA, followed by the post-hoc Newman-Keuls multiple comparison test.

To select the extracts and cell lines to use in the following experiments, two aspects were considered: 1) extracts should inhibit cell proliferation without significant direct cytotoxic effects; and 2) the IC_{50} value of the extract should be lower than 200 µg ml^{-1}. Having met

these two criteria, extracts E2 and E4 were selected and the anti-proliferative and pro-cell death effects were evaluated in HepG2, HCT116 and A375 cells.

The anti-proliferative effect of extracts E2, E4 and Dox in HepG2, HCT116 and A375 was also confirmed by a decrease of cell density and also by structural alterations, such as rounded and detached cells as observed in a phase contrast microscope (Figure 2).

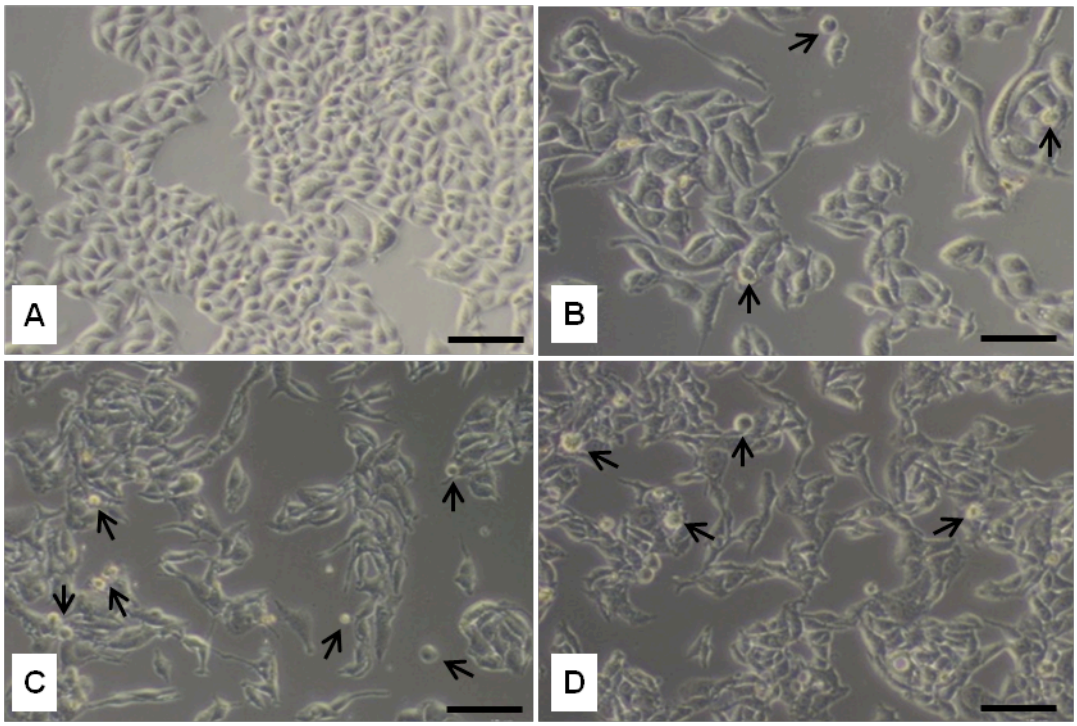

Figure 2. Morphology of HepG2 cells under phase contrast, after 48 h incubation with A) 0.5 % of DMSO, B) Dox, C) extract E2, and D) extract E4, all three at IC_{50}. Situation A) represents control cells, with normal morphology. In B), C) and D) arrows indicate rounded and detaching cells. Scale bar = 10 μm.

Evaluation of the effect of the extracts of marine-derived fungi on reproductive viability by the clonogenic assay

To evaluate the long-term anti-proliferative effects of the extracts, cells were pre-treated for 48 h with the extracts (IC_{50}) and then survival cells were allowed to grow for 10 days in fresh medium without extracts. Results from the clonogenic survival assay showed that extract E2 significantly decreased the proliferative ability of a single cell to form a viable colony in HepG2, HCT116 and A375 cells (Figure 3). Extract E4 significantly reduced the number of colonies in HepG2 and HCT116 cells when compared with control (cells pre-

treated with 0.5% DMSO) (Figure 3A and 3B). Meanwhile, no significant results were observed in A375 cells (Figure 3C). Cells pre-treated with Dox did not show ability to proliferate and form colonies.

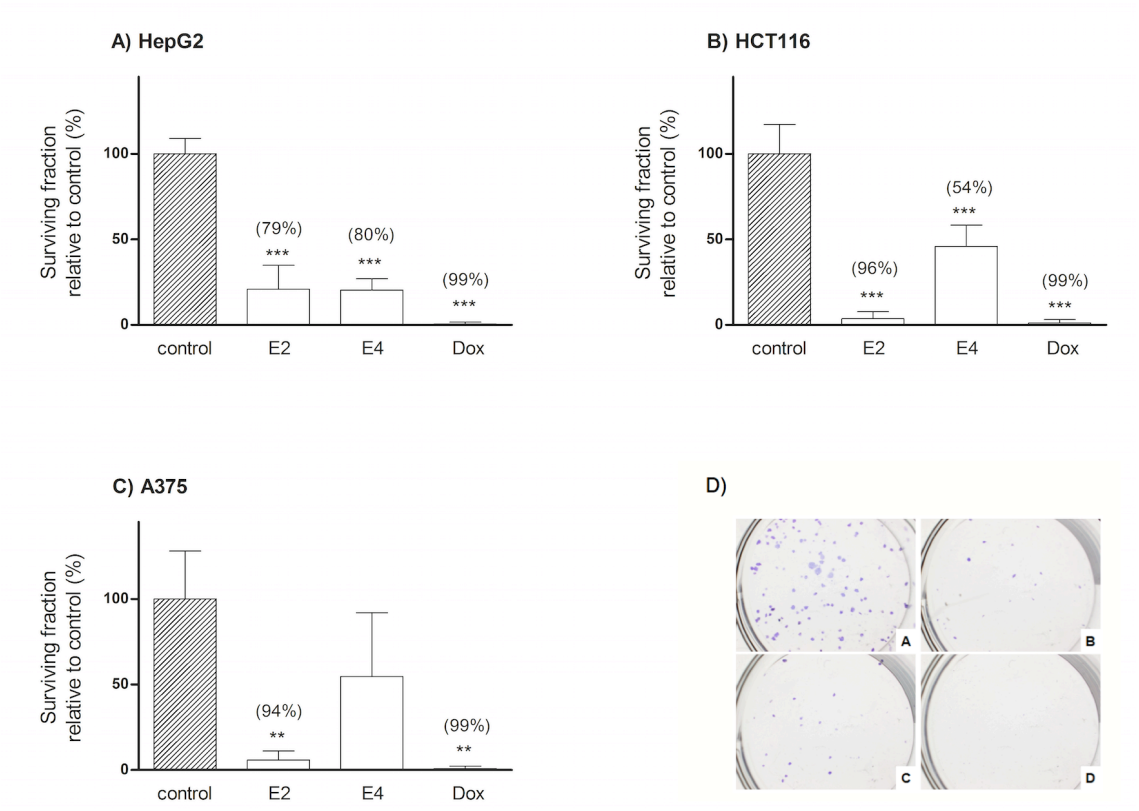

Figure 3. Clonogenic survival of HepG2 (A), HCT116 (B) and A375 (C) cells after pre-treatment with extracts E2, E4 and Dox (all three at IC_{50}) for 48 h, followed by 10 days in fresh medium. Results are expressed as mean ± SD of at least three independent experiments. Significant differences (* P≤0.05; ** P≤0.01 and *** P≤0.001) when compared with control cells were evaluated by one-way ANOVA, followed by the post-hoc Dunnett's test. D) Representative images of clonogenic assay in HepG2 cells after pre-treatment with a) 0.5 % of DMSO, b) extracts E2, c) extract E4 and d) Dox at IC_{50}, showing a decrease of the number of colonies formed relative to control (a).

Evaluation of the effect of the extracts of marine-derived fungi on DNA damage by the comet assay

To assess the genotoxic effect of the extracts, cells were incubated with them at an IC_{50} concentration for 4, 24 and 48 h, and DNA damage (SBs and alkali-labile sites) was assessed by the comet assay. Results were expressed as the percentage of DNA in tail. At 4 and 24 h of incubation, none of the extracts at the tested concentrations induced DNA

damage in HepG2, HCT116 or A375 cells (data not shown). However, after incubation for 48 h, extract E2 significantly increased by 16% the extent of DNA damage in the HepG2 cells (see white bars, Figure 4), when compared with control cells (treated with 0.5% DMSO). No effect was observed in HCT116 (grey bars) and A375 (black bars) cells when treated with extract E2. Moreover, extract E4 did not induce DNA damage detectable by comet assay at any of the tested experimental conditions. However, at 48 h, Dox evidenced a significant increase of DNA damage in HCT116 (by 25%) and A375 (by 9%) cells, when compared to the control (Figure 4).

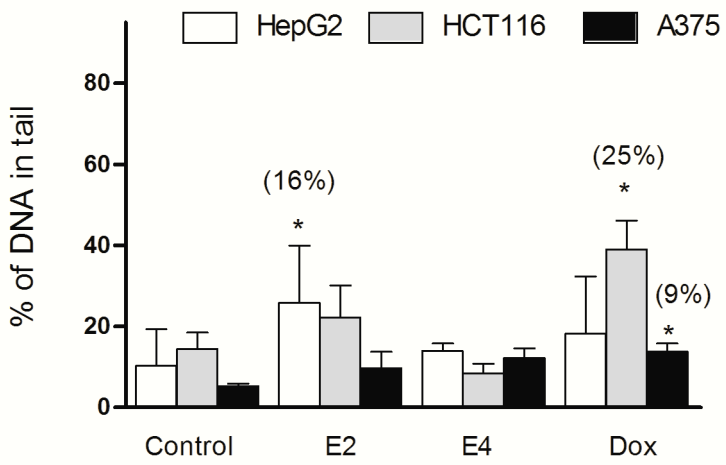

Figure 4. Effect of extracts E2, E4 and Dox (all three at IC_{50}) on DNA damage (SBs and alkali-labile sites) after 48 h in HepG2, HCT116 and A375 cells, assessed by comet assay. Values are mean ± SD of at least three independent experiments. Significant differences (* P≤0.05) when compared with control cells were judged by one-way ANOVA, followed by the post-hoc Dunnett's test. Additionally, a Student's *t*-test was selectively used to access significant differences in HepG2 (E2) and A375 (Dox) in relation to the respective control.

Evaluation of the effects of the extracts of marine-derived fungi on cell death by the nuclear condensation assay

Since the anti-proliferative effects of the extracts could be due to cell death, we evaluated nuclear condensation after 48 h cell incubation with extracts (at IC_{50}). As shown in Figure 5, extract E2 significantly induced nuclear condensation in HepG2 (Figure 5A) and HCT116 cells (Figure 5B). The number of cells with condensed nuclei was increased by 9% in HepG2 and 24% in HCT116 cells. Extract E4 also induced an increase of nuclear condensation, by 9% in HepG2 and 16% in HCT116 cells. Dox induced nuclear

condensation too, by 10% in HepG2 and by 8% in A375 cells. However, none of the tested extracts induced an increase of cells with nuclear condensation in A375 cells (data not shown). The HCT116 cells were the most sensitive to cell death induction by the extracts, being extract E2 the most effective against this cancer cell line.

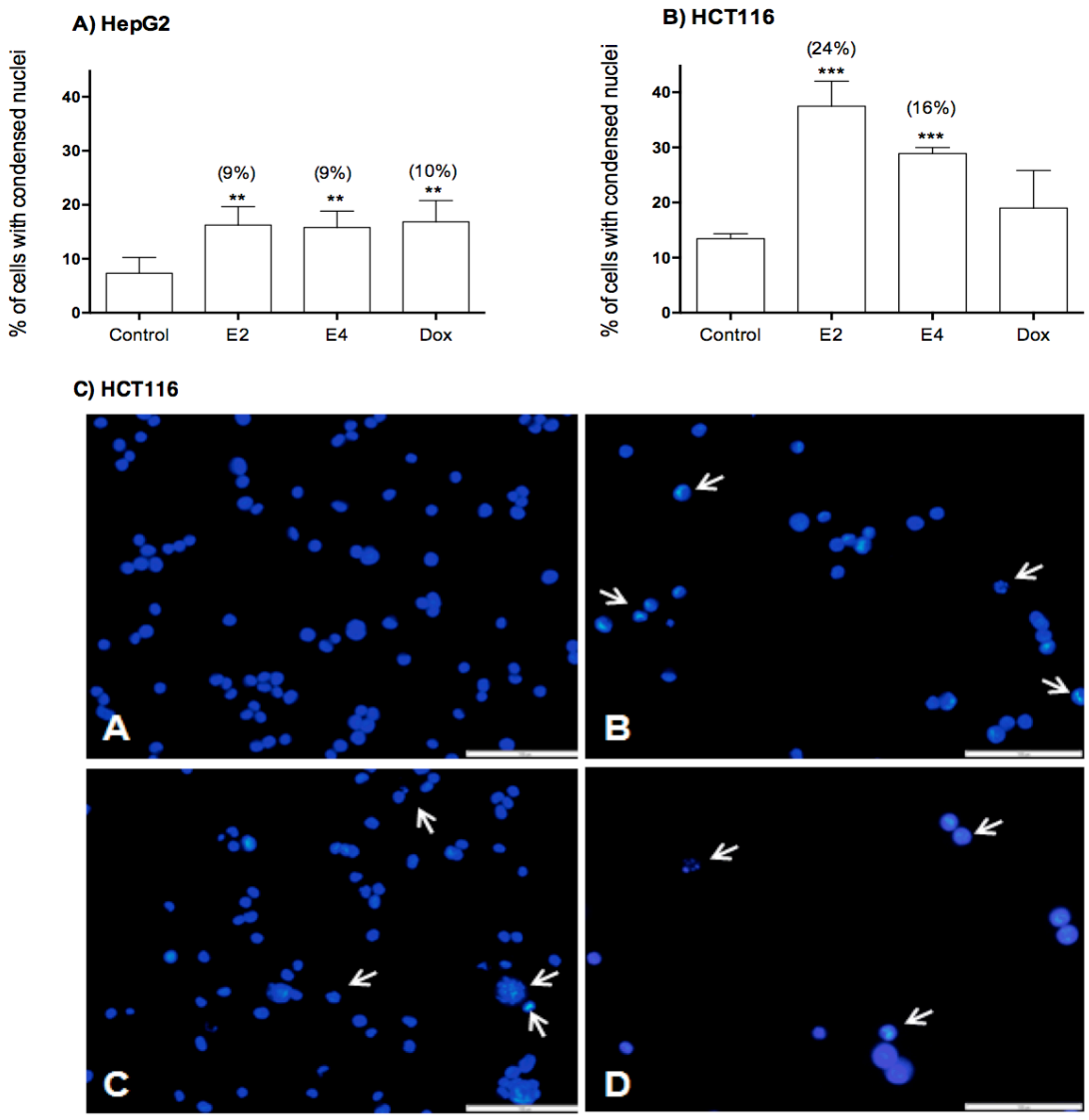

Figure 5. Effect of extracts E2, E4 and Dox (all three at IC_{50}) on the induction of nuclear chromatin condensation in HepG2 (A) and HCT116 cells (B) by the nuclear condensation assay after 48 h of incubation. Values are mean ± SD of at least three independent experiments. Significant differences (** P≤0.01 and *** P≤0.001) when compared with control cells were determined by one-way ANOVA, followed by the post-hoc Dunnett's test. C) Representative images of nuclear condensation in HCT116 cells after exposure to a) 0.5 % of DMSO, b) extracts E2, c) extract E4 and d) Dox at IC_{50}. Scale bar = 100 µm. Nuclear condensation (arrow).

Discussion

Marine-derived fungi biosynthesize a vast range of secondary metabolites with complex and unique structures (Bhakuni and Rawat 2005). However, very little is known about the biological activity of the extracts and compounds of these fungi. In this study, we investigated the *in vitro* anticancer activity of crude ethyl acetate extracts obtained from four marine-derived fungi *A. similanensis* (E1), *N. paulistensis* (E2), *N. siamensis* (E4) and *T. trachyspermus* (E3) on seven human cancer cell lines. The crude ethyl acetate extracts from *N. paulistensis* and *N. siamensis*, at concentrations that did not induce direct cytotoxicity, inhibited proliferation in HepG2, HCT116 and A375 cells, and caused cell death in HepG2 and HCT116 cells.

To the best of our knowledge this is the first report on the *in vitro* anticancer activity of marine-derived fungi *N. paulistensis* (KUFC 7897) and *N. siamensis* (KUFA 0017). (Buttachon et al. 2012) described that some isolated compounds from the culture of the strain of *N. siamensis* (KUFC 6349) isolated from soil, viz. sartorymesin, had anti-proliferative activity in U373 and Hs683 glioblastoma, A549 lung cancer, MCF-7 breast cancer and SK-MEL-28 melanoma cell lines.

In this study we showed that extracts E2 and E4 decrease the number of viable cells, with an IC_{50} lower than 200 μg ml^{-1} in HCT116, A375 and HepG2 cells. Extracts E1 and E3 exhibited lower activities, with an IC_{50} higher than 200 μg ml^{-1} in all of the tested cell lines. The effect of the extracts on cell proliferation was also determined by the MTT assay where positive values imply that the extracts have an anti-proliferative effect that is maybe due to cytotoxic and/or cytostatic effects. Negative values suggest that the number of viable cells after a 48 h exposure to extracts was less than the number of viable cells at the incubation beginning. This can occur due to a direct and short-term cytotoxic effect of the extracts, which may in turn indicate necrotic cell death (Kiesslich et al. 2013). Extracts E2 and E4 decreased cell proliferation in a dose-dependent manner and at concentrations greater than 200 μg ml^{-1}, exhibited a direct cytotoxic effect in HepG2, HCT116 and A375 cells. Some authors refer that intense insults (exposure to high concentrations) are able to induce uncontrolled cell death (necrosis) (Degterev and Yuan 2008). After the initial screening of anti-proliferative activity, the extracts — E2 and E4 — that showed an IC_{50} lower than 200 μg ml^{-1} were selected for the next studies.

The reduction of the number of viable cells could be due to an increase of cell death and/or decreased cell proliferation. In our study, the suggestive induction of cell death (apoptosis) by extracts was first noticed through the observation of morphological alterations such as cell shrinkage, membrane blebbing, and rounded and detached cells, in a phase

contrast microscope, and then confirmed by chromatin condensation using DAPI staining. The decrease of long-term cell proliferation is considered a cytostatic effect and may be evaluated by clonogenic cell survival assay. In summary, this assay measures the ability of a clonogenic cell to proliferate and form a viable colony after being exposed to a particular chemical agent for a certain amount of time, and then returned to fresh medium and left to proliferate (Rafehi et al. 2011).

Our data suggest that the *in vitro* anticancer effect of extract E4 in HepG2 and HCT116 cells could be due to both an increase of cell death and a decrease of long-term cell proliferation. In A375 cells, extract E4 decreased the number of viable cells, without an induction of cell death and presenting a slight decrease of colony formation. In this case, extract E4 seems to only inhibit cell proliferation at short-term; however at long-term the alterations induced by the extract seems to be reverted or repaired in A375 malignant melanoma cells. This lack of long-term inhibition may be explainable in view of what has been observed in other studies, which have shown that malignant melanoma is resistant to chemotherapy based on DNA damage induction, besides the normal function of p53 (Barckhausen et al. 2014). Such chemotherapy-resistance of melanoma cells seems to be related to the upregulation of some DNA repair genes (Li and Melton 2011).

Although until now no compounds have been isolated from the marine-derived strain of *N. siamensis* KUFA 0017, in a study by Buttachon et al. (2012), several compounds were isolated from a terrestrial strain *N. siamensis* KUFC 6349, namely, sartorymensin, tryptoquivaline, tryptoquivalines F, H, L and O, fiscalins A and C, *epi*-fiscalin A and C, *epi*-neofiscalin A, neofiscalin A and 4-dihydroxy-3- methylacetophenone. In the same study, selected compounds were screened for *in vitro* cytotoxic activity in a panel of cancer cell lines; results showed that sartorymensin, tryptoquivaline O and F showed an IC_{50} range between 72 and 91 μM in MCF-7 and U373 cells (Buttachon et al. 2012). Additionally, Sondgam et al. (2014) showed that tryptoquivaline L, *epi*-fiscalin A and C, isolated from *Xylaria humosa*, have cytotoxic effects in MCF-7 cells with an IC_{50} range between 21 and 33.6 μg ml^{-1} (Sodngam et al. 2014). If the nature of the compounds of the marine-derived strain is similar to the mentioned terrestrial counterpart, it is possible to hypothesize that some of the referred compounds may be responsible for the anticancer activity of extract E4. However, further studies should be addressed.

The *in vitro* anticancer activity of extract E2 in HepG2 and HCT116 cells was mainly due to the induction of cell death and decrease of long-term proliferation of survival cells. In HepG2 cells, the induction of cell death could be related with the ability of extract E2 to induce DNA damage. This characteristic is common to several chemotherapeutic drugs, which reveal an anticancer activity mainly due to their ability to induce DNA damages (Fu et

al. 2012). If such DNA damages are not properly repaired, their accumulation ultimately ensues in cell death (Roos and Kaina 2013). In agreement with our data, also other marine sponge-derived compounds such as scalaradial, cacospongionolide and 10-acetylirciformonin B increased DNA damage, which resulted in the induction of cell death by apoptosis in several cancer cell lines (De Stefano et al. 2012; Su et al. 2012). Gomes et al. (2014) have been isolated some compounds from the marine-derived fungus *Neosartorya paulistensis* KUFC 7897, namely, sartorypyrone C, tryptoquivalines H, L, F, 4(3H)-quinazolinone and 3′-(4-oxoquinazolin-3-yl) spiro [1H-indole-3,5′]-2,2′-dione (Gomes et al. 2014). As referred before tryptoquivaline L showed a cytotoxic activity against MCF-7 cells (Sodngam et al. 2014). It is thus possible to suggest that tryptoquivaline L and some others compounds may be in some measure responsible for the anticancer activity of extract E2.

Several compounds isolated from marine fungi have been reported as cell death inducers. Physcion, an anthraquinone isolated from the marine-derived fungus *Microsporum* sp., was found to exhibit a cytotoxic effect in HeLa cells by inducing apoptosis (Wijesekara et al. 2014). Fumigaclavine C, isolated from marine-derived fungus *Aspergillus fumigatus,* induced cell cycle arrest and apoptosis in MCF-7 cells. This compound seems to act by down-regulation of the NF-kB cell survival pathway (Li et al. 2013). Gliotoxin, isolated from several *Aspergillus, Penicillium* and *Gliocladium* species, inhibited cell proliferation and induced apoptosis via the mitochondrial pathway in HeLa and SW1353 cells (Nguyen et al. 2013). Bostrycin, a compound isolated from marine fungi in the South China Sea, inhibited cell proliferation and induced apoptosis in prostate, gastric and lung cancer cells (Sawadogo et al. 2013).

In A375 cells, extract E2 decreased long-term cell proliferation (diminished colony formation) without effects on induction of cell death; at the tested conditions. Decrease of clonogenic survival may be related with induction of senescence and mitotic castatrophe (Roninson et al. 2001; Luo et al. 2013).

Doxorubicin is an anticancer drug commonly used in the chemotherapeutic treatment of several different types of cancers. Its cytotoxic effect has been related with free radical formation, inhibition of DNA topoisomerase II and nucleotide intercalation, resulting in cell cycle arrest and apoptosis (Thorn et al. 2011). Some authors refer an activation of the tumor suppressor gene p53 after an exposure to Dox, and consequently p53 acts as a transcription factor of target genes, such as *PUMA* and *BAX*, inducing cell cycle arrest and apoptosis (Wang et al. 2013). In our study, the *in vitro* anticancer activity of Dox, used as a positive control, seems to be related with a decrease in long-term cell proliferation and induction of cell death. However, in accordance with other studies, in HCT116 cells Dox reduces the number of viable cells without an induction of apoptosis (Lüpertz et al. 2010).

Induction of cell death by Dox seems to depend on factors such as dose, exposure time and cell line used. Even so, facing our aims, Dox's exact mechanisms do not jeopardize it as control.

Genetic characteristics of cells, namely p53 status, influence its susceptibility to chemotherapy. p53 protein act as tumor suppressor, coordinating different activities such as cell death , cell cycle arrest, senescence, differentiation and DNA repair by interaction with different proteins (Oren 2003; Borges et al. 2007). Herein, the IC_{50} values for Dox were higher in HT29 and U251 cells (both p53 mutant) when compared with the other tested cell lines, demonstrating that these cells possess a more Dox resistant phenotype. Some studies have associated, in part, the Dox resistant phenotype to p53 status (Fojo and Bates 2003; Wang et al. 2013). Moreover, extracts E2 and E4 showed significant *in vitro* anticancer effects in three cell lines that are p53 wild-type. In fact, none of the tested extracts showed effects in p53 mutant cells, which suggests that the *in vitro* anticancer activity of the extracts could be p53 dependent. Both extracts also presented different ability to inhibit cell proliferation and induce cell death in the same cell line, strongly supporting that each extract acts through different molecular targets. Even so, the extracts' ability to decrease long-term cell proliferation and induce cell death is dependent of cell type, and thus it is plausible that other genetic features beyond the p53 status may influence these effects. Following this initial screening, more studies should be carried out to clarify the molecular mechanisms involved on the *in vitro* anticancer activity of extracts E2 and E4.

In summary, our study shows for the first time that crude ethyl acetate extracts obtained from marine-derived fungi *N. paulistensis* and *N. siamensis* have *in vitro* anticancer activities, by causing a decrease in cell proliferation and/or induction of cell death in hepatocellular carcinoma, colon carcinoma and melanoma cells. The inherent molecular targets and related signal transduction pathways should be researched in the future.

Conflict of interest

There are no conflicts of interest to report.

Acknowledgements

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References

Arasu, M.V., Duraipandiyan, V. and Ignacimuthu, S. (2013) Antibacterial and antifungal activities of polyketide metabolite from marine *Streptomyces* sp. AP-123 and its cytotoxic effect. *Chemosphere* **90**, 479-487.

Barckhausen, C., Roos, W., Naumann, S. and Kaina, B. (2014) Malignant melanoma cells acquire resistance to DNA interstrand cross-linking chemotherapeutics by p53-triggered upregulation of DDB2/XPC-mediated DNA repair. *Oncogene* **33**, 1964-1974.

Bhakuni, D. and Rawat, D. (2005) Bioactive metabolites of marine algae, fungi and bacteria. *Bioact Mar Nat Prod*, 1-25.

Borges, H.L., Linden, R. and Wang, J.Y. (2007) DNA damage-induced cell death: lessons from the central nervous system. *Cell Res* **18**, 17-26.

Buttachon, S., Chandrapatya, A., Manoch, L., Silva, A., Gales, L., Bruyère, C., Kiss, R. and Kijjoa, A. (2012) Sartorymensin, a new indole alkaloid, and new analogues of tryptoquivaline and fiscalins produced by *Neosartorya siamensis* (KUFC 6349). *Tetrahedron* **68**, 3253-3262.

Cai, M., Zhou, X., Lu, J., Fan, W., Niu, C., Zhou, J., Sun, X., Kang, L. and Zhang, Y. (2011) Enhancing aspergiolide A production from a shear-sensitive and easy-foaming marine-derived filamentous fungus *Aspergillus glaucus* by oxygen carrier addition and impeller combination in a bioreactor. *Bioresour Technol* **102**, 3584-3586.

Carvalho, C., Santos, R.X., Cardoso, S., Correia, S., Oliveira, P.J., Santos, M.S. and Moreira, P.I. (2009) Doxorubicin: the good, the bad and the ugly effect. *Curr Med Chem* **16**, 3267-3285.

Collins, A.R., Oscoz, A.A., Brunborg, G., Gaivão, I., Giovannelli, L., Kruszewski, M., Smith, C.C. and Štětina, R. (2008) The comet assay: topical issues. *Mutagenesis* **23**, 143-151.

De Stefano, D., Tommonaro, G., Malik, S.A., Iodice, C., De Rosa, S., Maiuri, M.C. and Carnuccio, R. (2012) Cacospongionolide and scalaradial, two marine sesterterpenoids as potent apoptosis-inducing factors in human carcinoma cell lines. *PloS one* **7**, e33031.

Debbab, A., Aly, A.H., Lin, W.H. and Proksch, P. (2010) Bioactive compounds from marine bacteria and fungi. *Microb Biotechnol* **3**, 544-563.

Degterev, A. and Yuan, J. (2008) Expansion and evolution of cell death programmes. *Nat Rev Mol Cell Biol* **9**, 378-390.

Fojo, T. and Bates, S. (2003) Strategies for reversing drug resistance. *Oncogene* **22**, 7512-7523.

Franken, N.A., Rodermond, H.M., Stap, J., Haveman, J. and Van Bree, C. (2006) Clonogenic assay of cells in vitro. *Nat Protoc* **1**, 2315-2319.

Fu, D., Calvo, J.A. and Samson, L.D. (2012) Balancing repair and tolerance of DNA damage caused by alkylating agents. *Nat Rev Cancer* **12**, 104-120.

Glass, N.L. and Donaldson, G.C. (1995) Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl Environ Microbiol* **61**, 1323-1330.

Gomes, N.M., Bessa, L.J., Buttachon, S., Costa, P.M., Buaruang, J., Dethoup, T., Silva, A. and Kijjoa, A. (2014) Antibacterial and Antibiofilm Activities of Tryptoquivalines and Meroditerpenes Isolated from the Marine-Derived Fungi *Neosartorya paulistensis*, *N. laciniosa*, *N. tsunodae*, and the Soil Fungi *N. fischeri* and *N. siamensis*. *Mar Drugs* **12**, 822-839.

Haefner, B. (2003) Drugs from the deep: marine natural products as drug candidates. *Drug Discov Today* **8**, 536-544.

Haloom, R., Christian, O., George T, G., Katherine, V., Assam, E.-O. and Tom C, K. (2011) Clonogenic Assay: Adherent Cells. *J Vis Exp*.

Hanahan, D. and Weinberg, Robert A. (2011) Hallmarks of Cancer: The Next Generation. *Cell* **144**, 646-674.

Henriquez, M., Vergara, K., Norambuena, J., Beiza, A., Maza, F., Ubilla, P., Araya, I., Chavez, R., San-Martin, A., Darias, J., Darias, M.J. and Vaca, I. (2014) Diversity of cultivable fungi associated with Antarctic marine sponges and screening for their antimicrobial, antitumoral and antioxidant potential. *World J Microb Biot* **30**, 65-76.

Jemal, A., Bray, F., Center, M.M., Ferlay, J., Ward, E. and Forman, D. (2011) Global cancer statistics. *CA Cancer J Clin* **61**, 69-90.

Jimeno, J., Faircloth, G., Sousa-Faro, J., Scheuer, P. and Rinehart, K. (2004) New Marine Derived Anticancer Therapeutics ─ A Journey from the Sea to Clinical Trials. *Mar Drugs* **2**, 14-29.

Kiesslich, T., Gollmer, A., Maisch, T., Berneburg, M. and Plaetzer, K. (2013) A Comprehensive Tutorial on In Vitro Characterization of New Photosensitizers for Photodynamic Antitumor Therapy and Photodynamic Inactivation of Microorganisms. *Biomed Res Int* **2013**, 17.

Kjer, J., Debbab, A., Aly, A.H. and Proksch, P. (2010) Methods for isolation of marine-derived endophytic fungi and their bioactive secondary products. *Nat Protoc* **5**, 479-490.

Kuml, D., Dethoup, T., Buttachon, S., Singburaudom, N., Silva, A.M. and Kijjoa, A. (2014) Spiculisporic acid E, a new spiculisporic acid derivative and ergosterol derivatives from the marine-sponge associated fungus *Talaromyces trachyspermus* (KUFA 0021). *Nat Prod Com* **9**, 1147-1150.

Li, W. and Melton, D. (2011) Cisplatin regulates the MAPK kinase pathway to induce increased expression of DNA repair gene ERCC1 and increase melanoma chemoresistance. *Oncogene* **31**, 2412-2422.

Li, Y.-X., Himaya, S., Dewapriya, P., Zhang, C. and Kim, S.-K. (2013) Fumigaclavine C from a Marine-Derived Fungus *Aspergillus fumigatus* Induces Apoptosis in MCF-7 Breast Cancer Cells. *Mar Drugs* **11**, 5063-5086.

Luo, H., Yang, A., Schulte, B.A., Wargovich, M.J. and Wang, G.Y. (2013) Resveratrol induces premature senescence in lung cancer cells via ROS-mediated DNA damage. *PloS one* **8**, e60065.

Lüpertz, R., Wätjen, W., Kahl, R. and Chovolou, Y. (2010) Dose-and time-dependent effects of doxorubicin on cytotoxicity, cell cycle and apoptotic cell death in human colon cancer cells. *Toxicology* **271**, 115-121.

Marmot, M., Atinmo, T., Byers, T., Chen, J., Hirohata, T., Jackson, A., James, W., Kolonel, L., Kumanyika, S. and Leitzmann, C. (2007) Food, nutrition, physical activity, and the prevention of cancer: a global perspective. *Washington, DC: World Cancer Research Fund/American Institute for Cancer Research*.

Munro, M.H., Blunt, J.W., Dumdei, E.J., Hickford, S.J., Lill, R.E., Li, S., Battershill, C.N. and Duckworth, A.R. (1999) The discovery and development of marine compounds with pharmaceutical potential. *J Biotechnol* **70**, 15-25.

Munshi, A., Hobbs, M. and Meyn, R.E. (2005) Clonogenic cell survival assay. *Chemosensitivity*, 21-28. Humana Press.

Nguyen, V.-T., Lee, J.S., Qian, Z.-J., Li, Y.-X., Kim, K.-N., Heo, S.-J., Jeon, Y.-J., Park, W.S., Choi, I.-W. and Je, J.-Y. (2013) Gliotoxin isolated from marine fungus *Aspergillus* sp. induces apoptosis of human cervical cancer and chondrosarcoma cells. *Mar Drugs* **12**, 69-87.

Oren, M. (2003) Decision making by p53: life, death and cancer. *Cell Death Differ* **10**, 431-442.

Pejin, B., Jovanovic, K.K., Mojovic, M. and Savic, A.G. (2013) New and highly potent antitumor natural products from marine-derived fungi: covering the period from 2003 to 2012. *Curr Top Med Chem* **13**, 2745-2766.

Portt, L., Norman, G., Clapp, C., Greenwood, M. and Greenwood, M.T. (2011) Anti-apoptosis and cell survival: A review. *Biochim Biophyis Acta* **1813**, 238-259.

Prompanya, C., Dethoup, T., Bessa, L.J., Pinto, M.M., Gales, L., Costa, P.M., Silva, A.M. and Kijjoa, A. (2014) New isocoumarin derivatives and meroterpenoids from the marine sponge-associated fungus *Aspergillus similanensis* sp. nov. KUFA 0013. *Mar Drugs* **12**, 5160-5173.

Qi, J., Shao, C.L., Li, Z.Y., Gan, L.S., Fu, X.M., Bian, W.T., Zhao, H.Y. and Wang, C.Y. (2013) Isocoumarin derivatives and benzofurans from a sponge-derived *Penicillium* sp. fungus. *J Nat Prod* **76**, 571-579.

Rafehi, H., Orlowski, C., Georgiadis, G.T., Ververis, K., El-Osta, A. and Karagiannis, T.C. (2011) Clonogenic assay: adherent cells. *J Vis Exp* **49**.

Ramos, A.A., Azqueta, A., Pereira-Wilson, C. and Collins, A.R. (2010) Polyphenolic compounds from *Salvia* species protect cellular DNA from oxidation and stimulate DNA repair in cultured human cells. *J Agric Food Chem* **58**, 7465-7471.

Ramos, A.A., Lima, C.F., Pereira, M., Fernandes-Ferreira, M. and Pereira-Wilson, C. (2008) Antigenotoxic effects of quercetin, rutin and ursolic acid on HepG2 cells: evaluation by the comet assay. *Toxicol Lett* **177**, 66-73.

Roninson, I.B., Broude, E.V. and Chang, B.-D. (2001) If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells. *Drug Resist Updat* **4**, 303-313.

Roos, W.P. and Kaina, B. (2013) DNA damage-induced cell death: from specific DNA lesions to the DNA damage response and apoptosis. *Cancer Lett* **332**, 237-248.

Sawadogo, W.R., Schumacher, M., Teiten, M.-H., Cerella, C., Dicato, M. and Diederich, M. (2013) A survey of marine natural compounds and their derivatives with anti-cancer activity reported in 2011. *Molecules* **18**, 3641-3673.

Shen, S., Li, W., Ouyang, M., Wu, Z., Lin, Q. and Xie, L. (2009) Identification of two marine fungi and evaluation of their antivirus and antitumor activities. *Wei Sheng Wu Xue Bao* **49**, 1240-1246.

Simmons, T.L., Andrianasolo, E., McPhail, K., Flatt, P. and Gerwick, W.H. (2005) Marine natural products as anticancer drugs. *Mol Cancer Ther* **4**, 333-342.

Sithranga Boopathy, N. and Kathiresan, K. (2010) Anticancer drugs from marine flora: an overview. *J Oncol* **2010**, 214186.

Sodngam, S., Sawadsitang, S., Suwannasai, N. and Mongkolthanaruk, W. (2014) Chemical constituents, and their cytotoxicity, of the rare wood decaying fungus *Xylaria humosa*. *Nat Prod Com* **9**, 157-158.

Su, J.-H., Chang, W.-B., Chen, H.-M., El-Shazly, M., Du, Y.-C., Kung, T.-H., Chen, Y.-C., Sung, P.-J., Ho, Y.-S. and Kuo, F.-W. (2012) 10-acetylirciformonin B, a sponge furanoterpenoid, induces DNA damage and apoptosis in leukemia cells. *Molecules* **17**, 11839-11848.

Thomas, T.R., Kavlekar, D.P. and LokaBharathi, P.A. (2010) Marine drugs from sponge-microbe association--a review. *Mar Drugs* **8**, 1417-1468.

Thorn, C.F., Oshiro, C., Marsh, S., Hernandez-Boussard, T., McLeod, H., Klein, T.E. and Altman, R.B. (2011) Doxorubicin pathways: pharmacodynamics and adverse effects. *Pharmacogenet Genomics* **21**, 440.

Tohme, R., Darwiche, N. and Gali-Muhtasib, H. (2011) A journey under the sea: the quest for marine anti-cancer alkaloids. *Molecules* **16**, 9665-9696.

Tsukada, M., Fukai, M., Miki, K., Shiraishi, T., Suzuki, T., Nishio, K., Sugita, T., Ishino, M., Kinoshita, K. and Takahashi, K. (2011) Chemical constituents of a marine fungus, *Arthrinium sacchari*. *J Nat Prod* **74**, 1645-1649.

Veer, P. and Kampman, E. (2007) Food, nutrition, physical activity and the prevention of cancer: a global perspective. *Washington, DC: World Cancer Research Fund/American Institute for Cancer Research*.

Wang, H., Yu, P., Bai, J., Zhang, J., Kong, L., Zhang, F., Du, G., Pei, S., Zhang, L. and Jiang, Y. (2013) Ocotillol enhanced the antitumor activity of doxorubicin via p53-dependent apoptosis. *Evid Based Complement Alternat Med* **2013**.

Wijesekara, I., Zhang, C., Van Ta, Q., Vo, T.-S., Li, Y.-X. and Kim, S.-K. (2014) Physcion from marine-derived fungus *Microsporum* sp. induces apoptosis in human cervical carcinoma HeLa cells. *Microbiol Res* **169**, 255-261.

Xavier, C.P., Lima, C.F., Preto, A., Seruca, R., Fernandes-Ferreira, M. and Pereira-Wilson, C. (2009) Luteolin, quercetin and ursolic acid are potent inhibitors of proliferation and inducers of apoptosis in both KRAS and BRAF mutated human colorectal cancer cells. *Cancer Lett* **281**, 162-170.

Zips, D., Thames, H.D. and Baumann, M. (2005) New anticancer agents: *in vitro* and *in vivo* evaluation. *In Vivo* **19**, 1-7.

CHAPTER III

CYTOTOXIC ACTIVITY OF COMPOUNDS ISOLATED FROM THE MARINE FUNGI *Neosartorya siamensis* IN HUMAN CANCER CELLS

This chapter is formatted according to the original manuscript to be submitted to the *Journal of Ethnopharmacology* or journals of adequate scope:

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3.1. Manuscript

Abstract

Aim of the study: To evaluate the anticancer activity of nine compounds isolated from the extract of marine sea fan fungus *Neosartorya siamensis* (KUFA 0017), obtained from the Similan Islands of Thailand.

Material and methods: Nine compounds isolated from the marine sea fan derived fungus *Neosartorya siamensis* (KUFA 0017), namely 2,4-dihydroxy-3-methylacetophenon (**C1**), nortryptoquivaline (**C2**), chevalone C (**C3**), tryptoquivaline H (**C4**), fiscalin A (**C5**), *epi*-fiscalin C (**C6**), *epi*-neofiscalin A (**C7**), *epi*-fiscalin A (**C8**) and tryptoquivaline F (**C9**) were tested for anti-proliferative activity by MTT assay, DNA damage induction by comet assay and induction of cell death by nuclear condensation assay on colon HCT116, liver HepG2 and melanoma A375 cancer cell lines.

Results: Compounds **C2**, **C3**, **C5**, **C6**, **C7** and **C8** presented IC_{50} values ranging from 24 to 153 µM in the selected cell lines. Cell death was induced in HCT116 by compounds **C2**, **C3** and **C5**. In HepG2, compounds **C2**, **C5**, **C7** and **C8** were able to induce significant cell death. This induction of cell death is possibly not related to genotoxicity since none of the compounds induced significant DNA damage.

Conclusion: Results suggest that compounds **C2**, **C3**, **C5**, **C6**, **C7** and **C8** present anti-proliferative activity and induce cell death, showing potential as future chemotherapeutic agents. Data justifies that further studies on mechanisms of action should be ensued.

Keywords: Anticancer activity; marine-derived fungi compounds; cancer cell lines; *Neosartorya siamensis*; cell death induction; cytotoxicity.

Abbreviations: Doxorubicin (Dox); Dimethyl sulphoxide (DMSO); 2,4-Dihydroxy-3-methylacetophenon (**C1**); Nortryptoquivaline (**C2**); Chevalone C (**C3**); Tryptoquivaline H (**C4**); Fiscalin A (**C5**); *Epi*-fiscalin C (**C6**); *Epi*-neofiscalin A (**C7**); *Epi*-fiscalin A (**C8**); Tryptoquivaline F (**C9**).

1. Introduction

According to the latest global census (GLOBOCAN 2012) there was an estimate of 14.1 million new cases of cancer in 2012 and 32.6 million people living with cancer, resulting in 8.2 million deaths. Colorectal and liver cancers are amongst the top ranking cancers in terms of diagnostic frequency, and with the highest mortality rates (Ferlay et al., 2013). Malignant melanoma, however, has shown a significant increase in the last decades, mostly in developed countries with populations with fairer skin (Erdmann et al., 2013). The increase of life expectancy, growth of population, exposure to hazardous environmental factors and engagement in risk behaviours, have given cancer the opportunity to become a global leading cause of death, and the number of cases is steadily increasing (Jemal et al., 2011). The development of cancer and the behavior of cancerous cells is characterized by several hallmarks: the induction of angiogenesis, the evading of growth suppressors, the sustaining of proliferative signaling, the enabling of replicative immortality, the activation of invasion and metastasis, and the resistance to cell death (Hanahan and Weinberg, 2011). Most desired for therapy are drugs that overcome the challenges inherent to cancer hallmarks and that exploit the vulnerabilities of cancer cells.

The search for anticancer drugs within natural sources has mobilized several research fields. New chemotherapeutic drugs are needed, in particular to attenuate the many harmful side effects observed with current common-use chemotherapeutics (Aluise et al., 2010; Nematbakhsh et al., 2012; Saad et al., 2014). While plants and terrestrial microorganisms have been in the limelight of natural product search for several decades, the marine environment is one of the current hotspots for the bio-prospection of new bioactive molecules (Montaser and Luesch, 2011). The vastness of the ocean hosts an impressive array of biodiversity, which is still largely unexplored. Nonetheless, several compounds of marine origin have been approved for clinical use, and several more are currently under clinical trials (Newman and Cragg, 2014; Schmidtko et al., 2010). Microorganisms have been some of the most prolific sources of bioactive compounds among marine organisms, much to the like of their terrestrial counterparts (Xiong et al., 2013). Marine fungi are now recognized as a prolific source of bioactive secondary metabolites, many of which are novel compounds presenting distinctive structural features. Metabolites of marine fungal origin have been described as having anticancer,

anti-inflammatory, antimicrobial and antiviral activity, amongst others (Elsebai et al., 2011; Lee et al., 2013; Li et al., 2013; Shushni et al., 2011).

Neosartorya siamensis (KUFA 0017) is a marine fungus associated to the *Rumphella* sp. sea fan. The *Neosartorya* belongs to the phylum Ascomycota, and species of that genus have been described to occur in the soil, air, organic materials, food, human habitations and also the marine environment (Yaguchi et al., 2010). In recent years, efforts have been made to isolate secondary metabolites from marine-derived *Neosartorya* sp., which have yielded several compounds with potential bioactivity (Eamvijarn et al., 2013; Gomes et al., 2014). In this quest, a recent study brought to light that the crude ethyl extract of *Neosartorya siamensis* (KUFA 0017) has anti-proliferative activity as well as cell death induction ability in three cancer cell lines (A375, HepG2 and HCT116) (Prata-Sena et al., 2014) having these results motivated this current study.

The aim herein was to assess the anticancer activity of nine compounds isolated from the marine sea fan derived fungus *Neosartorya siamensis* (KUFA 0017) — 2,4-dihydroxy-3-methylacetophenone (**C1**), four pyrazinoquinazoline derivatives, fiscalin A (**C5**), *epi*-fiscalin C (**C6**), *epi*-neofiscalin A (**C7**) and *epi*-fiscalin A (**C8**), three quinazoline derivatives, nortryptoquivaline (**C2**), tryptoquivaline H (**C4**) and tryptoquivaline F (**C9**), and the meroterpenoid chevalone C (**C3**) — on three selected human cancer cell lines, malignant melanoma (A375), hepatocellular carcinoma (HepG2) and colon carcinoma (HCT116), in accordance to results obtained in our previous study (Prata-Sena et al., 2014).

2. Experimental Section

2.1. *Reagents*

Roswell Park Memorial Institute medium (RPMI-1640), Minimum Essential Medium Eagle medium (MEM), Dulbecco's modified Eagle's medium (DMEM), sodium pyruvate, sodium bicarbonate, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), trypsin solution, 4,6-diamidino-2-phenylindole (DAPI), penicillin/streptomycin, doxorubicin, and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Biochrom KG (Berlin, Germany). Dimethyl sulfoxide (DMSO) was purchased from

AMRESCO LLC (Solon, SO, USA). All other reagents and chemicals used were of analytical grade.

2.2. *Fungal material*

Neosartorya siamensis (KUFA 0017) was isolated from sea fan (*Rumphella* sp.), collected from the coral reef at Similan Island, PhangNga province, Southern Thailand, by scuba diving at 10 m depth, in April 2010. Briefly, the sea fan tissue was cut into a piece of 0.5 cm x 0.5 cm, placed on the malt extract agar (MEA) with 70% sea water and incubated for 28ºC for 7 days. The fungus was identified by Professor Tida Dethoup, by morphological features, including the characteristic of ascospores and colonies. The identification was supported by sequence analysis of the β-tubulin gene, described in the previous reportas in Glass and Donaldson (1995), and the pure cultures were deposited as KUFA 0017 at Kasetsart University Fungal Collection, Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand.

2.3. *Extraction of metabolites*

The isolation of compounds from the marine-derived fungus *Neosartorya siamensis* (KUFA 0017) was performed by Professor Anake Kijjoa and collaborators from ICBAS and CIIMAR. The extraction of metabolites was made according to Buttachon et al. (2012).

2.4. *Cell culture*

The A375 cell line (human malignant melanoma) was acquired from the European Collection of Cell Cultures (ECACC). HCT116 cells (human colon carcinoma) were kindly provided by Professor Carmen Jerónimo from IPO, Porto. The HepG2 cells (human hepatocellular carcinoma) were kindly provided by Professor Rosário Martins from CIIMAR, Porto. Cells were maintained as monolayer cultures, respectively, in MEM for HepG2 cells, DMEM for HT29 cells, and RPMI for HCT116 cells. All mediums were supplemented with 10% FBS, 10 mM HEPES, 0.1 mM sodium pyruvate and 1% antibiotic solution (100 µg/ml streptomycin and 100 U/ml penicillin). Cells were maintained in a humidified incubator at 37ºC and 5% CO_2 atmosphere. The medium was changed every two days, and when reaching confluence, cells were trypsinised and sub-cultured.

Stock solutions of the compounds and doxorubicin were prepared in DMSO and stored at -20ºC. At the beginning of all experiments, compounds and doxorubicin were dissolved in fresh culture medium, with a final concentration of DMSO no higher than 0.5%. Negative controls were subjected only to culture medium with a maximum of 0.5% of DMSO (v/v), to assess the effect of the solvent.

2.5. *MTT colorimetric assay*

The MTT colorimetric assay was performed to assess the cell viability/proliferation effects of the tested compounds in HepG2, HCT116 and HT29 cell lines. In short, cells were plated in 96-multiwell culture plates at a density of 1 x 10^4 cells/well. After plating, cells were left to adhere for 24 h in a humidified incubator at 37ºC and 5% CO_2. Cells were incubated with fresh RPMI medium prepared with a ranging concentration of compounds (1, 10, 50, 100 and 200 µM) and doxorubicin (0.001, 0.01, 0.1, 1 and 10 µM) as a positive control. Negative controls contained only medium with 0.5% DMSO. All solutions when incubated with cells contained a maximum of 0.5% DMSO. For the initial control (t = 0), 0.5 mg/ml of MTT solution was added, left to incubate at 37ºC and 5% CO_2 for 2 hours and then the medium and MTT solution were removed. The cells treated with compounds and controls were then left to incubate at 37ºC and 5% CO_2 for the remaining 46 h of treatment. Subsequently, 0.5 mg/ml of MTT solution was added to all treatment conditions and incubated for 2 h at 37ºC. The formazan crystals formed during incubation were dissolved by adding 150 µl of DMSO:ethanol solution (1:1) (v/v), which was left to incubate in a plate shaker for 10 min. Absorbance (A) was read at 570 nm in a microplate reader (Multiskan EX, Labsystems, USA). The value of absorbance reflects the amount of formazan crystals produced by viable mitochondria in cells by the reduction of MTT, and is frequently used as an indicator of cell viability and cell proliferation (Vega-Avila & Pugsley, 2011). The half maximal inhibition concentration values (IC_{50}) of the compounds and doxorubicin were calculated by analysing dose-response data with GraphPad Prism v5.0 software (GraphPad Software, La Jolla, CA, USA).

2.6. *Alkaline single cell electrophoresis assay (Comet Assay)*

The genotoxic effects of the tested compounds were assessed by single cell electrophoresis assay, or comet assay. Cells were plated at a density of 0.1 x 10^6 cells/ml on 24-multiwell culture plates and left to adhere for 24 hours. Thereupon, cells were exposed to the respective IC_{50} concentrations (Table 1) of compounds and doxorubicin, and left to incubate for 48 h. After the incubation, an alkaline version of comet assay was

used to assess DNA damage in the form of alkali-labile sites and strand breaks (Collins et al., 2008; Olive and Banáth, 2006). After the conclusion of the treatment, cells were maintained on ice to avoid DNA repair. In summary, 5 x 10^4 cells were collected per sample and were resuspended with 0.5% (w/v) low melting agarose. Subsequently, cells were set on microscope slides coated with 1% (w/v) normal melting agarose and left to solidify at 4ºC for 10 min. Slides were then incubated for 1 h at 4ºC in lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10) with the addition of 1% (v/v) Triton X-100. In order to allow the DNA to unwind, slides were placed in a horizontal electrophoresis chamber and incubated in an alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13) for 40 min at 4ºC. Afterwards, an electrophoresis at 20 V was run for 20 min at 4ºC. Slides were washed twice in distilled water, fixed with 100% ethanol, left to air dry at room temperature and incubated with DAPI (1 µg/ml) for 10 min, protected from light. Samples were then observed by fluorescence microscopy (Olympus IX71), with a minimum of 100 randomly selected cells being observed per sample, and resulting images were analysed using the CometScore® software (CometScore, TriTek Corp.), to calculate the parameter "percentage of tail intensity".

2.7. *Nuclear condensation assay*

The effect of the compounds on the induction of cell death was analysed by a nuclear condensation assay, which is one of the features of apoptosis (Toné et al., 2007). Briefly, cells were plated at a density of 1 x 10^6 cells/ml in 24-multiwell culture plates and left to adhere for 24 h. Thereafter, cells were incubated with two or three selected concentrations, ranging from 10 to 150 µM, according to the cell line and respective IC_{50} value (Table 1) for each compound and doxorubicin for 48 h. After the incubation, the cells were washed and trypsinised, assuring that both adherent and detached cells were collected, then centrifuged several times and fixed with 4% (w/v) paraformaldehyde in PBS for 15 min at 37ºC. At the end of protocol, samples were stored at 4ºC in PBS until further use. Prior to use, cells were mounted on poly-L-Lysine-covered microscope slides by cytocentrifugation (Cell Spin Cytospin) and left to air dry at room temperature. Once dried, samples were circled with hydrophobic barrier pen and the slides were washed thrice for 5 min. To stain the nuclei, samples were incubated with DAPI solution (1µg/ml) for 10 min, protected from light. Condensed nuclei were observed by fluorescence microscopy (Olympus IX71). At least 3 different fields were observed, resulting in a minimum of 300 cells scored per sample. Results were analysed by applying the following equation: % of cell death = number of cells with condensed nuclei / total number of cells.

2.8. *Statistical analysis*

Analyses were made with the GraphPad Prism v6.0 software (GraphPad Software, La Jolla, CA, USA). Results were expressed as mean and standard deviation (SD) from at least 3 independent experiments. Outlier detection was performed using a ROUT test (Q=10%). Data were analysed for normal distribution and homogeneity of variances using the Kolmogorov-Smirnov test and Bartlett's test, respectively. One-way ANOVA was performed to assess significant differences ($p \leq 0.05$) between treatment conditions and the negative control, followed by *post hoc* multiple comparisons using the Newman-Keuls test. A Student's *t*-test was used to verify significant differences when appropriate.

3. Results

3.1. Evaluation of cell proliferation by MTT colorimetric assay

The assessment of the effect on cell viability and proliferation of the nine tested marine-derived compounds (2,4-dihydroxy-3-methylacetophenon (**C1**), nortryptoquivaline (**C2**), chevalone C (**C3**), tryptoquivaline H (**C4**), fiscalin A (**C5**), *epi*-fiscalin C (**C6**), *epi*-neofiscalin A (**C7**), *epi*-fiscalin A (**C8**) and tryptoquivaline F (**C9**)) was performed by an MTT colorimetric assay using three cancer cell lines, malignant melanoma (A375), hepatocellular carcinoma (HepG2) and colon carcinoma (HCT116). Each cell line was incubated with a varying concentration of compounds (1, 10, 50, 100 and 200 µM). Doxorubicin at the concentrations of 0.001, 0.01, 0.1, 1 and 10 µM was used as a positive control, whereas negative controls correspond to the cells incubated only with medium with 0.5% DMSO. The half maximal inhibitory concentration values (IC_{50}) values for each cell line were calculated by the analysis of dose-response curves. The IC_{50} values and respective 95% confidence intervals (CI) are summarized in Table 1.

Nortryptoquivaline (**C2**) was broadly effective in decreasing the viability in HCT116, HepG2 and A375, with IC_{50} values of 114 µM, 58 µM and 108 µM, respectively. *Epi*-fiscalin C (**C6**) also presented a broad effect across all cell lines, however, showing a greater inhibition of viability, with IC_{50} values of 86 µM for HCT116, 24 µM for HepG2 and 75 µM for A375 cell line. Chevalone C (**C3**) exhibited a significant decrease of cell viability in melanoma (A375) and colon carcinoma (HCT116) cell lines, with IC_{50} values of 98 µM and 153 µM, respectively. Fiscalin A (**C5**) also exhibited a considerable inhibition of cell viability in two cancer cell lines, with IC_{50} values of 123 µM for HCT116 and of 53 µM for HepG2 cells. *Epi*-neofiscalin A (**C7**) and *epi*-fiscalin A (**C8**) only presented a significant

decrease of cell viability in liver hepatocellular carcinoma cells (HepG2), with respective IC_{50} values of 76 µM and 115 µM. Tryptoquivaline H (**C4**) and tryptoquivaline F (**C9**) were overall ineffective in two cell lines, having the first only been narrowly inhibitory in HCT116 and the latter in HepG2. 2,4-Dihydroxy-3-methylacetophenon (**C1**) failed to cause significant alterations in cell viability in any of the cell lines, which prevented the calculation of an IC_{50}. Only the compounds presenting an IC_{50} of approximately equal of lower than 150 µM were selected for the following assays; this criterion is sustained by the need of assuring concentrations that are viable in further *in vivo* studies. The positive control, doxorubicin presented and IC_{50} of 0.13 µM for HCT116, 0.11 µM for HepG2 and 0.08 µM for A375.

The effect of the compounds on cell proliferation was assessed by an MTT assay, where the total of viable cells at the beginning of the experiment (t = 0 h) was subtracted from the total of viable cells at the end of the experiment (t = 48 h). Hence, results were expressed in percentage, considering 100% proliferation that equal to the negative control (0.5% DMSO) at 48 h, and 0% proliferation that equal to the negative control at 0 h. Compound treatments with proliferation percentages ranging from 0% to under 100% are considered as presenting an anti-proliferative effect while negative percentages imply direct cytotoxic effects of compounds.

In HCT116 cell line, the compounds **C2, C3,** and **C5** slightly decrease cell proliferation when tested at 50 µM, showing cell proliferation inhibitions of 19%, 16% and 22%, respectively. Compound **C6** exhibited a significant inhibition of cell proliferation around 58% (Table 2). At 200 µM, the compounds **C2**, **C3**, and **C5** showed a direct cytotoxic effect on HCT116 cells (Figure 1A).

In HepG2 cell line, compounds **C2, C5,** and **C6** presented a strong cell proliferation inhibition of 83, 77 and 99%, respectively, at 50 µM. Compounds **C7** and **C8** also significantly inhibit cell proliferation, but with more moderate activity, obtaining 59 and 50% of inhibition at 50 µM (Table 2). The compound C6 at 1 µM already showed a significant decrease of cell proliferation as observed in Figure 1B. Compounds, C2, C5 and C6 at 100 and 200 µM showed direct cytotoxic effect (observed by negative values) (Figure 1B).

In the A375 melanoma cell line, compounds **C2, C3** and **C6** caused a significant inhibition of cell proliferation at 50 µM, by 27, 39 and 53%, respectively (Table 2). Figure 1C shows that the three compounds, at 200 µM, showed direct cytotoxic effects. Though the compound **C6** showed a very promising anti-proliferative effect, due to exhaustion of the isolated stock it was impossible to make with further studies within this study scope. Doxorubicin, the positive control, caused a decrease in cell proliferation in a dose-dependent manner in all tested cell lines (data not shown). Doxorubicin at 0.1 µM significantly inhibited proliferation of HCT116, HepG2 and A375 cells, by 60, 82 and 87%,

respectively (Table 2). The anti-proliferative effect of the compounds was also observed by phase contrast microscopy, where alterations in cell morphology, density and cell detachment were detected, as shown in Figure 2.

Table 1 – Half maximal inhibitory concentrations (IC_{50}), in μM, and respective 95% confidence intervals of compounds **C1** to **C9** and doxorubicin (positive control) in three cell lines as determined by the MTT assay.

	Cancer cell lines					
	HCT116		HepG2		A375	
Compounds	**IC_{50}** (μM)	*95% confidence interval*	**IC_{50}** (μM)	*95% confidence interval*	**IC_{50}** (μM)	*95% confidence interval*
Doxorubicin	0.13	(0.08 – 0.21)	0.11	(0.04 – 0.28)	0.08	(0.05 – 0.14)
C1	-	-	-	-	-	-
C2	**114**	(94 – 139)	**58**	(45 – 75)	**108**	(92 – 127)
C3	**153**	(120 – 196)	190	(61 – 586)	**98**	(65 – 146)
C4	202	(130 – 312)	-	-	-	-
C5	**123**	(100 – 151)	**53**	(34 – 84)	169	(118 – 242)
C6	**86**	(62 – 118)	**24**	(13 – 44)	**75**	(57 – 99)
C7	203	(149 – 275)	**76**	(44 – 132)	160	(121 – 212)
C8	277	(151 – 507)	**115**	(75 – 176)	-	-
C9	-	-	235	(105 – 530)	-	-

The "-" means "not determined".

Table 2 – Percentage of cell proliferation inhibition, relative to the negative control, of the compounds, at 50 μM, and doxorubicin, at 0.1 μM, in HCT116, HepG2 and A375 cells.

	% of cell proliferation inhibition (relative to control)		
Compound (50 μM)	**HCT116**	**HepG2**	**A375**
C2	19	83	27
C3	16	-	39
C5	22	77	-
C6	58	99	53
C7	-	59	-
C8	-	50	-
Doxorubicin (0.1 μM)	60	82	87

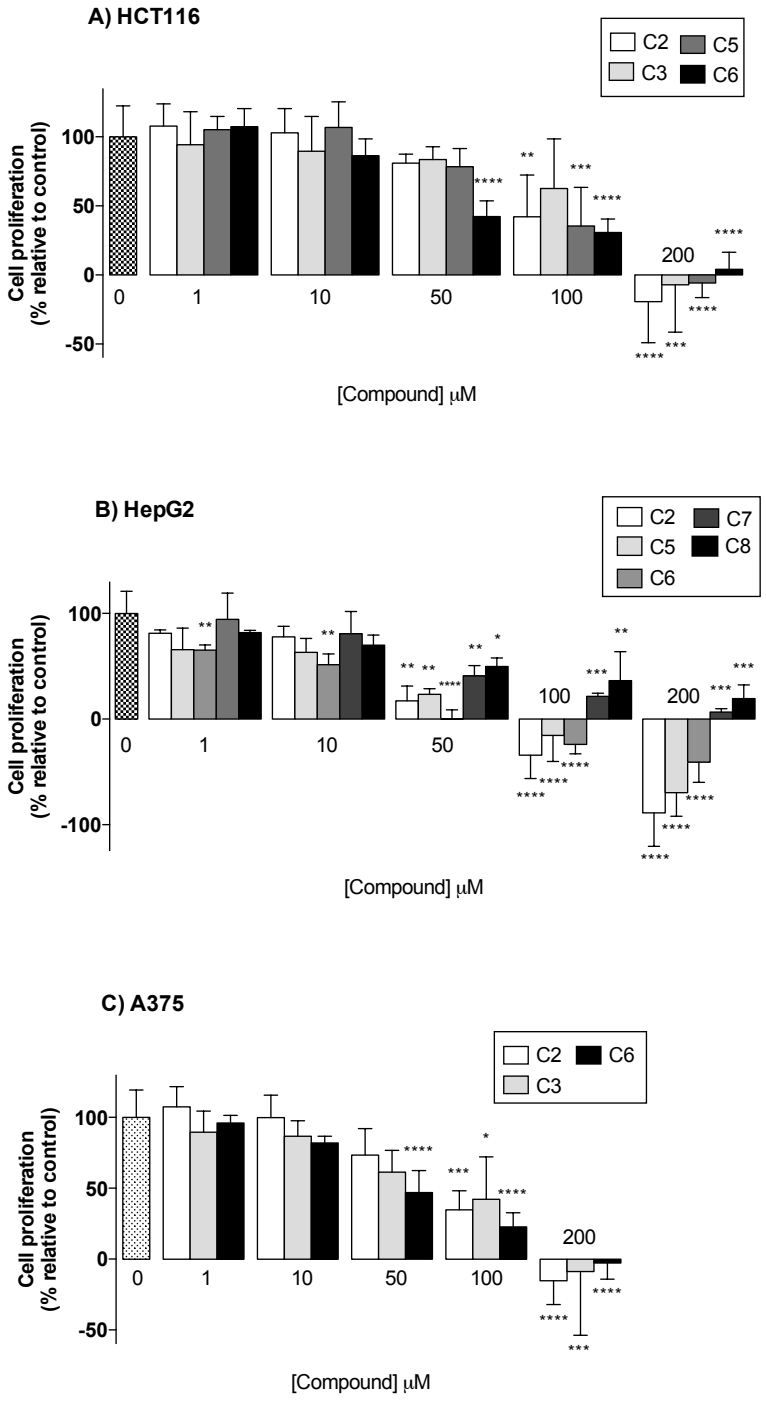

Figure 1 – Effect of compounds on cell proliferation in HCT116 (A), HepG2 (B) and A375 (C) cells after 48 h, evaluated by MTT assay. Results are expressed as mean + SD of at least three independent experiments, in duplicate. Significant differences (*$p \leq 0.05$; ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$) when compared with control cells were determined by one-way ANOVA, followed by a Newman-Keuls Multiple comparison test.

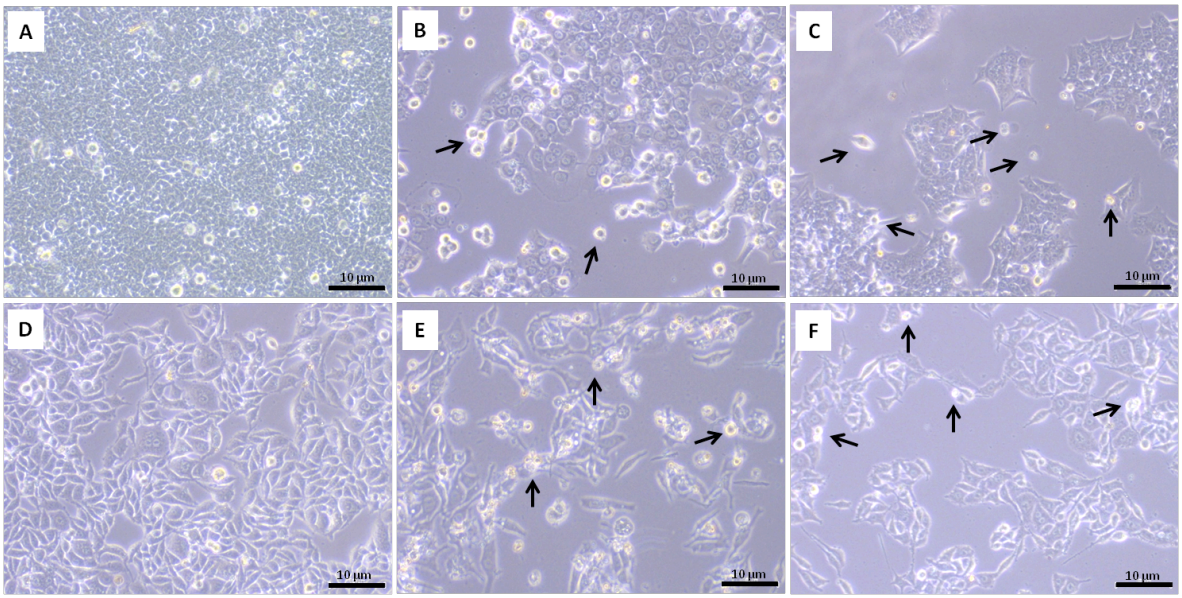

Figure 2 - Phase contrast morphology of HCT116 cells treated with (A) 0.5% DMSO (negative control); (B) doxorubicin at 0.1 µM; (C) compound **C2** at 150 µM and HepG2 cells treated with (D) 0.5% DMSO (negative control); (E) doxorubicin at 0.1 µM; (F) compound **C2** at 150 µM. Rounded and detached cells (arrows).

3.2. *Evaluation of genotoxic effects by single cell electrophoresis assay*

The analysis of DNA damage of alkali-labile sites and strand breaks was assessed by comet assay regarding a 48 h exposure to the IC_{50} value of the compounds presenting an $IC_{50} \leq 150$ µM in HCT116, HepG2 and A375 cancer cell lines. The analysis of the results revealed that none of the compounds causes significant damage to the DNA detectable by comet assay (data not shown).

3.3. *Evaluation of cell death induction by nuclear chromatin condensation assay*

Considering that the observed anti-proliferative effect induced by the compounds may be due to the induction of cell death, a nuclear chromatin condensation assay was performed after a 48 h exposure of the three cell lines (HCT116, HepG2 and A375) to the compounds that exhibited an $IC_{50} \leq 150$ µM (Figure 3).

The concentrations of compounds to which the cell lines were exposed in the nuclear condensation assay and the comet assay were based on the IC_{50} values obtained from the MTT assay, thus in general three concentrations were used, one concentration approximate to the IC_{50} value, one above and one below. HCT116 and A375 were both exposed to the compounds **C2** and **C3** at a range of concentrations of 50, 100 and 150

μM, while HCT116 was also exposed to the compound **C5** at 50 and 150 μM. HepG2 was exposed to the compounds **C5** and **C7** at a range of concentrations of 10, 50 and 100 μM, as well as to **C2** and **C8** at 50, 100 and 150 μM. All experiments had as a positive control 0.1 μM of doxorubicin and as a negative control medium with 0.5% of DMSO.

In A375 cells, only doxorubicin exhibited a significant ($p < 0.001$) increase of cells with condensed nuclei by 21%. The compounds tested (**C2** and **C3**) evidenced no statistically significant variations in the amount of cells with condensed nuclei — this at the tested concentrations and when compared with the negative control; accordingly, neither was it possible to observe a clear dose-response effect (data not shown).

HCT116 cells were sensitive to the induction of nuclear condensation in a dose-response manner by all the three compounds (Figure 3A). Compounds **C2** and **C5** were the most potent compounds regarding this cell line, and at a concentration of 150 μM demonstrated an increase of 11% and 10%, respectively, in relation to the negative control. Compound **C3** exhibited an increase of 6% and 8% at 100 μM and 150 μM, respectively. In HCT116 cells, doxorubicin did not present a clear significant difference in relation to the negative control ($p = 0.0549$). However, the proximity to $p \leq 0.05$ may indicate marginal significance, and thus considering, doxorubicin revealed a 7% increase in relation to control.

All the tested compounds induced nuclear condensation in hepatocellular carcinoma cell line at one or more concentrations (Figure 3B). Compound **C7** exhibited a significant effect in all three of the tested concentrations, with an increase of 4%, 6% and 8%, respectively, at 10 μM, 50 μM and 100 μM. Compound **C2** displayed a significant increase of cells with condensed nuclei at two concentrations, presenting 5% and 6% at 100 μM and 150 μM, respectively. Compound **C5** showed an increase of 5% of cells with condensed nuclei at 100 μM. Meanwhile, compound **C8** presented an 8% increase in relation to control at 150 μM. Doxorubicin induced an increase of 12% in relation to the negative control.

HepG2 cells were clearly the most sensitive to the compounds' activity, being affected by all four tested compounds, and in one case having had induction of chromatin condensation in a concentration as low as 10 μM.

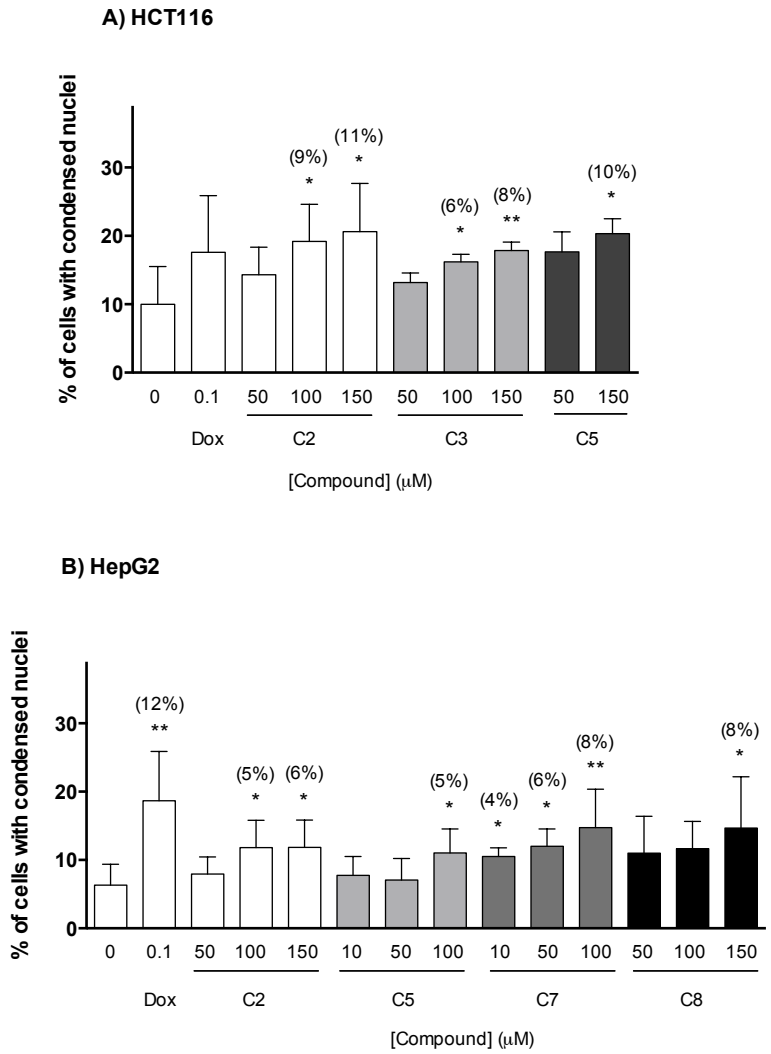

Figure 3 – Effect of compounds **C2**, **C3**, **C5**, **C7** and **C8**, accordingly, on the induction of nuclear chromatin condensation in (A) HCT116 and (B) HepG2 cells, as assessed by a nuclear condensation assay. Percentages refer to the increment of cells with condensed nuclei in relation to the negative control. Results are the mean + SD of at least three independent experiments. Significant differences (*$p \leq 0.05$; ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$) when compared with the negative control were determined by a One-way ANOVA followed by a Newman-Keuls Multiple comparison test. Additionally, an unpaired Student's *t*-test was selectively used to assess significant differences in relation to the negative control in HepG2 for **C2** (100 and 150 μM), **C5** (100 μM), **C7** (10 μM) and doxorubicin (0.1 μM).

4. Discussion

To our knowledge, this is the first time that compounds are isolated from the marine sea fan derived fungus *Neosartorya siamensis* (KUFA 0017). Although these compounds are not novel isolates, it is the first time that their anti-proliferative activity is described in

HepG2 hepatocellular carcinoma, HCT116 colon carcinoma and A375 malignant melanoma cell line, accompanied by mechanistic assessment of genotoxicity (detection of DNA damage, such as strand breaks and alkali-labile sites) and induction of cell death.

In our study, effects on cell viability/proliferation were assessed by an MTT assay and all compounds tested, in exception to compounds **C1**, **C4** and **C9** significantly decreased cell viability and proliferation in one or more of the cell lines tested. The anti-proliferative effect of the compounds could be due to induction of cell death and/or cell cycle arrest. Morphological features of cell death by apoptosis, such as membrane blebbing, cell shrinkage, rounding and detachment, were visible by phase contrast microscopy after an incubation of 48 h with compounds, and these features are corroborated by the observation of nuclear chromatin condensation. The nuclear condensation assay is used to detect nuclear chromatin condensation, which in turn is one of the key features of apoptosis (Kroemer et al., 2009). In normal conditions, chromatin is heterogenous and spaciously packaged in nuclei, becoming more condensed in the event of mitosis, however, upon the unraveling of apoptosis, nuclear chromatin becomes exceptionally condensed and fragmented, and alongside nuclear proteins, ends up forming what is commonly known as apoptotic bodies (Toné et al., 2007). Thus, the increase of cells with condensed nuclei suggests an increase of cell death, most probably by apoptosis.

HepG2 hepatocellular carcinoma cell line was by far the most sensitive line to the panel of compounds. In fact, almost all the compounds, except **C1 and C4**, proved to have an anti-proliferative effect in a dose-dependent manner in this cell line. Notwithstanding, the IC_{50} values observed regarding HepG2 were also the most promising, as the values were inferior to 100 μM in four cases (**C2**, **C5**, **C6** and **C7**), reaching the lowest concentration of 24 μM for compound **C6**. Inclusively, the compound **C6** was able to inhibit cell proliferation at the smallest tested concentration (1 μM) with an inhibition of > 35%, which increased consistently at higher concentrations. This is particularly interesting since an anti-proliferative activity at such low doses may possibly imply a lesser amount of side effects. Compound **C3** yielded an IC_{50} value above the pre-determined threshold (≤ 150 μM) and was thus not considered for further assays. The 2,3-dihydrosorbicillin compound, obtained from the terrestrial endophytic fungi *Penicillium* sp. P-1, exhibited an anti-proliferative activity on HepG2 cells, with an IC_{50} of 44 μM. In addition, resveratrodehyde C, obtained from the marine mangrove endophytic fungi *Alternaria* sp. R6, obtained an IC_{50} of 41.86 μM in HepG2 cell line. Both these fungal compounds show a degree of cytotoxic activity similar to our own compounds against this hepatocellular carcinoma cell line. Furthermore, our results indicated that the compounds

C2, **C5**, **C7** and **C8** acted also by inducing cell death, since all compounds caused a significant increase of cells with condensed nuclei, to a varying extent and mostly in accordance with increasing dose. However, the induction of cell death in HepG2 was to some extent lower than that in HCT116. This may imply that in the liver cancer cell line the compounds' action may be also strictly related to inhibition of proliferative mechanisms.

In HCT116 colon carcinoma cell line, the effect of the compounds on cell viability/proliferation was in general weaker than that observed in HepG2. The compounds showing a suitable effect, **C2**, **C3**, **C5** and **C6**, presented IC_{50} values ranging from 86 to 153 μM. Once more, it can be observed that compounds **C5** and **C6**, both belonging to the fiscalin family, also play an interesting role in HCT116 cells, alongside compound **C2**, which is also responsible for a generalist activity by acting in the three cell lines tested. These three compounds obtained the highest cell proliferation inhibition percentages (Figure 1). The colon carcinoma cell line seems to be positively influenced by compounds **C2**, **C3** and **C5** in terms of an increment of cells with condensed nuclei, ultimately indicating an increase of cell death in a concentration-dependent manner (Figure 3). This is consistent to the results obtained from the proliferative screening, and interestingly this cell line seems to be slightly more affected by the induction of cell death than the other tested cell lines. This may suggest that the compounds are successfully interfering in cell death mechanisms, most probably by promoting a pro-apoptotic response. Once more, there is no evidence that the compounds have the ability to induce DNA damage, at least considering damage detectable by the alkaline version of the comet assay, in the form of strand breaks or alkali-labile sites.

A375 melanoma cell line was overall the most selective in terms of compound sensitivity. Only three compounds, **C2**, **C3** and **C6**, resulted in fitting IC_{50} values (≤ 150 μM) and with values similar to those observed in HCT116 cells. The remaining compounds **C4**, **C7** and **C8** did not reach the pre-established threshold for the IC_{50}. In A375 cells, the compounds **C2** and **C3** exhibited a better percentage of cell proliferation inhibition than that observed in HCT116. In spite of the promising anti-proliferative activity, the malignant melanoma cell line seems unaltered regarding an increase of cells with nuclear chromatin condensation when exposed to compounds **C2** and **C3**; this may suggest that the induction of cell death can occur by a mechanism differing from apoptosis, or that the decrease of cell proliferation may be due to an inhibition of proliferative pathways. In A375 cells, the anti-proliferative effect of compounds is also apparently not influenced by the compound's ability to damage DNA, since results from the comet assay did not indicate feasible DNA damage. Current literature also reports

studies with similar compounds, such as the study by Eamvijarn et al. (2013), which screened an analogue of chevalone C (**C3**), sartorypyrone B, obtained from *Neosartorya tsunodae* (KUFC 9213) in A375-C5 melanoma cell line and obtained a half maximal growth inhibitory concentration (GI_{50}) of 25 μM (Eamvijarn et al., 2013).

Compounds **C1** and **C9** were showed no significant anti-proliferative activity in any of the tested cell lines, thus further studies with these compounds were not carried out in our study. However, more assays could be done to test their anti-proliferative (or other kind of) effects in other cell models before discarding the biomedical potential of the two isolates.

Buttachon et al. (2012) isolated several identical compounds from a terrestrial strain of *Neosartorya siamensis* (KUFC 6349). Besides other isolated compounds specific to the terrestrial strain, compounds **C4**, **C8** and **C9** which are common to our marine strain were assessed for anti-proliferative activity by Buttachon et al. (2012) in cancer cell lines MCF-7 (breast adenocarcinoma), A549 (non-small cell lung cancer), SK-MEL-28 (malignant melanoma), and U373 and Hs683 (glioblastoma). Results indicated that only compound **C9** had effect upon a cell line, namely U373, with an IC_{50} of 90 μM, otherwise, none of the remaining compounds exhibited relevant anti-proliferative effects on any of the tested lines. In our study, compound **C9** did not show any significant activity in liver, skin or colon cancer cell lines, thus hinting that compound **C9** has a potential activity against a very restrictive cell spectrum.

Like herein, the anti-proliferative effects of compound **C3** were also seen by Kanokmedhakul et al. (2011), based on the fungus *Eurotium chevalieri*, from which chevalone C (**C3**) was first isolated, revealing that compound **C3** exhibited an IC_{50} value of 8.7 μg/ml in BC1 (breast cancer) cell line. The cited study also described the activity of other chevalone derivatives (chevalones A, B and D), where chevalone B was active in KB (mouth cancer) and NCI-H187 (small-cell lung cancer) cell lines and chevalone D in BC1 cell line, with IC_{50} values of 2.9, 9.8 and 7.8 μg/ml, respectively (Kanokmedhakul et al., 2011).

Meanwhile, Sodngam et al. (2014) have also described variable cytotoxic activity from compounds chevalone C (**C3**), fiscalin A (**C5**), *epi*-fiscalin C (**C6**) and *epi*-fiscalin A (**C8**), isolated from wood decomposing fungus *Xylaria humosa* in NCI-H187 (small cell lung cancer), MCF-7 (breast adenocarcinoma) and KB (mouth epidermal carcinoma) cancer cell lines (Sodngam et al., 2014). In the study by Sodngam et al. (2014), compound **C3** was active in KB and NCI-H187, with an IC_{50} of 49.4 and 17.7 μg/ml, respectively. Both compounds **C6** and **C8** were active in MCF-7, with an IC_{50} of 21 and 24.4 μg/ml,

respectively. Compound **C5** showed no activity in lung, breast and mouth cancer cell lines, whereas our results clearly indicate an effect of this compound in hepatic and colon cancer cells. The occurrence of activity in different cancer cell types/lines validates the interest and potential of such fungal compounds as prospective chemotherapeutic agents.

In general, the pyrazinoquinazoline derivatives of the fiscalin family (**C5**, **C6**, **C7** and **C8**) exhibited a broader anti-proliferative action, having all four compounds acted in at least one cell line, and one of the compounds (**C6**) was effective in all three lines. Overall, *epi*-fiscalin C (**C6**) was the most effective and promising compound in the inhibition of proliferation in all tested cell lines, moreover, this compound held the lowest IC_{50} values in regard to each cell line (from 24 μM to 86 μM). Unfortunately, the stock limitations of compound **C6** withheld its further testing for the induction of DNA damage and cell death herein. When observing results regarding cell death induction, three compounds of the fiscalin family induced cell death in two cell lines, HepG2 and HCT116. Overall, the pyrazinoquinazoline derivatives (**C5**, **C6**, **C7** and **C8**), as well as the quinazoline derivative nortryptoquivaline (**C2**) and the meroterpenoid chevalone C (**C3**) exhibited promising results in terms of anti-proliferative activity and induction of cell death in the cell line tested. Since the molecular mechanisms involved remain unknown, further studies are worthwhile in the light of our data.

4. Conclusions

The results obtained with this study demonstrate that the compounds obtained from the marine-derived fungus *Neosartorya siamensis* (KUFA 0017) have significant anticancer properties, as evaluated by the means of anti-proliferative activity and cell death induction in hepatocellular carcinoma, colon carcinoma and malignant melanoma. Compounds of the fiscalin family are of specific interest as they present prominent anticancer bioactivity. The present data justify further studies to address the nature of the molecular targets and the transduction pathways involved in the bioactive mechanisms of these compounds.

Conflicts of Interest

The authors declare no conflict of interest.

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References

Aluise, C.D., Sultana, R., Tangpong, J., Vore, M., Clair, D.S., Moscow, J.A., Butterfield, D.A., 2010. Chemo brain (chemo fog) as a potential side effect of doxorubicin administration: role of cytokine-induced, oxidative/nitrosative stress in cognitive dysfunction, Chemo Fog. Springer, pp. 147-156.

Buttachon, S., Chandrapatya, A., Manoch, L., Silva, A., Gales, L., Bruyère, C., Kiss, R., Kijjoa, A., 2012. Sartorymensin, a new indole alkaloid, and new analogues of tryptoquivaline and fiscalins produced by *Neosartorya siamensis* (KUFC 6349). Tetrahedron 68, 3253-3262.

Collins, A.R., Oscoz, A.A., Brunborg, G., Gaivão, I., Giovannelli, L., Kruszewski, M., Smith, C.C., Štětina, R., 2008. The comet assay: topical issues. Mutagenesis 23, 143-151.

Eamvijarn, A., Gomes, N.M., Dethoup, T., Buaruang, J., Manoch, L., Silva, A., Pedro, M., Marini, I., Roussis, V., Kijjoa, A., 2013. Bioactive meroditerpenes and indole alkaloids from the soil fungus *Neosartorya fischeri* (KUFC 6344), and the marine-derived fungi *Neosartorya laciniosa* (KUFC 7896) and *Neosartorya tsunodae* (KUFC 9213). Tetrahedron 69, 8583-8591.

Elsebai, M.F., Kehraus, S., Lindequist, U., Sasse, F., Shaaban, S., Gütschow, M., Josten, M., Sahl, H.-G., König, G.M., 2011. Antimicrobial phenalenone derivatives from the marine-derived fungus *Coniothyrium cereale*. Organic & Biomolecular Chemistry 9, 802-808.

Erdmann, F., Lortet-Tieulent, J., Schüz, J., Zeeb, H., Greinert, R., Breitbart, E.W., Bray, F., 2013. International trends in the incidence of malignant melanoma 1953–2008—are recent generations at higher or lower risk? International Journal of Cancer 132, 385-400.

Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F. (2013) GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; 2013. Available from: http://globocan.iarc.fr, accessed on 9/09/2014.

Glass, N.L. and Donaldson, G.C., 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Applications in Environmental Microbiology 61, 1323-1330.

Gomes, N.M., Bessa, L.J., Buttachon, S., Costa, P.M., Buaruang, J., Dethoup, T., Silva, A., Kijjoa, A., 2014. Antibacterial and Antibiofilm Activities of Tryptoquivalines and Meroditerpenes Isolated from the Marine-Derived Fungi *Neosartorya paulistensis*, *N. laciniosa*, *N. tsunodae*, and the Soil Fungi *N. fischeri* and *N. siamensis*. Marine Drugs 12, 822-839.

Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. Cell 144, 646-674.

Jemal, A., Bray, F., Center, M.M., Ferlay, J., Ward, E., Forman, D., 2011. Global cancer statistics. CA: A Cancer Journal for Clinicians 61, 69-90.

Kanokmedhakul, K., Kanokmedhakul, S., Suwannatrai, R., Soytong, K., Prabpai, S., Kongsaeree, P., 2011. Bioactive meroterpenoids and alkaloids from the fungus *Eurotium chevalieri*. Tetrahedron 67, 5461-5468.

Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E.S., Baehrecke, E.H., Blagosklonny, M.V., El-Deiry, W.S., Golstein, P., Green, D.R., Hengartner, M., Knight, R.A., Kumar, S., Lipton, S.A., Malorni, W., Nunez, G., Peter, M.E., Tschopp, J., Yuan, J., Piacentini, M., Zhivotovsky, B., Melino, G., 2009. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death & Differentiation 16, 3-11.

Lee, D.-S., Jang, J.-H., Ko, W., Kim, K.-S., Sohn, J.H., Kang, M.-S., Ahn, J.S., Kim, Y.-C., Oh, H., 2013. PTP1B inhibitory and anti-inflammatory effects of secondary metabolites isolated from the marine-derived fungus *Penicillium* sp. JF-55. Marine Drugs 11, 1409-1426.

Li, Y.X., Himaya, S.W.A., Dewapriya, P., Zhang, C., Kim, S.K., 2013. Fumigaclavine C from a Marine-Derived Fungus *Aspergillus fumigatus* Induces Apoptosis in MCF-7 Breast Cancer Cells. Marine Drugs 11, 5063-5086.

Min, F.-L., Zhang, H., Li, W.-J., Gao, Q.-X., Zhou, G.-M., 2005. Effect of exogenous wild-type p53 on melanoma cell death pathways induced by irradiation at different linear energy transfer. *In Vitro* Cellular & Developmental Biology-Animal 41, 284-288.

Montaser, R., Luesch, H., 2011. Marine natural products: a new wave of drugs? Future Medicinal Chemistry 3, 1475-1489.

Nematbakhsh, M., Ashrafi, F., Pezeshki, Z., Fatahi, Z., Kianpoor, F., Sanei, M.-H., Talebi, A., 2012. A histopathological study of nephrotoxicity, hepatoxicity or testicular toxicity: Which one is the first observation as side effect of Cisplatin-induced toxicity in animal model? Journal of Nephropathology 1, 190.

Newman, D.J., Cragg, G.M., 2014. Marine-Sourced Anti-Cancer and Cancer Pain Control Agents in Clinical and Late Preclinical Development. Marine Drugs 12, 255-278.

OConnor, P.M., Jackman, J., Bae, I., Myers, T.G., Fan, S.J., Mutoh, M., Scudiero, D.A., Monks, A., Sausville, E.A., Weinstein, J.N., Friend, S., Fornace, A.J., Kohn, K.W., 1997. Characterization of the p53 tumor suppressor pathway in cell lines of the National Cancer Institute anticancer drug screen and correlations with the growth-inhibitory potency of 123 anticancer agents. Cancer Research 57, 4285-4300.

Olive, P.L., Banáth, J.P., 2006. The comet assay: a method to measure DNA damage in individual cells. Nature Protocols 1, 23-29.

Prata-Sena, M., Ramos, A., Castro-Carvalho, B., Dethoup, T., Buttachon, S., Kijjoa, A., & Rocha, E., 2014. Anti-proliferative and pro-apoptotic activities of two marine sponge-derived fungi extracts in HepG2, HCT116 and A375 cancer cell lines. Planta Medica, 80(16), P1N3. doi: 10.1055/s-0034-1394594

Saad, M., Tafani, C., Psimaras, D., Ricard, D., 2014. Chemotherapy-induced peripheral neuropathy in the adult. Current Opinion in Oncology 26, 634-641.

Schmidtko, A., Lotsch, J., Freynhagen, R., Geisslinger, G., 2010. Ziconotide for treatment of severe chronic pain. Lancet 375, 1569-1577.

Shushni, M.A., Singh, R., Mentel, R., Lindequist, U., 2011. Balticolid: A new 12-membered macrolide with antiviral activity from an Ascomycetous fungus of marine origin. Marine Drugs 9, 844-851.

Sodngam, S., Sawadsitang, S., Suwannasai, N., Mongkolthanaruk, W., 2014. Chemical constituents, and their cytotoxicity, of the rare wood decaying fungus *Xylaria humosa*. Natural Product Communications 9, 157-158.

Toné, S., Sugimoto, K., Tanda, K., Suda, T., Uehira, K., Kanouchi, H., Samejima, K., Minatogawa, Y., Earnshaw, W.C., 2007. Three distinct stages of apoptotic nuclear condensation revealed by time-lapse imaging, biochemical and electron microscopy analysis of cell-free apoptosis. Experimental Cell Research 313, 3635-3644.

Vega-Avila, E., Pugsley, M.K., 2011. An overview of colorimetric assay methods used to assess survival or proliferation of mammalian cells, Proceedings of the Western Pharmacology Society, pp. 10-14

Xiong, Z.Q., Wang, J.F., Hao, Y.Y., Wang, Y., 2013. Recent Advances in the Discovery and Development of Marine Microbial Natural Products. Marine Drugs 11, 700-717.

Yaguchi, T., Matsuzawa, T., Tanaka, R., Abliz, P., Hui, Y., Horie, Y., 2010. Two new species of *Neosartorya* isolated from soil in Xinjiang, China. Mycoscience 51, 253-262.

CHAPTER IV

CONCLUSIONS AND FUTURE PERSPECTIVES

4.1. Conclusions and future perspectives

The aim of this study was to assess the *in vitro* anticancer bioactivity of crude ethyl extracts obtained from sponge-derived fungi *Aspergillus similanensis* KUFA 0013 (**E1**), *Neosartorya paulistensis* KUFC 7897 (**E2**), *Talaromyces trachsypermus* KUFC 0021 (**E3**) and sea fan-derived fungi *Neosartorya siamensis* KUFA 0017 (**E4**), as well as of the nine compounds – of which eight alkaloids and one terpenoid – isolated from the latter fungus.

The crude ethyl extracts of marine-derived fungi (**E1**, **E2**, **E3** and **E4**) were tested on a panel of seven human cancer cell lines, in particular, non-small-cell lung carcinoma (A549), hepatocellular carcinoma (HepG2), glioblastoma (U251), breast adenocarcinoma (MCF-7), colorectal carcinoma (HT29, HCT116), and malignant melanoma (A375) cells. Both extracts *Neosartorya paulistensis* KUFC 7897 (**E2**) and *Neosartorya siamensis* KUFA 0017 (**E4**) presented *in vitro* anticancer activity by decreasing cell proliferation, in most cases exhibiting an inhibition of long-term cell proliferation and induction of cell death in melanoma, hepatocellular carcinoma and colon carcinoma.

In an attempt to identify the compounds responsible for the anticancer effect observed in the extract of *Neosartorya siamensis* KUFA 0017 (**E4**), nine compounds were isolated, namely, 2,4-dihydroxy-3-methylacetophenone (**C1**), nortryptoquivaline (**C2**), chevalone C (**C3**), tryptoquivaline H (**C4**), fiscalin A (**C5**), *epi*-fiscalin C (**C6**), *epi*-neofiscalin A (**C7**) and *epi*-fiscalin A (**C8**), and tryptoquivaline F (**C9**). Although these compounds are not novel and have been isolated from other fungi of terrestrial origin (Buttachon et al., 2012; Sodngam, Sawadsitang, Suwannasai, & Mongkolthanaruk, 2014) and assessed for anticancer activity in selected cancer cell lines, it is the first time that these compounds were screened for anticancer activity in colon, liver and skin cancer cell lines, including analysis of cell death induction and assessment of genotoxicity.

Results demonstrated that the great majority of the compounds, in particular, **C2**, **C3**, **C5**, **C6**, **C7** and **C8**, displayed a significant anti-proliferative activity against the selected cell lines, with IC_{50} values that ranged from 24 to 153 μM, indicating that these compounds possess a more enhanced cytotoxic effect in comparison to extract **E4**. Additionally, compounds **C2**, **C5**, **C7** and **C8** were able to induce cell death in hepatocellular carcinoma cells, whereas compounds **C2**, **C3** and **C5** were able to induce cell death in colon carcinoma cell lines. None of these compounds were observed to act by the induction of DNA strand breaks and alkali-labile sites. In parallel, none of the compounds managed to induce significant cell death in malignant melanoma, in spite of

the strong anti-proliferative effect of compounds **C2** and **C3**, observed by the MTT assay. The anti-proliferative effect may possibly not be related to DNA damage associated to DNA strand breaks, as these were not observed by comet assay. It is thus possible to suggest that the anti-proliferative activity of these compounds in A375 cell line is linked to inhibition of proliferative pathways and not to a pro-cell death stimulus. The overall results obtained screening of anticancer activity of these nine compounds were in accordance to those obtained with the extract, thus it is plausible to conclude that these may be some of the main compounds acting in the anticancer activity of the extract **E4**.

Future perspectives regarding this work should further approach the compounds isolated as a result of the *in vitro* anticancer screening, with particular emphasis on reaffirming some of the results obtained with a higher degree of detail, as well as pinpointing molecular targets and signal transduction pathways.

It would be of particular interest to further fundament that the induction of cell death is occurring by apoptosis, as well as distinguishing the pathways and gene interactions that are occurring, with consequent analysis of DNA fragmentation, mitochondrial membrane potential, cytochrome *c* release, reactive oxygen species (ROS) generation, alongside the expression of genes that are highly relevant in apoptosis, such as *TP53*, *Bax* and *Bcl-2*, as well as the expression of initiator and effector caspases and proteins involved on DNA repair. The analysis of morphological alterations in the cells by electron microscopy may also be evaluated. The analysis of whether the extracts and compounds' anti-proliferative activity may be dependent on cell cycle arrest should also be evaluated. In spite of the compounds absence of induction of DNA strand breaks and alkali-labile sites, other types of DNA damage, such as oxidative and alkylating damage, may be observed in order to further conclude about the compound's overall genotoxicity.

Considering the results given in Chapter II, that may be considered as very promising, there is high relevance in the pursuit of isolating compounds from *Neosartorya paulistensis* KUFC 7897 (**E2**), with successive screening for anticancer activity and for its underlying mechanisms.

Further studies concerning potential synergistic effects from the combination of isolated compounds with commonly used chemotherapeutic drugs seem also relevant to carry out. In fact, such approaches have been initiated by our work group, and have so far achieved positive and promising results.

APPENDIX

EXPERIMENTAL PROTOCOLS

P1. Cell culture

Principle

The culture of animal cells is based on the maintenance *in vitro* of disaggregated cells, which may have normal (non-neoplastic) or neoplastic origin. Neoplastic cell lines are originally extracted from tumours and are then grown *in vitro* with the addition of appropriate media and other additives to ensure proliferation. The control of other conditions, such as temperature, pH, CO_2 and O_2 tension are essential to maintain durable cultures. Cell lines may be maintained for extended passages, provided that they are appropriately sub-cultured when reaching confluence and that adequate physiological and physiochemical conditions are verified at all times. When cell lines are stabilized and growth of successive passages is ensured, cells may then be used in the desired experimental assays, this process is initiated by collecting cells and analyzing cell density and viability prior to seeding in culture plates (Freshney 2010; Cree 2011).

References

Cree, I. A. (2011). Principles of Cancer Cell Culture, *Cancer Cell Culture*, Vol. 731, pp. 13-26.

Freshney, R. I. (2010) Subculture and Cell Lines. *Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications*, 6th Edition, John Wiley & Sons, Inc., Hoboken, NJ, USA. doi: 10.1002/9780470649367.ch12.

Materials	Assay solutions and reagents
T25 cell culture flasks	PBS (Phosphate Buffered Saline) buffer
Humidified, controlled temperature/CO_2 incubator	Cell culture medium (depending on cell line, e.g. DMEM, RPMI, MEM, etc.)
Laminar flow hood	Fetal Bovine Serum (FBS)
Inverted phase contrast microscope	Antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin)
Neubauer chamber	Sodium pyruvate
	Sodium bicarbonate
	HEPES
	0.25% Trypsin/EDTA solution

Assay solutions

Prepare the following assay solutions:

(1) PBS buffer (1X)

	Final concentration	Weight
NaCl	137 mM	8 g
KCl	2.7 mM	0.2 g
Na_2PO_4	10 mM	1.44 g
KH_2PO_4	2 mM	0.24 g

Dissolve the reagents above in 800 ml of ultrapure H_2O. Adjust pH to 7.4. Add necessary H_2O for a final volume of 1 L. Autoclave the solution and store at 4ºC.

(2) Cell culture medium (MEM) [1] [2]

	Final concentration	Weight / Volume
Minimum Essential Medium Eagle's (MEM)	9.6 g/L	4.8 g
Sodium bicarbonate	2.2 g/L	1.1 g
Sodium pyruvate	110 mg/L	55 mg
HEPES	10 mM	1.192 g
FBS	10%	50 ml
Penicillin / Streptomycin	1%	5 ml
Ultrapure H_2O	-	445 ml

Weight the reagents and add into an appropriate glass container. Add ultrapure H_2O to a final volume of 445 ml and mix until completely dissolved. Inside the laminar flow hood, sterilize the

[1] Only applicable for MEM cell culture medium. Each medium has a different final composition. FBS and antibiotics % are common for all mediums.
[2] Pre-prepared medium is available from several manufacturers. In this case, only FBS and antibiotics need to be added.

solution by filtering using a filter with a 0.2 µM pore. Add the FBS and antibiotics. Store tightly sealed at 4ºC. It is recommendable to aliquot the medium into 50 ml tubes in order to preserve the stock solution. Be sure to keep one aliquot for each respective cell line. Observe the medium periodically in order to avoid contamination.

GENERAL GUIDELINES

Cell maintenance [3]

Starting a new culture

1. To start a new culture of cells, thaw an aliquot of frozen cells by mixing with appropriate cell culture medium. This process must be fast in order to avoid cytoxicity from the DMSO used in the freezing medium.
2. Pipette the cells into a sterile cell culture flask.
3. Add 4 ml of warm medium into the flask. Gently shake the flask to ensure the cell solution covers the entire bottom of the flask.
4. Incubate at 37ºC and 5% CO_2 in a humidified incubator. Cells will have adhered within 24 h.
5. It is preferable to change the medium the next day in order to remove all traces of DMSO in the culture.
6. The medium must be changed every two days, and cells should be trypsinized at least once a week, when reaching confluence.

Changing medium

1. The medium must be changed every two days. Prior to manipulation, always check the culture for confluence and contamination under an inverted microscope.
2. In a water bath (ensure that the water is not contaminated), pre-heat PBS and appropriate cell culture medium to 37ºC.
3. Remove the medium from the flask; be careful not to scrape the bottom of the flask.

[3] Cell manipulation must always be done under sterile conditions. Process all steps under a laminar flow hood and use aseptic manipulation techniques.

4. Wash with 1 ml of PBS, this step may be done twice if desired or if there are plenty of cell debris.
5. Remove the PBS and add 5 ml of cell culture medium.
6. Incubate at 37ºC and 5% CO_2 in a humidified incubator.

Trypsinization (Sub-culturing)

1. When the cell culture has reached a confluence of about 80 % or more, the cells must be trypsinized.
2. Pre-heat a trypsin solution in a water bath at 37ºC. Both medium and PBS can be left at room temperature.
3. Carefully remove the medium from the flask. Wash twice with 1 ml PBS and remove the PBS.
4. Add 1 ml of trypsin solution and leave to incubate at 37ºC for 5-7 min.
5. Observe under a microscope to ensure that cells have detached and are fairly loose from each other.
6. Add 4 ml of medium and resuspend with care.
7. Remove a given amount of cell solution (depending on the growth speed of the cell line and desired confluence date), e.g. sub-cultivation ratio of 1:8 (600 µl into a new flask).
8. Add medium up to 5 ml.
9. Incubate at 37ºC and 5% CO_2 in a humidified incubator.

P2. MTT colorimetric assay

Principle

The MTT colorimetric assay is used to assess cell proliferation and viability. This assay is based on the reduction of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT), a yellow water-soluble substrate, into non-soluble purple formazan crystal by the enzymatic activity of the cell's mitochondria (Vega-Avila & Pugsley 2011). Subsequent analysis by reading optical density of each sample translates the cellular metabolic activity, which can be used to directly and proportionally infer the total number of live cells in a given sample (Van Merloo et al. 2011). The data obtained from this assay can be used to perform the calculation of the IC_{50}, which corresponds to the concentration of a given compound that decreases the number of viable cells by 50% (Dias 2013).

References

Dias, T. A., Duarte, C. L., Lima, C. F., Proença, M. F., Pereira-Wilson, C. (2013). Superior anticancer activity of halogenated chalcones and flavonols over the natural flavonol quercetin. *Eur J Med Chem*, 65, 500-510

Van Meerloo, J., Kaspers, G. J., & Cloos, J. (2011). Cell sensitivity assays: the MTT assay. *Cancer Cell Culture*, pp. 237-245: Springer.

Vega-Avila, E., & Pugsley, M. K. (2011). An overview of colorimetric assay methods used to assess survival or proliferation of mammalian cells. Paper presented at the *Proc West Pharmacol Soc*.

Materials	Assay solutions and reagents
96-multiwell culture plates	PBS (Phosphate Buffered Saline*)* buffer
Microplate reader	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) stock solution
	DMSO (dimethyl sulfoxide):ethanol (1:1) solution

Assay solutions

Prepare the following assay solutions:

(1) PBS buffer (1X)

	Final concentration	Weight
NaCl	137 mM	8 g
KCl	2.7 mM	0.2 g
Na_2PO_4	10 mM	1.44 g
KH_2PO_4	2 mM	0.24 g

Dissolve the reagents above in 800 ml of ultrapure H_2O. Adjust pH to 7.4. Add necessary H_2O for a final volume of 1 L. Autoclave the solution and store at 4ºC.

(2) MTT stock solution [4]

	Final concentration	Weight
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	5 mg/ml	50 mg

Add 50 mg of MTT and add PBS (1X) to a final volume of 10 ml. Dissolve the solution in an ultrasound bath, remember to avoid overheating. Store at -20ºC.

(3) DMSO:ethanol (1:1) [5]

Add 250 ml of dimethyl sulfoxide (DMSO) and 250 ml of pure ethanol (100%, v/v). Beware of DMSO toxicity, manipulate with care in a fume hood. Store at room temperature and protected from light.

PROTOCOL

Cell preparation [6]

[4] MTT is carcinogenic, handle with care.
[5] DMSO is toxic, beware of any vapours and handle with care. Use rubber or nitrile gloves for extended manipulation.

1. In sterile conditions, incubate 100 µl ($0.1x10^6$ cells/well) of cell suspension in a 96-multiwell culture plate and allow cells to adhere for 24 h in a humidified incubator at 37ºC and 5% CO_2.
2. Treat cells with the desired experimental design. Add 10 µl of MTT solution (0.5 µg/ml) to the initial control cells (t = 0).
3. Incubate in a humidified incubator at 37ºC and 5% CO_2 for 2 hours.
4. Remove the medium of the control cells (t = 0 h) gently, in order to avoid losing the formazan crystals. If there are crystals in suspension, the medium must be collected into a tube and centrifuged.
5. Incubate in a humidified incubator at 37ºC and 5% CO_2 for the remaining 46 hours (for a 48 h assay, depending on cell and treatment type).
6. Add 10 µl of MTT solution (0.5 µg/ml) to the remaining cells, both final control (t = 48 h) and treated cells.
7. Incubate in a humidified incubator at 37ºC and 5% CO_2 for 2 hours.
8. Remove the medium of all cells, as before (point 2).
9. Add 150 µl of DMSO:ethanol solvent to dissolve the formazan crystals.
10. Place the culture plate in a plate shaker and let shake slowly for 10 – 20 min to dissolve the crystals. This process must be undergone while protecting the plate from exposure to light.
11. Once the crystals are completely dissolved, measure the absorbance in a microplate reader at 570 nm.

Calculations

The absorbance (A) values obtained for both the initial control (t = 0 h) and final control (t = 48 h) allow the calculation of the percentage of cell proliferation, by using the following equation:

$$\text{Cell proliferation} = (A_{\text{sample}} - A_{\text{initial control}} / A_{\text{final control}} - A_{\text{initial control}})$$

[6] This protocol must be processed under sterile conditions.

P3. Comet assay

Principle

The single cell gel electrophoresis assay, also known as comet assay, is a method developed for measuring the level of DNA damage in single cells (Olive & Banáth 2006). When submitted to an electric field, loops and fragments of DNA migrate through an agarose gel, subsequently DNA migration is directly proportional to DNA damage. The alkaline version (pH > 13) of this assay allows the specific detection of alkali-labile sites and DNA strand breaks (Collins 2006). The observation of results relies on DNA staining with a fluorochrome such as DAPI and observation under a fluorescence microscope. Appropriate software for imaging analysis, such as CometScore®, allows the scoring for percentage of DNA damage and the percentage of DNA in the comet tail.

References

Collins, A. R. (2004). The comet assay for DNA damage and repair. *Mol Biotech, 26*(3), 249-261.

Olive, P. L., & Banáth, J. P. (2006). The comet assay: a method to measure DNA damage in individual cells. *Nat Prot, 1*(1), 23-29.

Materials	Assay solutions and reagents
Horizontal electrophoresis tank	PBS (Phosphate Buffered Saline) buffer
Electric power supply	0.25% Trypsin/EDTA solution
Fluorescence microscope	Lysis solution, pH 10
	Electrophoresis Buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13)
	1% Normal Melting Point Agarose (w/v)
	0.5% Low Melting Point Agarose (w/v)
	DAPI staining solution (1 µg/ml)

Assay solutions

Prepare the following solutions:

(1) PBS buffer (1X)

	Final concentration	Weight
NaCl	137 mM	8 g
KCl	2.7 mM	0.2 g
Na_2PO_4	10 mM	1.44 g
KH_2PO_4	2 mM	0.24 g

Dissolve the reagents above in 800 ml of ultrapure H_2O. Adjust pH to 7.4. Add necessary H_2O for a final volume of 1 L. Autoclave the solution and store at 4ºC.

(2) Lysis solution, pH 10

	Final concentration	Weight
NaCl	2.5 M	146.1 g
Na_2EDTA	100 mM	37.22 g
Tris Base	10 mM	1.211 g
NaOH to pH of 10		Approx. 7 g

Add the reagents and ultrapure H_2O to a final volume of 990 ml. Store at 4ºC. Prior to use, immediately add 1% Triton X-100 (v/v).

(3) Electrophoresis Buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13)

	Final concentration	Weight
NaOH	3 M	120 g

Add NaOH and distilled water to a final volume of 1 L. Store at room temperature or 4ºC.

Na_2EDTA	200 mM	3.722 g

Add Na_2EDTA and distilled water to a final volume of 50 ml. Store at room temperature or 4ºC.

The electrophoresis buffer must be prepared immediately before use. To prepare 1 L of solution, mix 895 ml of distilled water, 5 ml of 200 mM Na_2EDTA and 100 ml of 3 M NaOH. Recommended pH is ≥13. It is advisable to use this solution only once per set of gels run.

(4) 1% Normal Melting Point Agarose (w/v)

Dissolve 200 mg of normal melting point (NMP) agarose in 20 ml of distilled water. Heat the solution for 1 – 2 min in a microwave (full power) until completely dissolved. Store at 4ºC and when required, melt prior to use.

(5) 0.5% Low Melting Point Agarose (w/v)

Dissolve 100 mg of low melting point (LMP) agarose in 20 ml of PBS. Heat the solution for 1 – 2 min in a microwave (full power) until completely dissolved. Aliquot 2 ml of the solution into heat-resistant cryotubes and store at 4ºC. Prior to use, melt and maintain at 37ºC in a water bath.

(6) DAPI staining solution (1 µg/ml) [7]

Prepare a working solution of 1 µg/ml from a stock solution of 100 µg/ml. Dilute in distilled water. Store at -20ºC, protected from light.

PROTOCOL

Slide preparation

Label conventional microscope slides, preferably in pencil, with the appropriate assay information on the glazed area. Melt 1% NMP agarose (w/v) and dip conventional microscope slides in this solution. Allow the slides to dry horizontally at room temperature overnight; remember to keep the glazed side upwards. Prepared slides may be stored for a few days before use.

Cell preparation [8]

1. In sterile conditions, incubate 1000 µl ($0.1x10^6$ cells/well) of cell suspension in a 24-multiwell culture plate and allow cells to adhere for 24 h in a humidified incubator at 37ºC and 5% CO_2.
2. Treat cells with the desired experimental design.

[7] DAPI is carcinogenic, handle with care.
[8] This protocol must be processed under sterile conditions up to step 3.

3. Incubate in a humidified incubator at 37ºC and 5% CO_2 for 48 hours (depending on your experimental design).
4. Wash cells with warm PBS, remove the PBS and add 150 µl of warm trypsin for 3 – 5 minutes.
5. Stop the action of the tryspin by adding 400 µl of culture medium. Resuspend the cells with care. Place the plate on ice.
6. Collect approximately 50.000 cells per well into marked tubes, maintain the tubes on ice.

Comet assay

7. Centrifuge the cells for 1 min at 5000 rpm.
8. Remove the supernatant and gently resuspend cells in 100 µl of LMP agarose; the agarose must be no warmer than 37ºC, in order to preserve cell integrity.
9. On the NMP side of a pre-coated and labeled microscope slide, apply a 75 µl drop of the cell suspension/LMP agarose mixture.
10. Immediately cover the drop with a 22 x 22 mm coverslip and place the slides at 4ºC.
11. Prepare the lysis solution, add Triton X-100.
12. Remove the coverslips with care and place slides in an adequate slide container. Add the lysis solution until the slides are fully immersed.
13. Incubate at 4ºC for at least 2 h, protected from light.
14. Prepare the electrophoresis buffer and use it to appropriately fill the horizontal electrophoresis tank. Be sure to keep the tank leveled. Maintain the tank at 4ºC.
15. Remove slides from the lysis solution and carefully rinse the slides in distilled water.
16. Transfer the slides into the horizontal electrophoresis tank. Set the labeled side towards the anode. Proceed with care in order to avoid losing the agarose gel from the slides, as it can easily peel off.
17. To allow the DNA to unwind, incubate the slides in the electrophoresis buffer for 40 min at 4ºC.
18. Set the power supply to 20 V (1 V/cm) and turn it on. Run the electrophoresis for 20 minutes at 4ºC.
19. Remove the slides and immerse in distilled water for 5 minutes, washing twice more for 5 minutes.

20. Dehydrate the slides twice in 100% ethanol for 5 minutes.
21. Leave the slides to air dry. When ready for analysis, apply 20 µl of working DAPI staining solution to each sample and apply a coverslip. From this point on, keep slides away from light.
22. The samples are now ready to evaluate under a fluorescence microscope. A minimum of 4 independent experiments is recommended in order to produce feasible results.

Evaluation of DNA damage

The samples may now be observed under a fluorescence microscope, and resulting images analysed by a quantitative method, by the use of appropriate image analysis software (e.g. CometScore®), Software analysis expresses results in various forms, namely % of DNA in tail, tail length and tail moment. In alternative, the comets may be assessed semiquantitatively by manual scoring, refer to Collins *et al* (1995) for a thorough description. Consider the guidelines below when processing your samples.

1. The samples should be analysed by one person only.
2. Cells should be observed by using a 20x or 40x objective.
3. It is advisable to score at least 100 cells per sample.
4. Base your analysis on the scanning of all fields of the sample (see point 5 and 6). Take care not to repeat the same areas, to avoid this, analyse your sample from top to bottom.
5. Be careful not to analyse cells around the edges of the gel, as these may often appear to have high levels of damage that do not result from treatment induction.
6. If the comets seem to have a non-horizontal orientation, there has probably been a problem during the electrophoresis. Keep the tank leveled and the slides as horizontal as possible while running the gel. Be sure to repeat the procedure for these samples.

P4. Nuclear condensation assay

Principle

Cell death by apoptosis is characterized by a series of morphological features such as cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation (Kroemer et al. 2009). In normal conditions, chromatin is loosely packaged in the nucleus. In the event of mitosis, chromatin is condensed and organized into chromosomes. However, during the induction of apoptosis, chromatin is heavily condensed alongside nuclear proteins and forms the hallmark apoptotic bodies. This assay comprises the staining of cell nuclei with DAPI (4',6-diamidino-2-phenylindole), a fluorochrome which binds to the A-T regions of the DNA, and allows the observation of nuclear chromatin condensation (Toné et al. 2007).

References

Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E. S., Baehrecke, E. H., . . . Melino, G. (2009). Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death Differ, 16*(1), 3-11. doi: Doi 10.1038/Cdd.2008.150

Toné, S., Sugimoto, K., Tanda, K., Suda, T., Uehira, K., Kanouchi, H., et al. (2007). Three distinct stages of apoptotic nuclear condensation revealed by time-lapse imaging, biochemical and electron microscopy analysis of cell-free apoptosis. *Exp Cell Res, 313*(16), 3635-3644.

Materials	Assay solutions and reagents
24-multiwell culture plates	PBS (Phosphate Buffered Saline*)* buffer
Poly-L-Lysine microscope slides	0.25% Trypsin/EDTA solution
Hydrophobic barrier pen	4% Paraformaldehyde (PFA) (w/v)
Centrifuge	DAPI staining solution (1 µg/ml)
Cytocentrifuge filters	50% Glycerol solution
Cytocentrifuge	
Fluorescence microscope	

Assay solutions

Prepare the following solutions:

(1) PBS buffer (1X)

	Final concentration	Weight
NaCl	137 mM	8 g
KCl	2.7 mM	0.2 g
Na_2PO_4	10 mM	1.44 g
KH_2PO_4	2 mM	0.24 g

Dissolve the reagents above in 800 ml of ultrapure H_2O. Adjust pH to 7.4. Add necessary H_2O for a final volume of 1 L. Autoclave the solution and store at 4ºC.

(2) 4% Paraformaldehyde (w/v) [9]

	Final concentration	Weight
Paraformaldehyde (PFA)	4%	4 g

Add 4 g of PFA and PBS up to a final volume of 100 ml. Place the solution in a 60ºC water bath. Beware of any vapours, keep the lid tightly closed. Stir the solution periodically. When completely dissolved, let the solution cool down and adjust the pH to 7.4. Aliquot as desired and store at -20ºC.

(3) DAPI staining solution (1 µg/ml) [10]

Prepare a working solution of 1 µg/ml from a stock solution of 100 µg/ml. Dilute in distilled water. Store at -20ºC, protected from light.

(4) 50 % Glycerol solution

Add 5 ml of pure glycerol and 5 ml of PBS (1X). The solution must be vortexed thoroughly, as it is difficult to dissolve glycerol. Store at 4ºC.

[9] PFA solution is toxic, beware of vapours and handle with care.
[10] DAPI is carcinogenic, handle with care.

PROTOCOL

Cell preparation [11]

1. In sterile conditions, incubate 1000 µl ($0.1x10^6$ cells/well) of cell suspension in a 24-multiwell culture plate and allow cells to adhere for 24 h in a humidified incubator at 37ºC and 5% CO_2.
2. Treat cells with the desired experimental design.
3. Incubate in a humidified incubator at 37ºC and 5% CO_2 for 48 hours (depending on your experimental design).
4. Collect the medium into a centrifuge tube labeled with the sample number. Wash cells with 1 ml warm PBS; recover the PBS into the respective tube. Add 150 µl of warm trypsin to the cells for 3 – 5 minutes.
5. Stop the action of the tryspin by adding 400 µl of culture medium. Resuspend the cells with care.
6. Recover the medium and cells into the tube. Wash the wells with 1 ml PBS and recover into the respective tube.
7. Centrifuge the samples at 2000 rpm for 10 min.
8. Discard all the supernatant until 500 µl and resuspend the pellet with 3 ml of PBS.
9. Centrifuge the samples once more at 2000 rpm for 10 min.
10. Discard all the supernatant until 500 µl and resuspend the pellet with 2 ml of PFA.
11. Incubate at 37ºC for 15 – 20 min.
12. Discard all the supernatant until 500 µl and resuspend the pellet with 4 ml of PBS.
13. Centrifuge the samples at 2000 rpm for 10 min.
14. Discard all the supernatant until 500 µl, collect this supernatant into labeled tubes and store at 4ºC. The samples can be stored at 4ºC for a few months.
15. Label poly-L-Lysine microscope slides accordingly. Prepare filters and mount the slides onto the cytocentrifuge frames. Pipette 80 µl (depending on cell density) of cell sample into the respective cytocentrifuge tube. Each slide can fit two samples, one on each side. Run the cytocentrifuge at 500 rpm for 5 min.

[11] This protocol must be processed under sterile conditions up to step 3.

16. Unmount the frames and leave the slides to air dry.
17. Circle the samples with a hydrophobic barrier pen.
18. Wash the slides three times with PBS, for 5 min each turn.
19. Incubate each sample with 20 µl of DAPI staining working solution for 10 min in the dark.
20. Add a 6 µl drop of 50 % glycerol solution to each sample and cover with a 22 x 22 mm coverslip. Store the samples at -20ºC, protected from light.

Analysis of nuclear condensation

Observe the samples under a fluorescence microscope. Observe at least 300 cells per sample, in at least 3 different areas. Count both the total number of cells and the number of cells presenting condensed nuclei. The percentage of cells with condensed nuclei is determined according to the ratio between total number of cells presenting condensed nuclei and the total number of cells.

P5. Clonogenic assay

Principle

The clonogenic assay is a simple and effective cell survival assay by which it is possible to determine the long-term ability of a single cell to form a colony (Munshi et al. 2005). This assay is mainly used to assess the whether a chemical agent presents cytostatic activity, thus definitively arresting indefinite proliferation of cells by reducing reproductive viability (ability of a single cell to form a colony of 50 or more cells) in treated cells in relation to untreated cells (control) (Franken et al. 2006).

References

Franken, N. A., Rodermond, H. M., Stap, J., Haveman, J., & Van Bree, C. (2006). Clonogenic assay of cells *in vitro*. *Nat Prot, 1*(5), 2315-2319.

Haloom, R., Christian, O., George T, G., Katherine, V., Assam, E-O., & Tom C, K. (2011). Clonogenic Assay: Adherent Cells. *J Vis Exp* (49).

Munshi, A., Hobbs, M., & Meyn, R. E. (2005). Clonogenic cell survival assay. *Chemosensitivity* (pp. 21-28): Springer.

Materials	Assay solutions and reagents
12 and 24-multiwell culture plates	PBS (Phosphate Buffered Saline*)* buffer
Stereomicroscope	0.25% Trypsin/EDTA solution
Laminar flow hood	4% Paraformaldehyde (PFA) (w/v)
	0.05% Crystal violet (w/v)

Assay solutions

Prepare the following solutions:

(1) PBS buffer (1X)

	Final concentration	Weight
NaCl	137 mM	8 g
KCl	2.7 mM	0.2 g
Na_2PO_4	10 mM	1.44 g
KH_2PO_4	2 mM	0.24 g

Dissolve the reagents above in 800 ml of ultrapure H_2O. Adjust pH to 7.4. Add necessary H_2O for a final volume of 1 L. Autoclave the solution and store at 4ºC.

(2) 4% Paraformaldehyde (w/v) [12]

	Final concentration	Weight
Paraformaldehyde (PFA)	4%	4 g

Add 4 g of PFA and add PBS up to a final volume of 100 ml. Place the solution in a 60ºC water bath. Beware of any vapours, keep the lid tightly closed. Stir the solution periodically. When completely dissolved, let the solution cool down and adjust the pH to 7.4. Aliquot as desired and store at -20ºC.

[12] PFA solution is toxic, beware of vapours and handle with care.

(3) 0.05% Crystal violet (w/v)

	Final concentration	Weight
Crystal violet	0.05%	5 mg

Add 5 mg of crystal violet and PBS up to a final volume of 100 ml. Mix thoroughly until completely dissolved. Store at room temperature, protected from light.

PROTOCOL

Cell preparation [13]

1. In sterile conditions, incubate 1000 µl ($0.1x10^6$ cells/well) of cell suspension in a 24-multiwell culture plate and allow cells to adhere for 24 h in a humidified incubator at 37ºC and 5% CO_2.
2. Treat cells with the desired experimental design.
3. Incubate in a humidified incubator at 37ºC and 5% CO_2 for 48 hours (depending on your experimental design).
4. Wash cells with warm PBS. Add 150 µl of warm trypsin to the cells for 3 – 5 minutes.
5. Stop the action of the tryspin by adding 400 µl of culture medium. Resuspend the cells with care.
6. Prepare serial dilutions for a final dilution of 200 cells in 1 ml of culture medium. Be sure to resuspend thoroughly.
7. Plate 1 ml with 200 cells per well in 12-multiwell culture plates.
8. Incubate in a humidified incubator at 37ºC and 5% CO_2 for 10 days.
9. After 10 days of culture, remove the medium and wash with 2 ml of warm PBS.
10. Fix the cells *in situ* with 2 ml of 4% PFA. Incubate for 15 min at 37ºC.

[13] This protocol must be processed under sterile conditions up to step 9.

11. Remove the PFA and wash with 2 ml of warm PBS. Remove the PBS.
12. Incubate with 2 ml 0.05% crystal violet for 30 min at room temperature.
13. Wash with distilled water, remove the water and leave to air dry.
14. Count colonies with more than 50 individual cells using a stereomicroscope.

Calculations

The plating efficiency (PE) is calculated from the ratio between the number of colonies (> 50 cells) counted and the number of cells plated. Subsequently, the surviving fraction (relative to control) is calculated by using the following equation:

$$\% \text{ Surviving fraction (SF)} = (PE_{\text{treated cells}} / PE_{\text{control}}) \times 100$$