CATARINA VIZETTO GUERREIRO DUARTE

BIOMEDICAL PROPERTIES OF *CYSTOSEIRA* SPECIES: INSIGHTS INTO NUTRA- AND PHARMACEUTICAL APPLICATIONS

2016

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BIOMEDICAL PROPERTIES OF *CYSTOSEIRA* SPECIES: INSIGHTS INTO NUTRA- AND PHARMACEUTICAL APPLICATIONS

PhD in Biological Sciences

Specialty in Cellular and Molecular Biology

Thesis supervised by

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UNIVERSIDADE DO ALGARVE

FACULDADE DE CIÊNCIAS E TECNOLOGIA

2016

BIOMEDICAL PROPERTIES OF *CYSTOSEIRA* SPECIES:

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Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam na listagem de referências incluída.

Catarina Vizetto Duarte

This work was funded by the Portuguese Foundation for Science and Technology (FCT) through the PhD fellowship (Grant SFRH/BD/81425/2011).

ACKNOWLEDGMENTS

Pela primeira vez sentimo-nos desassossegados. As palavras ganham um novo sentido quando não se tornam previsíveis; ganham nova vida quando propõem olhares e despertam perplexidades, quando inquietam o pensamento. E tornam-se sempre insuficientes quando com elas queremos dizer o que nos vai para lá da alma. Como agora.

Esta Tese resulta não apenas de extensas horas de estudo, reflexão e trabalho durante as diversas etapas que a constituem. É igualmente o culminar de um objetivo académico a que me propus e que não seria possível sem a ajuda de um vasto número de pessoas.

Estou especialmente agradecida ao Prof. Dr. João Varela, orientador principal desta tese, em primeiro lugar por me ter acolhido no grupo MarBiotech e por me ter dado a possibilidade de realizar o presente trabalho. Mas o meu agradecimento vai para muito além disso. Quero agradecer por ser uma pessoa que realmente se preocupa com os alunos, não só a nível académico como a nível pessoal. Toda a sua ajuda, motivação e apoio na superação dos diversos obstáculos foram imprescindíveis na realização deste trabalho. Muito obrigada!

Estou também agradecida aos meus orientadores Prof.ª Dra. Amélia Pilar Rauter e Prof. Dr. Fernando Albericio por me terem acolhido nos respetivos grupos de trabalho e me terem proporcionado a oportunidade, as condições necessárias para a elaboração deste trabalho e por terem tempo para se reunirem comigo e estarem interessados em discutir e orientar o trabalho. À vossa hábil direção, instrução e apoio, o meu muito obrigado.

Quero igualmente agradecer à Dra. Luísa Custódio e à Prof.ª Dra. Luísa Barreira que, não sendo minhas orientadoras "no papel", foram exatamente isso na prática sempre discutindo ideias e possibilidades, corrigindo artigos e debatendo resultados e perspectivas futuras. Obrigado por sempre me terem apoiado, muito para além das obrigações profissionais. O vosso apoio, críticas e sugestões foram indispensáveis na construção deste trabalho.

Cheguei ao grupo MarBiotech no CCMAR, tendo acabado de concluir a minha tese de mestrado no Centro de Patogénese Molecular, no iMed – FFUL ("*Reactivity of human fetal and adult immortalized hepatocytes to potentially toxic bilirubin and bile acid species*"). Senti como se tivesse sido recebida de braços abertos numa família. Literalmente. Tendo relativamente longe a minha própria família, eles fizeram esse papel, sendo acarinhada e chamada à atenção pelo Professor e Professora como se estivessem no papel dos meus pais e pela Luísa sempre sábia, resolvida e prática como uma irmã mais velha, com a máxima "não existem problemas, só existem soluções". E assim começou uma bolsa de técnica de investigação (BTI) no projeto SEABIOMED onde obtivemos os resultados preliminares necessários para surgir a ideia de uma tese de doutoramento, a qual ganhei no segundo ano que concorri. No primeiro ano que concorri não ganhei; trabalhei durante esse ano para construir um currículo melhor e voltei a concorrer no ano seguinte depois duma insistência feroz por parte do Hugo Pereira e Luísa Custódio. Eles não me deixaram desistir quando eu estava com receio de voltar a concorrer e "passar a vergonha" de não ganhar outra vez. No final, fiquei em 3º lugar a nível nacional quando concorri nesse ano graças à vossa insistência e, portanto, muito obrigada!!

Pouco tempo depois juntou-se ao nosso grupo também com bolsa de doutoramento a Carolina Bruno de Sousa, companheira neste processo todo, desde a colheita das algas (chegámos várias vezes a fazer colheitas às 6 da manhã com a água gelada por ser quando a maré estava mais baixa), até vários dias de escrita em casa da Carolina onde ela me acolheu durante várias noites de trabalho. Obrigada por todo o companheirismo e apoio!

Agradeço também aos colegas do grupo MarBiotech, Hugo Pereira, Dr. Katkam, Maria Rodrigues, Catarina Pereira, Ivo e Eunice, Cristina Polo, Eva Resek, Vlad Enache, Luís, Marta, Peter, Tamára, Mariana Cardoso e todos os estudantes que passaram pelo laboratório do MarBiotech. Obrigada pela boa disposição e sentido de entreajuda que fazem do nosso local de trabalho um lugar onde existe prazer em estar. Gostava de deixar aqui uma lembrança especial ao Fernando Soares, um estudante impecável que infelizmente faleceu poucos anos depois de ter realizado a tese de mestrado connosco.

Aos restantes grupos e colegas do CCMAR gostaria de agradecer a forma acolhedora como me receberam e como sempre ajudaram quando necessário, fosse algum reagente, equipamento ou mesmo espaço de bancada! Obrigada também aos grupos do CBMR pelas mesmas razões, havendo sempre um espírito de entreajuda na Universidade do Algarve, e obrigada em particular ao Prof. Nuno Santos por nos permitir realizar as análises de *Western blot* no seu laboratório e pela constante disponibilidade.

Quero igualmente agradecer aos que contribuíram directamente na identificação e amostragem das algas nomeadamente Javier Cremades Ugarte, Aschwin Engelen, Ricardo Bermejo, Rita Jacinto, Mafalda Tavares e Tânia Pereira.

Ao grupo "PharmaMar" e "Combinatorial chemistry for the discovery of new compounds" do IRB – Institute for Research in Biomedicine (Barcelona), em particular ao Gerardo Acosta, José Pastor, Miriam Royo, Carolina, Daniel Pulido do laboratório de Rodolfo Lavilla (Unitat de química combinatória - Laboratori biosyner), Marta Paradís (ANIMOOOO!), Belen, Iván Ramos, Jesús Herraiz, Júlia Pindado e às pessoas que se tornaram amigos mais próximos Carolina Velasco, Alvaro Siano, Brunello Nardone e Salvatore Bongarzone com quem ainda mantenho contacto ativo hoje em dia. Um muito obrigada em especial para o meu co-orientador o Prof. Dr. Fernando Albericio por ter possibilitado o contacto com este instituto incrível.

Ao grupo "Cancer Drug Resistance" do IPATIMUP – Instituto de Patologia e Imunologia Molecular da Universidade do Porto, nomeadamente Prof. Helena Vasconcelos, Raquel Lima, Diana Sousa, Vanessa Rodrigues e Filipa Reis por me terem recebido neste grupo, permitido a colaboração com esta instituição e ajudado na realização de mais testes biológicos que enriqueceram este trabalho.

Ao "Carbohydrate Chemistry Group" do Centro de Química e Bioquímica (CQB) da Faculdade de Ciências da Universidade de Lisboa (FCUL), em especial à Prof. Amélia Pilar Rauter e à Drª Alice Martins por me terem apoiado não só durante a minha estadia no grupo como em todo o percurso desde que as conheci. Obrigada à Catarina Dias, Ana Marta Matos, Vasco Cachatra, João Pedro Pais, Linda Thaï, Nuno Xavier, Andreia,

Daniela, Vânia e Rafael por sempre me ajudarem em tudo o que necessitei e pelo bom ambiente que se vive neste grupo de investigação.

Um obrigada muito especial ao Prof. João Lago da Universidade de São Paulo no Brasil por me ter auxiliado numa altura que estava a desesperar com a identificação das moléculas a partir do zero e, graças ao seu conhecimento, experiência e disponibilidade, conseguimos avançar neste trabalho. Quero também agradecer a sua constante motivação e boa disposição!

Obrigada a todos os co-autores que ajudaram no trabalho experimental, escrita e correcções dos artigos.

Obrigada ao repórter Hugo Rodrigues e à revista SUL INFORMAÇÃO pela divulgação na comunicação social de parte do trabalho aqui apresentado.

Aos meus amigos de longa data pois sempre me apoiaram e fizeram sorrir nos bons e maus momentos. Não posso nem consigo enumerar todos os que deram tantas diferentes contribuições ao longo deste processo. Muito obrigada!

Chegando ao final desta reflexão sobre os últimos anos, não podia deixar de agradecer às pessoas mais importantes da minha vida. À minha família.

À minha mana Fernanda, obrigada por partilhares a minha vida desde sempre, me ajudares a crescer e a me tornar no que sou e por me apoiares de forma incondicional. Sem ti, não era eu. Ao meu cunhado Fernando, obrigado pelo apoio e por sempre me fazer ver a vida pelo lado otimista. Aos meus sobrinhos, os meus amores, Tomás e Diogo, um muito obrigada por fazerem-me reviver a infância e darem um novo sentido à minha vida.

Aos meus avós, João e Florinda, que faleceram enquanto eu estava no Algarve a realizar esta tese, por sempre me apoiarem e ajudarem em tudo. Adoro-vos e estão sempre presentes no meu coração e na minha memória!

Finalmente, quero agradecer aos meus queridos pais, Rui e Fátima, a quem dedico esta tese, tudo o que sempre fizeram por mim. Por me incutirem o amor ao estudo, à ciência e à realização profissional, entre outros valores que regem a minha vida. Obrigado por toda a vossa ajuda, pelo vosso amor, compreensão e carinho. O meu obrigado vai obviamente para além do que as palavras conseguem exprimir. Obrigada por sempre me apoiarem incondicionalmente!

Os agradecimentos já vão longos e portanto peço desculpa ao leitor mas não foi possível expressar mais resumidamente tudo pelo que estou grata nestes 4 anos. Tem sido uma grande aventura!

Obrigada a todos.

OUTLINE OF THE THESIS

This thesis is divided into seven chapters. Chapter 1 corresponds to a literature review of the main subjects of the thesis. Chapters 2 to 5 describe the publications achieved with experimental work and the main results accomplished. Chapter 6 includes an overall discussion and Chapter 7 describes the conclusions and future perspectives of this research. This thesis was designed to be based on research articles and a book chapter that have been published or accepted for publication.

ABSTRACT

Brown algae contain interesting nutritional profiles, usually with low levels of lipids but relatively high amounts of polyunsaturated fatty acids (PUFA) and minerals. Moreover, brown algae have secondary metabolites that display several biological activities. However, studies on the chemical composition, bioactive compounds and respective biological activities of brown algae are scarce. Here it is shown that *Cystoseira* species have potential biotechnological applications. Among the species evaluated, *C. tamariscifolia* and *C. baccata* were those that in general had the highest ash, protein and lipid contents, while the highest levels of moisture and total carbohydrates were detected in *C. nodicaulis* and *C. compressa*. *Cystoseira* species had also high amounts of K, Ca and Fe, and a favorable Na/K ratio. *C. tamariscifolia*, *C. compressa* and *C. nodicaulis* stood out for their high polyunsaturated/saturated fatty acids (PUFA/SFA) and low n-6/n-3 PUFA ratios as well as appropriate unsaturation, atherogenicity and thrombogenicity indices, suggesting a high nutritional value. *C. tamariscifolia* hexane extract had the highest antioxidant and anti-proliferative activities against a panel of tumoral cells. This extract was particularly selective for hepatocarcinoma cells (HepG2) when compared to non-tumoral cells. HepG2 cells presented pro-apoptotic features and disaggregation on 3D multicellular tumor spheroids after incubation with the extract. Demethoxy cystoketal chromane was isolated and identified as an anti-proliferative compound, selective towards HepG2 cells. Furthermore, isololiolide was isolated for the first time also from *C. tamariscifolia* hexane extract. The latter compound exhibited significant cytotoxic activity against three human tumoral cell lines, namely HepG2 cells, whereas no cytotoxicity was found in non-malignant human fibroblasts. Isololiolide disrupted the HepG2 normal cell cycle and induced apoptosis. Moreover, it altered the expression of proteins that are important in the apoptotic cascade, increasing PARP cleavage and p53 protein expression, and decreasing procaspase-3 and Bcl-2 expression levels. Taken together, the results here presented highlight the potential of *Cystoseira* macroalgae as sources of products for nutra- and pharmaceutical applications.

Keywords: apoptosis; biological activities; *Cystoseira*; natural compounds; nutrition; PUFA.

RESUMO

Desde o início da história da humanidade que os organismos marinhos proporcionam alimentos e subprodutos alimentares. Hoje em dia, são também considerados como uma valiosa fonte de metabolitos secundários com estruturas únicas e aplicações biomédicas importantes. As algas castanhas possuem perfis químicos interessantes do ponto de vista nutricional e biotecnológico, geralmente com baixos níveis de lípidos, mas enriquecidos em ácidos gordos polinsaturados, minerais e metabolitos secundários bioativos. No entanto, são escassos os estudos sobre a sua composição química, compostos bioativos e respetivas atividades biológicas. Assim, este trabalho visou estudar o perfil nutricional de macroalgas castanhas do género *Cystoseira*. Para além disso, foram analisadas as atividades biológicas de extratos orgânicos de diferentes espécies deste género, com especial ênfase no isolamento e caracterização de compostos bioativos e mecanismos de ação. A composição nutricional das algas *C. humilis*, *C. tamariscifolia, C. nodicaulis, C. compressa* e *C. baccata* foi determinada pela primeira vez relativamente à humidade, cinza, conteúdo total em proteína, lípidos, glúcidos e perfil de minerais. Em geral, as espécies *C. tamariscifolia* e *C. baccata* apresentaram maior teor de cinza, proteínas e lípidos. *C. nodicaulis* e *C. compressa* demonstraram conter mais humidade e glúcidos. Todas as espécies apresentaram um elevado conteúdo em minerais, especialmente K, Ca e Fe. Possuem também uma razão Na/K considerada benéfica para a saúde humana. A análise do perfil de ácidos gordos de seis espécies do género *Cystoseira*, nomeadamente *C. compressa*, *C. humilis*, *C. tamariscifolia, C. nodicaulis, C. baccata* e *C. barbata*, foi também realizada pela primeira vez. Os ácidos gordos polinsaturados corresponderam a 29-46% do total de ácidos gordos detetados. As espécies *C. compressa*, *C. tamariscifolia* e *C. nodicaulis* destacaram-se por uma elevada proporção de ácidos gordos polinsaturados em relação aos saturados, uma razão baixa entre os ácidos gordos n-6 e n-3, e índices de insaturação, aterogenicidade e de trombogenicidade favoráveis à saúde humana. Estes resultados sugerem que as espécies estudadas possuem um elevado valor nutricional e que poderão ter, efetivamente, potenciais aplicações na indústria alimentar e de nutracêuticos.

Para além do potencial nutricional, vários estudos descrevem as algas castanhas como ricas em compostos secundários bioativos com importantes propriedades biológicas como atividade antioxidante e antiproliferativa, duas características importantes para compostos com potencial utilização farmacêutica. Neste trabalho, foram realizados

extratos orgânicos de três espécies de *Cystoseira* (*C. humilis*, *C. tamariscifolia* e *C. usneoides*). Estes extratos foram avaliados relativamente ao seu conteúdo em compostos fenólicos totais e actividade antioxidante contra o radical 1,1-difenil-2-picrilhidrazil (DPPH) e o ácido 2,2'-azino-bis(3-etilbenzotiazolina-6-sulfónico) ou ABTS. Além disso, os mesmos extratos foram testados em relação à sua atividade antiproliferativa contra várias linhas celulares de origem tumoral. O extrato de hexano de *C. tamariscifolia* apresentou o teor mais elevado de compostos fenólicos e também a maior actividade antioxidante, quando comparado com os outros extratos estudados. O extrato de hexano de *C. tamariscifolia* apresentou também a mais elevada actividade antiproliferativa contra uma linha celular de hepatocarcinoma (HepG2, IC_{50}=2.31 µg/mL), tendo sido selecionado para uma caraterização química bio-guiada. De modo a averiguar a sua seletividade, o extrato foi aplicado em quatro linhas celulares tumorais adicionais (adenocarcinoma cervical HeLa; adenocarcinoma gástrico AGS; adenocarcinoma colorretal HCT-15; e neuroblastoma SH-SY5Y), e duas linhas celulares não-tumorais (células de estroma de medula óssea de murganho S17 e células endoteliais humanas do cordão umbilical HUVEC). O extrato de hexano de *C. tamariscifolia* reduziu significativamente a viabilidade celular de todas as linhas celulares tumorais estudadas mas, em particular, das células HepG2. Este efeito foi seletivo, especialmente quando comparando o valor de IC_{50} obtido para as células HepG2 com o valor obtido para as células não-tumorais. As células HepG2 incubadas com o extrato de hexano de *C. tamariscifolia* apresentaram evidências significativas de atividade pro-apoptótica, como o aumento da externalização da fosfatidilserina (analisado por citometria de fluxo pela ligação de anexina V à membrana celular) e alterações morfológicas visíveis ao microscópio após a marcação de células com 4',6-diamidino-2-fenilindole (DAPI). Para além disso, ocorreu uma clara desagregação celular no modelo de esferoides multicelulares tumorais 3D após a incubação com o referido extrato. Posteriormente ao fracionamento do extrato, foi isolado o composto dimetoxi cistoquetal cromano, um derivado do meroditerpenoide cistoquetal, o qual apresentou actividade antiproliferativa e seletiva contra as células HepG2.

Por fim, foi descrito pela primeira vez o isolamento do isololiólido, um metabolito da degradação de carotenoides, a partir do extrato de hexano da alga *C. tamariscifolia*. Este composto apresentou actividade citotóxica significativa contra três linhas celulares tumorais, nomeadamente contra as células de hepatocarcinoma HepG2, sem apresentar

citotoxicidade significativa contra os fibroblastos humanos MRC-5 e HFF-1. Análises de citometria de fluxo demonstraram que o isololiólido causou disrupção total do ciclo celular e induziu a apoptose das células HepG2. Além disso, alterou significativamente a expressão de proteínas envolvidas em importantes vias de sinalização apoptóticas. A análise *western* de células incubadas com este composto revelou que o isololiólido causou um aumento da clivagem da PARP, aumentou a expressão da proteína supressora de tumores p53, promoveu a clivagem da procaspase-3 e reduziu os níveis de expressão da proteína anti-apoptótica Bcl-2. Assim, verificámos que o isololiólido não só inibe a progressão do ciclo celular, como também induz a morte programada de células de hepatocarcinoma humano através da modulação de vias de sinalização importantes para a ativação da apoptose.

Em conclusão, esta tese contribuiu para um conhecimento mais alargado acerca da composição química, metabolitos secundários e atividades biológicas do género *Cystoseira*, nomeadamente das espécies *C. compressa*, *C. humilis*, *C. tamariscifolia, C. nodicaulis, C. usneoides, C. baccata* e *C. barbata*. Todas as espécies demonstraram ter potencial para poderem ser usadas nas indústrias nutra- e farmacêutica. Especificamente, a espécie *C. tamariscifolia* mostrou ser a mais promissora em relação ao isolamento de compostos com potencial anticancerígeno, devido à sua forte atividade antioxidante, antiproliferativa e pro-apoptótica em células de hepatocarcinoma. Além disso, foi desta espécie que foram isolados os dois compostos estudados, demonstrando que esta macroalga da costa portuguesa possui um elevado potencial na obtenção de metabolitos de valor terapêutico. Estudos futuros incluem avaliação da absorção, distribuição, metabolismo e excreção (ADME), tanto em modelos *in vitro* como *in vivo*, das moléculas aqui identificadas, assim como informação sobre a sua farmacocinética e farmacodinâmica, visando a sua aplicação.

Palavras-chave: ácidos gordos polinsaturados; apoptose; atividades biológicas; compostos naturais; *Cystoseira*; nutrição.

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1D and 2D NMR	one-dimensional and two-dimensional nuclear magnetic resonance
^{1}H and ^{13}C NMR	proton and carbon-13 nuclear magnetic resonance
AA	arachidonic acid
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ANOVA	one-way analysis of variance
APAF-1	apoptotic protease activating factor 1
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2–associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-W	Bcl-2-like protein 2
Bcl-XL	B-cell lymphoma-extra large
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
CDK	cyclin-dependent kinases
CNT	compound-induced cytotoxicity on non-tumoral cells
CT	compound-induced cytotoxicity on tumoral cells
CTH	*Cystoseira tamariscifolia* hexane extract
CVD	cardiovascular diseases
DAD	diode-array detector
DAPI	4',6-diamidino-2-phenylindole
DHA	docosahexaenoic acid
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
DPX	depex-polystyrene in xylene
DW	dry weight
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EPA	eicosapentaenoic acid
EtOAc	ethyl acetate

FA	fatty acid
FBS	fetal bovine serum
F-C	Folin-Ciocalteu
FDA	Food and Drug Administration
FITC	fluorescein isothiocyanate
GC	gas chromatography
HCC	hepatocellular carcinoma
HPLC	high performance liquid chromatography
HRESIMS	high-resolution electrospray ionization mass spectrometry
HRMS	high resolution mass spectrometry
HRP	horseradish peroxidase
HSD	honest significant difference
IA	index of atherogenicity
IC_{50}	half maximal inhibitory concentration
IT	index of thrombogenicity
LDL	low-density lipoprotein
m/z	mass-to-charge ratio
MeOH	methanol
MS	mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide
NCBI	National Center for Biotechnology Information
NED	N-(1-Naphthyl) ethylenediamine dihydrochloride
NMR	nuclear magnetic resonance
NOS	nitrogen species
NP-40	Nonidet P-40
p53	protein 53
PAR	poly (ADP-ribose)
PARP	poly (ADP-ribose) polymerase
PBS	phosphate-buffered saline
PG	propyl gallate
PI	propidium iodide
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid
ROS	reactive oxygen species
RPMI	Rosewell Park Memorial Institute

RT	room temperature
SAR	structure-activity relationship
SDS	sodium dodecyl sulfate
SEM	standard error of mean
SFA	saturated fatty acid
TBHQ	*tert*-butylhydroquinone
TBS	Tris-buffered saline
TLC	thin layer chromatography
TMS	tetramethylsilane
TPC	total phenolic content
T-TBS	Tris-buffered saline containing Tween-20
UV	ultraviolet
VIS	visible
WHO	World Health Organization

CHAPTER I

GENERAL INTRODUCTION

Partially included in the book chapter:

Vizetto-Duarte C, Bruno de Sousa C, Rodrigues M, Custódio L, Barreira L and Varela J. 2017. Marine algae bioactivies. *In*: F. Xavier Malcata, Isabel Sousa Pinto and A. Catarina Guedes. "Marine Macro- and Microalgae: An Overview". CRC press, Taylor & Francis publishers, UK (in the publisher).

1 Marine macroalgae: sources of food products and novel bioactive compounds

Since the beginning of human history marine macroalgae have been viewed as a major source of food and food products and, more recently, of bioactive compounds to be used in different areas such as pharmaceuticals and cosmetics (Faulkner 2002).

Macroalgae, commonly known as seaweeds, are defined as photosynthetic multicellular eukaryotic organisms with a wide variety of cell morphologies and life cycles. Their morphological diversity results from their polyphyletic origin within the eukaryotic tree of life (Keeling 2013). In their natural environment macroalgae grow on rocky substrates and form stable, multi-layered, perennial vegetation (Luning and Pang 2003). Several classifications for macroalgae have been proposed over the years. They are usually divided into three main groups, according to the occurrence of several photosynthetic pigments that influence the color of their thalli. As such, they can be classified as green (Chlorophyta), red (Rhodophyta) and brown (Stramenopiles, Phaeophyceae) algae. Brown algae are the only macroalgae belonging to the Stramenopiles-Alveolata-Rhizaria evolutionary line. Their green and red counterparts are currently seen as part of the Archaeplastida megagroup, which includes the land plants (Keeling 2013). Whilst the taxonomic classification of several algal evolutionary lines is still being worked out, the one that gathers the most consensus amongst the scientific community is the one proposed by Keeling (2013). Other taxonomists (Cavalier-Smith and Chao 2006, Riisberg et al. 2009) have proposed that brown algae should be classified as belonging to the Ochrophyta, an unranked taxon within the Stramenopiles phylum (also known as Heterokonta or Heterokontophyta).

Macroalgae play a major role as primary producers in the oceans. About 150 macroalgal species are consumed as food and 250 have other commercial uses such as the extraction of industrial gums and chemicals (Kumari et al. 2011). Macroalgae are considered to be an excellent natural source of primary and secondary metabolites that could lead to the development of innovative food and novel compounds with a diverse array of biological activities.

1.1 Phaeophyceae: General characteristics

Brown algae (Fig. 1.1) belong to the Phaeophyceae class, which consists of approximately 2000 known species of macroscopic organisms inhabiting mostly marine

waters. These species have adapted to a great variety of marine ecological niches, including all the tidal and intertidal zones, rock pools but also relatively deep waters, usually under 100 meters. A high number of Phaeophyceae are found in the intertidal or upper littoral area, where environmental factors such as temperature and salinity can change greatly in a 24-hour period.

Fig. 1.1 – Phaeophyceae from the genera *Laminaria* (on top) and *Cystoseira* (on bottom) during low tide at Almograve beach, Odemira, south of Portugal (photo by the author).

In general, brown algae are composed of a holdfast (root-like structure), thallus and lamina. Pneumatocysts are commonly found in brown algae. They exert an upward force that allows the organism to float and thus receive more sunlight for photosynthesis (Martin and Gutow 2005). A prominent feature of Phaeophyceae algae is their brown or yellowish-brown color, resulting from their main carotenoid fucoxanthin. Along with fucoxanthin, the main photosynthetic and photoprotective pigments of brown algae are chlorophylls *a*, *c*1 and *c*2, β-carotene, violaxanthin and diatoxanthin. Their brown color is also influenced by the presence of phlorotannins stored in vesicles called physodes (Goodwin 1974). Contrary to Chlorophyta and Rhodophyta, their main storage product is not starch but laminarin, mannitol and oils. Their cell walls are composed of cellulose, alginate and sulfated polysaccharides (such as fucoidan), that have commercial purposes (Balboa et al. 2013). In fact, many Phaeophyceae have been

researched extensively, especially due to its commercial importance. Alginate, for instance, is used as thickening agent in the textile, cosmetic and food industries.

1.1.1 The genus *Cystoseira*

The genus *Cystoseira* was first described by C. Agardh (1820) and initially included 37 species. Nowadays *Cystoseira* encompasses about 40 species and 20 infraspecific taxa, the majority occurring in the Mediterranean or Atlantic-Mediterranean (Guiry and Guiry 2016). Indeed, *Cystoseira* is a solely marine genus of worldwide distribution with about 80% of the species occurring along the Mediterranean, Adriatic and Atlantic coasts. In the Atlantic, the genus extends from the Cape Verde Islands, through the Canaries, Madeira and Azores, along the coasts of Morocco, Portugal, Spain and France, to the English Channel and up the west coast of the British Isles (Draisma et al. 2010).

From a morphological point of view *Cystoseira* is characterized by a partially perennial thallus that can vary from 30 cm to several meters in length, with a cylindrical or flat appearance. The thalli are attached to the rocky substrate by a conical fibrous base disk. Their profusely branched morphology imparts a tree-like feature to these algae. The lateral branches are repeatedly branched in a once, twice or thrice pinnate fashion and sometimes bear short spine-like or filiform appendages. Despite these common features, there is an emphasis on the variability of the genus, not merely as between species but also between individuals of a single species and, seasonally, within a single individual (Roberts 1967).

Noteworthy is also the fact that certain species (e.g. *C. tamariscifolia* and *C. nodicaulis*) can present a blue, purple or green iridescence when viewed under water (Fig. 1.2). The iridescence may vary in different individuals of the same species at different latitudes, or in a single individual at different times of the year (Ercegovir 1952). Studies revealed that the iridescence is due to physode-like cellular inclusions that are proteinaceous and contain also polysaccharides and phenolic compounds such as phlorotannins (Ragan and Craigie 1976, Pellegrini 1980). Ercegovic (1952) claimed that such iridescence provided protection against excess light. Besides the proposed sunscreen effect, other authors suggest that the presence of phlorotannins in these

cellular inclusions may be an indication of herbivore deterrence or antimicrobial activity (Jegou et al. 2015). However, its precise function remains to be discovered.

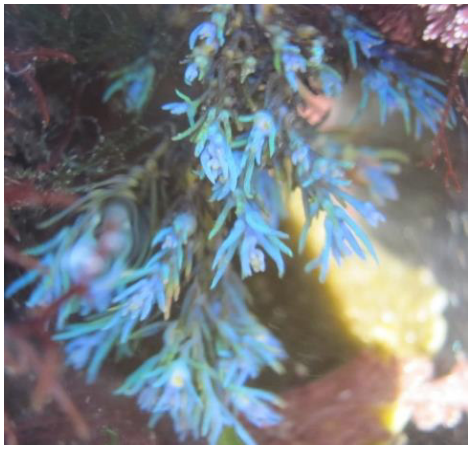

Fig. 1.2 – Detail from *C. tamariscifolia* iridescence (photo by the author).

Cystoseira are indicators of high quality coastal waters (Bermejo et al. 2013), according to the criteria of the Water Framework Directive of the European Union (WFD 2000/60/EC). In addition, species of the *Cystoseira* genus may have great interest when incorporated in food products, nutraceutical and pharmaceutical preparations (Andrade et al. 2013).

1.2 Marine macroalgae: food source for a hungry world?

It is estimated that the world population will grow up to 9 billion by 2050, placing substantial demands on the food supply. Moreover, the ability of traditional agriculture to contribute and satisfy the increasing demand has been questioned due to low yields, increased land use and loss of crops due to climate change (Badgley and Perfecto 2007). On the other hand, consumer health awareness continues to grow with the widespread availability of health-related information and increased incidence of lifestyle-related ailments such as heart disease. It has therefore become highly important to find new solutions to face the major challenges caused by growing populations without compromising environmental integrity and public health.

Macroalgae could be part of a key solution to the expectable food crisis. Macroalgae have played an important role in Asian diet since ancient times and are also widely used by the food industry. The use of macroalgae in food and/or food products

has grown steadily since the early 1980s, particularly in western countries (Besada et al. 2009), which had formerly been reluctant to consume such products. The use of ingredients with added-value properties constitutes an important source for the design of novel food products and is one of the strongest and most sustainable health-driven markets in the world (Buono et al. 2014). Traditionally, seaweed has been incorporated into diets through sushi, salads or soups. Recently, fresh and dried seaweeds have enjoyed growing popularity in gourmet cuisine as side dishes, garnishes and condiments. In 1990, the French government published regulations on the use of marine algae (including several brown algae such as *Ascophyllum nodosum*, *Fucus vesiculosus*, *Fucus serratus* and *Undaria pinnatifida*) as raw or semi-processed ingredients in prepared and processed foods. Several species are in fact consumed after undergoing only minor processing such as drying.

1.2.1 Nutritional proximate composition of macroalgae

Macroalgae are considered to have high nutritional value due to their chemical composition. The chemical composition of seaweed depends on various factors such as the species, time of collection, geographic habitat, water temperature, light intensity and nutrient availability in water (Mabeau and Fleurence 1993, Marinho-Soriano et al. 2006). It is often found that large differences in composition can occur among macroalgae of the same genus and species (Martínez and Rico 2002, Dawczynski et al. 2007), making comparisons difficult. However, it is important to have an estimate of the nutritional profile of edible macroalgae.

One common feature of fresh macroalgae is that they contain large amounts of water. Macroalgae are likely to deteriorate rapidly within a few days upon harvesting and therefore drying is an essential step to preserve them. Drying delays microbial growth, helps to preserve desirable qualities and reduces storage volume (Rodrigues et al. 2015).

Macroalgae contain significant amounts of proteins (Lourenço et al. 2002), which are important and essential factors establishing the nutritional value of food. It has been reported that red and green seaweeds have relatively high protein concentrations averaging 10–30% of dry matter (Mabeau and Fleurence 1993, Burtin 2003). On the other hand, in brown algae, the protein content is usually lower, varying

between 5 and 15% (Burtin 2003, Dawczynski et al. 2007). There have been some exceptions reported however: in *Fucus serratus* and *Undaria pinnatifida*, protein contents of 44% and 21.3% have been reported, respectively (Marsham et al. 2007, Mišurcová et al. 2012).

Lipids play diverse and critical roles in metabolism. The lipid content of macroalgae represents only 1–5%, and thus the contribution of this class of molecules as an energy source appears to be limited (Burtin 2003). However, Phaeophyceae such as *Cystoseira* are among the algae with higher lipid contents, in particular in terms of polyunsaturated fatty acids (PUFA), when compared to algae belonging to other phyla (Colombo et al. 2006, Pereira et al. 2012). PUFA account for almost half of this lipid fraction with a significant amount of it occurring in the form of "omega-3" (n-3) and "omega-6" (n-6) fatty acids such as eicosapentaenoic (EPA) and arachidonic (AA) acids, respectively (MacArtain et al. 2007). PUFA regulate a wide range of functions in the body, such as blood pressure, blood clotting, and the correct development and function of the brain and nervous systems (Patterson et al. 2012). Furthermore, PUFA have a role in regulating inflammatory responses through the production of eicosanoids, which are known inflammatory mediators (Calder 2006). In particular, the *cis* (Z) stereochemistry of the double bond causes a kink in the alkyl chain that has consequences in the physical properties of the molecules, like fluidity, which is important in a biological context, namely in cellular membranes. Brown algae are particularly rich in the n-3 fatty acids EPA and α-linolenic acid, and in the n-6 fatty acids AA and linoleic acid, along with relatively high levels of palmitic and oleic acids (Dawczynski et al. 2007). Brown algae have a balanced n-6/n-3 ratio (0.6–5.1:1), considering that in a healthy human diet the ratio of n-6/n-3 should not exceed 10:1 (van Ginneken et al. 2011).

Macroalgal carbohydrate content is considered high. However, digestibility of these carbohydrates is low (Bocanegra et al. 2009). Standard polysaccharides in brown algae are laminarin, cellulose, alginates, mannitol and fucoidan. Most of these polysaccharides are not digestible by the human gastrointestinal tract and therefore can be regarded as dietary fibers (Dawczynski et al. 2007). Storage polysaccharides such as agar, carrageenans and alginates, are the most commercially exploited components in seaweeds. These storage polysaccharides exhibit textural and stabilizing properties

(MacArtain et al. 2007). Hence they are used in thickening aqueous solutions, gels, water-soluble films and stabilizers.

The ash content of macroalgae is generally high, especially when compared to that of terrestrial vegetables. It is known that ash levels are associated with the amount of mineral elements. Minerals are an essential part of human diet and more than 95% of mineral intake originates from food. Minerals play an important role in the human body as they are structural materials for building tissues and also significant factors in vital reactions such as cofactors of many metalloenzymes. Macroalgae are known as a significant source of minerals due to their capacity to absorb inorganic ions from the environment, mainly iron, potassium, calcium and sodium (Misurcova et al. 2011).

1.3 Nutraceuticals *vs*. Pharmaceuticals

In recent years, lifestyle shifts such as the consumption of diets rich in highly saturated fats, sugars, and salt (often named as "fast foods") have significantly increased the risk of diseases such as atherosclerosis, stroke or type-2 diabetes. In this sense, "nutraceuticals" arose in the last decades as a way of improving human health through the diet and the intake of added-value food products.

Stephen DeFelice, MD, founder and chairman of the Foundation for Innovation in Medicine (FIM), used the term "nutraceutical" for the first time in 1989, merging the word "nutrition" with "pharmaceutical". However, there is no common internationally recognized definition of what a nutraceutical is (Aronson 2016). The European Nutraceutical Association defines nutraceuticals as nutritional products that have an effect on the health of humans or animals and that are neither synthetic substances nor chemical compounds formulated for specific indications. Currently, "nutraceutical" can be defined as a comprehensive term which includes isolated nutrients, food, herbal products or dietary supplements that provide medical or health benefits, including the prevention and/or treatment of disease (Russo et al. 2016). Among the different nutraceuticals, applicability of marine-derived nutraceuticals is a blooming sector of the food industry, and macroalgae are playing a major role in it.

On the other hand, "pharmaceuticals" are defined by the European Union law (Directive 2001/83) as "any substance or combination of substances presented as having properties for treating or preventing disease in human beings" or "any substance or

combination of substances which may be used in or administered to human beings either with a view of restoring, correcting or modifying physiological functions by exerting a pharmacological, immunological or metabolic action, or by making a medical diagnosis". Pharmaceuticals are commonly referred to as molecules with established therapeutical effect with specific indications, in a formulation where the concentration and route of administration is well defined. The search for pharmaceuticals in marine organisms, including algae, has greatly increased in the past decades as many of the drugs sold today are copies or simple synthetic modifications of substances found in nature (Hussain et al. 2012).

1.4 Primary and secondary bioactive metabolites

In order to understand the term "secondary metabolite" one must first differentiate between primary and secondary metabolism. Primary metabolism can be summarized as the synthesis and breakdown of compounds namely carbohydrates, proteins, lipids and nucleic acids, that are vital for the growth of all organisms (Dewick 2002). The compounds involved are usually referred to as "primary metabolites". Secondary metabolism, on the other hand, is the process by which biomolecules frequently found to be specific to an organism, or of the expression of the individuality of a limited number of species, are produced (Sarker et al. 2001). These "secondary metabolites", often designated by "natural products", are generally not essential for the growth, development or reproduction of an organism, but are produced either as a result of adaptations to its surrounding environment or as a defense mechanism against predators (Dewick 2002). Secondary metabolism typically depends on the primary metabolism for carbon skeletons in the form of, for example, amino acids or acetate. These compounds can then be diverted to secondary metabolic pathways and produce "shunt metabolites". These intermediates have apparently adopted an alternate biosynthetic route, leading to the production of secondary metabolites (Sarker et al. 2001). The biosynthesis and accumulation of this wide array of natural products provide unique chemical structures with unusual biological activities.

Marine algae are among the richest sources of chemically diverse natural products (Dias et al. 2012), although their potential in drug discovery has remained

largely unexplored. Nevertheless, great effort has been done in the last decades in order to investigate this resource.

1.5 Biologically active natural products from marine macroalgae

Biological activity can be defined as the specific effect of, or a reaction to, the exposure of a living organism/tissue/cell/enzyme to a given compound or mix of compounds (e.g. extracts). Humans have explored different terrestrial plants as sources of biologically active compounds for centuries. Historically, marine organisms have had a limited number of reported applications in traditional medicine as compared to their terrestrial counterparts (Dias et al. 2012). In the past decades, however, this trend has changed. Intense research has been performed due to the discovery of novel and diverse bioactive molecules that have become good candidates for the development of innovative drugs.

Biodiversity is vital in the screening for new chemical entities in drug discovery research. In this context, the search of new biologically active compounds from marine organisms can be seen as an almost unlimited field. Marine organisms produce a large amount of secondary metabolites that are not found in terrestrial ones. Extreme and different environmental conditions have led to the development of chemical defense strategies that resulted in a significant diversity of compounds.

Recent surveys of drugs from natural sources have shown that algae are promising organisms that provide new biochemically active compounds with beneficial effects on human health and nutrition (Rocha-Santos and Duarte 2014). More than 22000 compounds have been isolated from marine organisms and algae account for 14% of these (Fig. 1.3).

Several compounds with biological activities isolated from macroalgae have been reported. Intertidal macroalgae live in complex habitats submitted to extreme conditions (e.g. fluctuations in salinity, temperature, nutrients, UV-Vis irradiation, and pressure). Hence, they produce a great variety of secondary metabolites with different chemical structures (Cardozo et al. 2007).

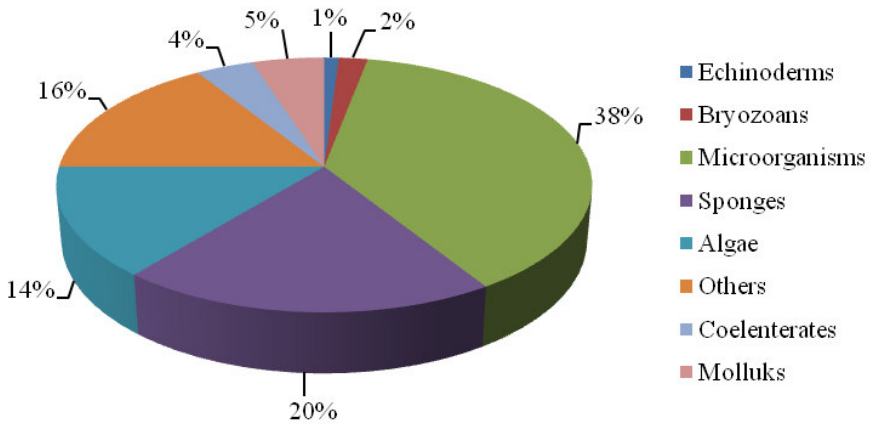

Fig. 1.3 – Contribution of different sources of marine natural products in the discovery of novel bioactive compounds from 2010 to 2013 (adapted from Rocha-Santos and Duarte 2014).

1.6 Extracts and compounds from macroalgae with antioxidant properties

Extracts and compounds from macroalgae with antioxidant properties are being intensively investigated due to the current growing demand from the food and pharmaceutical industries for antioxidant agents. An antioxidant is a substance capable of preventing or slowing down the oxidation of other molecules (Flora 2009). Oxidation is a chemical reaction that can produce free radicals, leading to chain reactions that may damage cells. In an organism or tissue, oxidation may cause oxidative stress. Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the ability of a biological system to readily detoxify the reactive intermediates or to repair the resulting damage. The end result is typically the generation of free radicals that need to be scavenged in order to prevent cell damage. Indeed, a free radical is defined as a chemical species capable of independent existence, having one or more unpaired electrons. These unpaired electrons make free radicals unstable and highly reactive towards other substances, and even towards themselves. Examples of ROS include superoxide (O_2^-), singlet oxygen ($^1O_2$), hydroxyl (HO•), peroxyl (ROO•) and nitric oxide (NO). ROS are continuously produced during physiological events and can initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides (Gülçin 2010). In addition to the biological processes that produce endogenous forms of ROS, the human organism is also exposed to ROS

generated by the exposition to external factors such as UV radiation, tobacco smoke, certain pollutants, organic solvents and pesticides (Lobo et al. 2010). If ROS are not effectively scavenged, they can stimulate free radical chain reactions, subsequently damaging cellular biomolecules such as proteins and lipids. Importantly, reaction of ROS with nucleic acids of the nuclear and mitochondrial genomes can lead to mutations and disease (Taylor and Turnbull 2005). However, this imbalance can be shifted when levels of antioxidants are increased.

In food and food products, protection against oxidation is essential to maintain nutritional value and organoleptic properties such as flavor, color, odor and texture. In these products, autoxidation of lipids by chemical, thermal, electromagnetic and/or enzymatic processes is a frequent event that leads to an increase in free radicals. Lipid hydroperoxides, for instance, are unstable and easily converted to secondary oxidation products (e.g. aldehydes, ketones, alcohols, hydrocarbons) that affect food quality (Balboa et al. 2013). Several synthetic antioxidants as for example butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and *tert*-butylhydroquinone (TBHQ) have been used to avoid oxidation of food and other products, although they have been associated with undesirable side effects such as toxicity and carcinogenicity (Lanigan and Yamarik 2002). Therefore, the safety issues have caused an increasing need to study and identify alternative sources of antioxidants to be used in the food industry (Cho et al. 2011).

Brown algae encompass the highest number of macroalgal families that are rich in bioactive compounds with antioxidant properties, including Sargassaceae (Zubia et al. 2009). Several extracts, fractions and compounds with antioxidant activity have been isolated from brown algae, most of them belonging to the phenolic fraction (Balboa et al. 2013). Regarding the *Cystoseira* genus, it was previously identified as antioxidant the dichloromethane/methanol extract (1:1) of *C. tamariscifolia* (Zubia et al. 2009), super-critical water extract of *C. abies-marina* (Plaza et al. 2010), methanol extract of *C. hakodatensis* (Airanthi et al. 2011), chloroform extract from *C. crinita* (Mhadhebi et al. 2011) and tetraprenyltoluquinol derivatives from *C. crinita* (Fisch et al. 2003).

1.7 Macroalgae-derived compounds and anti-proliferative activities: general aspects and specific applications

1.7.1 Cancer cells proliferation: cell survival *vs*. cell death under pathological conditions

The balance between cell division and cell death is a basic feature in the development and maintenance of homeostasis. Disturbances in this balance can cause disease: too much cell death can cause injury; too little cell death is a prerequisite for the development of cancer. Thus, a tight control of the equilibrium between cell death and proliferation is necessary. Under typical conditions this balance is maintained by tightly regulating both processes. However, when one or both processes are deregulated, cancer may ensue.

Andreeff et al. (2003) stated that cancer is primarily the accumulation of clonal cells, leading to therapies that consist in trying to reduce the number of tumor cells and preventing their accumulation. These approaches are implemented either by cytotoxic (stimulating cancer cell death) or via cytostatic effects. Briefly, anti-proliferative mechanisms preventing carcinogenesis may include up-regulation of apoptosis, inhibition of DNA synthesis and cell cycle progression. The inactivation of apoptosis is central to the development of cancer and, in the last decades, targeting apoptosis for the treatment of cancer has become an increasingly attractive strategy.

1.7.2 Apoptosis: Morphological and biochemical changes in the cell

The word “apoptosis” was proposed by Kerr et al. (1972) to describe a controlled physiologic process of removing individual unnecessary components of an organism without destruction or damage to the organism. Apoptosis was initially confirmed as a specific form of programmed cell death that served to eliminate excessive or unwanted cells during embryonic development and normal tissue growth (Williams 1991), but at a later stage this process was also linked to cellular injury (Haslett 1992). Deregulation of the apoptotic program is a complex pathophysiological underpinning involved in the development of chronic diseases, including cancer (Wang 2014).

From the morphological point of view, apoptotic cells show cytoplasmic cell shrinkage, budding of plasma membrane, exposure of membrane phosphatidylserine

(PS) on the extracellular side, chromatin condensation and DNA fragmentation. The plasma membrane maintains intact throughout the whole process. The expression of PS in the outer leaflet of the cell membrane is an early process that allows recognition of dead cells by macrophages, resulting in phagocytosis without a significant release of pro-inflammatory cellular components (Elmore 2007). The typical morphological features demonstrated at a later stage are the consequence of a series of precisely regulated events that are frequently altered in tumor cells (Kasibhatla and Tseng 2003). These events provide the opportunity for selective clinical intervention to bring about the death of the tumor cell by apoptosis without damaging the surrounding tissues.

Apoptosis is primarily executed by a family of proteases known as caspases (cysteinyl, aspartate-specific proteases) that cleave and inactivate or activate target substrates within a cell. Caspases are synthesized as inactive zymogens, which must be cleaved at two (or three in some cases) aspartate residues to generate the active mature enzyme (Amirkhiz et al. 2013). The generation of active caspases forms a cascade in which the initiator caspases cleave the executioner caspases that perform critical proteolysis of specific cellular substrates, resulting in the final apoptotic cell death. Caspases are central to the mechanism of apoptosis, as they are both initiators (caspase-2, -8, -9 and -10 are primarily responsible for the beginning of the apoptotic pathway) and executioners (caspase-3, -6 and -7, responsible for the definite cleavage of cellular components) of cell death (Tan et al. 2009). Among them, caspase-3 has been identified as a key mediator of apoptosis in mammalian cells. Caspase-3 is one of the pro-apoptotic executioner caspases that are activated by upstream initiator caspases and are responsible for the cleavage of key proteins, such as cytoskeletal proteins, which give rise to the typical morphological changes observed in cells undergoing apoptosis (Elmore 2007).

Although apoptosis can be triggered by several different stimuli, apoptotic signaling is mainly transduced by two major molecular pathways: an extrinsic pathway mediated by death receptors on the cell surface, and an intrinsic pathway, which is triggered at the mitochondrial level (Fig. 1.4). Both pathways culminate in the activation of executioner caspases (such as caspase-3) cleaving various substrates that ultimately cause the morphological and biochemical changes seen in apoptotic cells (Tait and Green 2010).

Many of the signals eliciting apoptosis converge in the mitochondria, which responds to pro-apoptotic signals by releasing cytochrome *c*, an apoptogenic factor that triggers the formation of the apoptosome, along with APAF-1 (apoptotic protease activating factor 1), promoting caspase-9 activation (Czabotar et al. 2014). Members of the Bcl-2 family of proteins, that can have either pro-apoptotic (such as Bax and Bak) or anti-apoptotic (Bcl-2, Bcl-XL, Bcl-W) function, act in part by governing mitochondrial death signaling through cytochrome *c* release.

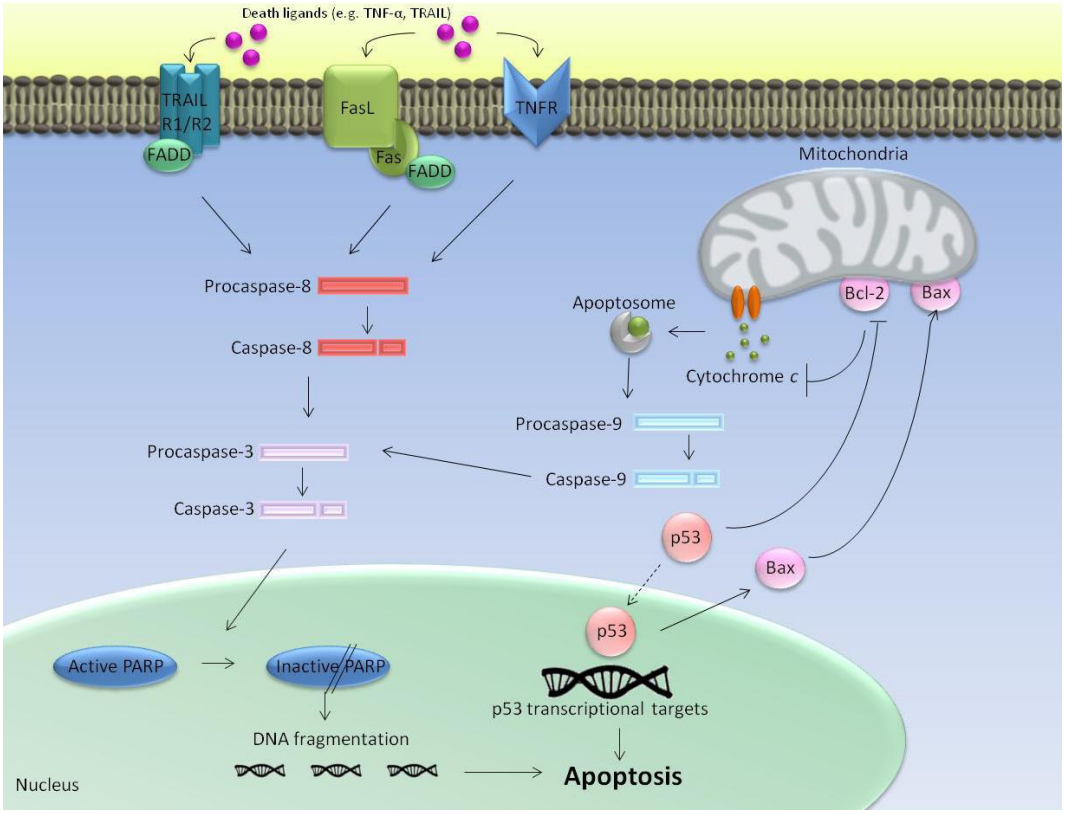

Fig. 1.4 – Overview of the apoptotic pathways studied in this thesis. Caspase-dependent apoptosis has been dichotomously discussed as extrinsic (death receptors) and intrinsic (mitochondrial) pathways. In the extrinsic pathway caspase-8 is activated, while in the intrinsic pathway caspase-9 is triggered. Both pathways culminate in the activation of caspase-3. Caspase-3 cleaves several substrates such as PARP, inactivating it and originating DNA fragmentation and apoptosis. On the other hand, p53 may increase pro-apoptotic Bax expression and relocation, increasing the release of cytochrome *c* from mitochondria, which will produce the apoptosome that will activate caspase-9. Moreover, p53 can also down-regulate the anti-apoptotic Bcl-2. Bcl-2 prevents the release of cytochrome *c* from the mitochondria, inhibiting the intrinsic pathway.

One of the most widely studied negative regulators of apoptosis is Bcl-2, thought to prevent the release of cytochrome *c* from the mitochondria. Bcl-2 is expressed in a wide variety of fetal tissues. However, in the adult organism, expression seems to be

confined to cells that are rapidly dividing and differentiating (Kirkin et al. 2004). Up-regulation of the anti-apoptotic Bcl-2 protein ensures that over-proliferating cells survive to acquire further mutations promoting tumorigenesis.

Poly (ADP-ribose) polymerase (PARP) is a molecular sensor of DNA breaks and has a key role in the spatial and temporal organization of their repair. It is a highly conserved multifunctional enzyme (Megnin-Chanet et al. 2010). Through its physical association with, or by the poly(ADP-ribosyl)ation (PAR) of partner proteins, it regulates chromatin structure and DNA metabolism (Fig. 1.5). These partner proteins include histones, topoisomerases I and II, DNA helicases, single-strand break repair and base-excision repair factors, and various transcription factors (Schreiber et al. 2006). There is increasing evidence that a deficiency of PARP leads to DNA repair defects, genomic instability, failure of induction of cell death and modulation of gene transcription.

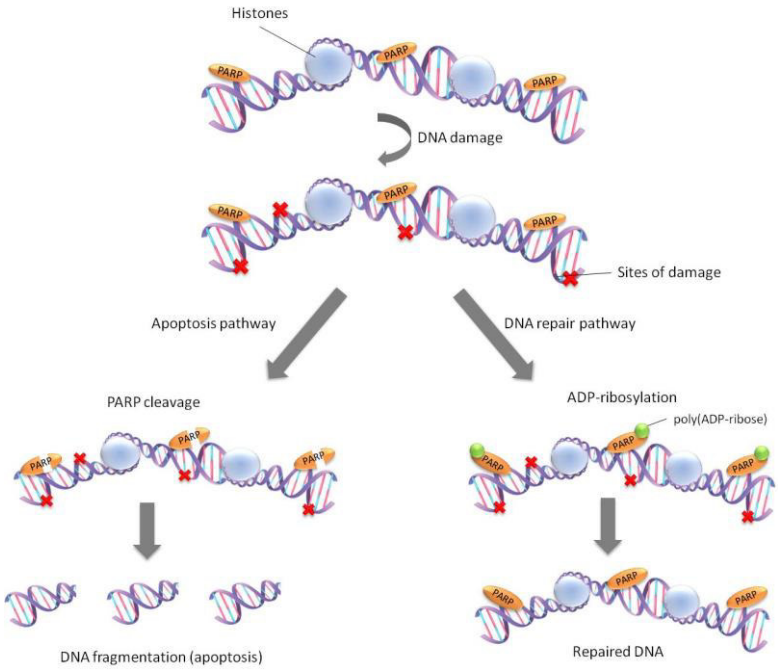

Fig. 1.5 – Poly (ADP-ribose) polymerase (PARP) is an abundant enzyme present in all somatic cells that detects and signals DNA damage to other gene products involved in DNA repair. Once PARP detects DNA damage, it binds to the DNA and begins the synthesis of a poly (ADP-ribose) chain (PAR) as a signal for other DNA-repairing enzymes. After DNA repair, the PAR chains are degraded. Conversely, PARP is cleaved and inactivated in cells undergoing apoptosis.

Caspase-3 is primarily responsible for the cleavage of PARP, inactivating it during cell death (Megnin-Chanet et al. 2010). Furthermore, the sequence at which caspase-3 cleaves PARP is very well conserved in the PARP protein from very distant species, indicating the potential importance of PARP cleavage in apoptosis (Boulares et al. 1999).

p53 (also known as protein 53 or tumor suppressor protein 53) is a potent transcription factor playing a critical role in the cellular stress response in cancer. p53 induces either growth arrest, which prevents the replication of damaged DNA, or apoptosis, eliminating defective cells. It is usually responsible for activating DNA repair proteins when DNA has sustained damage, blocking the cell cycle at regulation checkpoints in DNA damage recognition or initiating apoptosis if DNA damage proves to be irreparable (Amaral et al. 2010). Indeed, development of a full malignant phenotype in many cell types requires deletion or inhibition of p53 functions to bypass senescence (Kirkin et al. 2004). Approximately 50% of all human tumors express mutated p53, which make this protein an important target for cancer therapy (Rivlin et al. 2011).

1.7.3 Cell cycle arrest

Tumor cells accumulate mutations that result in constitutive mitogenic signaling, triggering mitosis and defective responses to anti-mitogenic signals that contribute to unscheduled and increased proliferation. In addition, most tumors acquire genomic instability that leads to additional mutations as well as chromosomal instability, a defect responsible for changes in chromosomes. These alterations result not only in proliferative advantages but also in increased susceptibility to the accumulation of additional genetic mutations that contribute to tumor progression and acquisition of more aggressive phenotypes (Giam and Rancati 2015). These cell cycle defects are mediated, directly or indirectly, by misregulation of cyclin-dependent kinases (CDK). p21 is an inhibitor of most of the CDK which regulates the cell cycle and it is an important target of p53 (Fig. 1.6). Proper progression through the cell cycle is monitored by checkpoints that sense possible defects during DNA synthesis and chromosome segregation. Since p21 inhibits both the cyclin-dependent G1 and the G2/M-specific kinases, p53 is capable of controlling both G1 and G2/M checkpoints

(Pucci et al. 2000). Activation of these checkpoints induces cell cycle arrest through modulation of CDK activity. Cell cycle arrest allows cells to properly repair these defects, thus preventing their transmission to the resulting daughter cells. As a result, DNA damage checkpoints protect cells from accumulating mutations in the DNA.

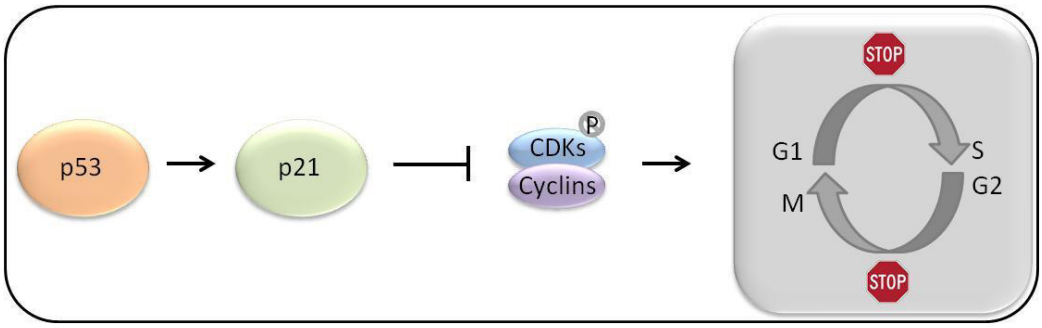

Fig. 1.6 – A simplified scheme of p53-induced cell cycle arrest in G1 and G2/M phases. A key transcriptional target of p53 is p21, an inhibitor of CDK, which inhibits cyclin-CDK complexes and thereby causes cell cycle arrest in G1 and G2/M phases.

A few cell cycle arrest promoter compounds are now in clinical trials (Tao et al. 2013). However, there is still the need to improve existing therapies as well as searching for novel drugs that provide higher survival rates and lower the impact of side effects, especially in p53 or p21-mutated tumor cells.

A coordinate modulation of apoptosis and cell cycle, ordered in space and time, orchestrates the complex response to injury by inducing genes that regulate cell survival, proliferation, differentiation and tissue specific functions. On this basis, pharmacological or molecular modulation of these pathways is currently under consideration as an approach to therapy of neoplastic conditions (Surova and Zhivotovsky 2013).

1.7.4 Anti-proliferative properties from brown macroalgae: specific applications

Compounds from Phaeophyceae macroalgae have been reported to possess bioactive properties including anti-proliferative activities (Dias et al. 2012). Several molecules with anti-proliferative activity were identified, such as polysaccharides, quinones, sterols, terpenoids and others, and are presented in Table 1.1.

Ale et al. (2011), for instance, found that fucose-containing sulfated polysaccharides extracted from the seaweeds *Sargassum henslowianum* and *Fucus vesiculosus* decreased the proliferation of melanoma cells in a dose-response fashion via induction of apoptosis involving the activation of caspase-3.

Khanavi et al. (2012) identified fucosterol, the most abundant phytosterol from the hexane fraction of *Sargassum angustifolium*, as the responsible for the cytotoxic effect of this extract against human ductal breast epithelial tumor (T47D) and human colon carcinoma (HT29) cell lines.

A phloroglucinol derivative, dioxinodehydroeckol, isolated from *Ecklonia cava* inhibited the proliferation of human MCF-7 breast cancer cells. Treatment with dioxinodehydroeckol induced an increase in caspase (-3 and -9) activity and PARP cleavage (Kong et al. 2009).

Carotenoids are well known terpenoids with a strong anti-proliferative effect. Zeaxanthin, for instance, has shown to induce apoptosis in human melanoma cells through members of the Bcl-2 family (Bi et al. 2013). Fucoxanthin, on the other hand, is commonly found in brown macroalgae and it has been shown to have antioxidant, anti-inflammatory and anti-proliferative properties. Additionally, it exhibited pro-apoptotic, cell cycle arrest and anti-angiogenesis activities (Peng et al. 2011, Rengarajan et al. 2013). Interestingly, even the metabolites of carotenoids seem to have anti-proliferative activities as well. For example, fucoxanthinol, a fucoxanthin metabolite, displayed remarkable anti-proliferative effects on human adult T-cell leukemia cells *in vitro* (Ishikawa et al. 2008) and also in breast cancer cell lines MCF-7 and MDA-MB-231 (Rwigemera et al. 2015).

Table 1.1 Anti-proliferative compounds from brown macroalgae.[1]

Fraction / Compound	Organism		Reference(s)
Structure / Name	**Family**	**Species**	
Carboxylic acid			
Turbinaric acid	Sargassaceae	*Turbinaria ornata*	Asari et al. 1989
Polysaccharides			
Fucose-containing sulfated polysaccharide	Sargassaceae	*Sargassum henslowianum*	Ale et al. 2011
Fucose-containing sulfated polysaccharide	Fucaceae	*Fucus vesiculosus*	Ale et al. 2011
Quinones			
Bis-prenylated quinones	Sporochnaceae	*Perithalia capillaris*	Blackman et al. 1979
Sterols			
6 -hydroxy-24-ethylcholesta-4,24(28)-dien-3-one	Sargassaceae	*Turbinaria conoides*	Sheu et al. 1999
24 -hydroperoxy-6 -hydroxy-24-ethylcholesta-4,28(29)-dien-3-one	Sargassaceae	*Turbinaria conoides*	Sheu et al. 1999
24-ethyl-cholesta-4,24(28)-dien-3-one	Sargassaceae	*Turbinaria conoides*	Sheu et al. 1999
Fucosterol	Sargassaceae	*Sargassum angustifolium*	Khanavi et al. 2012
Terpenoids			
4-acetoxydictyolactone	Dictyotaceae	*Dictyota dichotoma*	Ishitsuka et al. 1988
Atomarianones A and B	Dictyotaceae	*Taonia atomaria*	Abatis et al. 2005
Bifurcadiol	Sargassaceae	*Bifurcaria bifurcata*	Guardia et al. 1999
Cystoseirol monoacetate	Sargassaceae	*Cystoseira myrica*	Ayyad et al. 2003
Dictyol F monoacetate	Sargassaceae	*Cystoseira myrica*	Ayyad et al. 2003
Dictyone acetate	Sargassaceae	*Cystoseira myrica*	Ayyad et al. 2003
Dictyotalide A	Dictyotaceae	*Dictyota dichotoma*	Ishitsuka et al. 1988
Dictyotalide B	Dictyotaceae	*Dictyota dichotoma*	Ishitsuka et al. 1988
Dolabellane	Dictyotaceae	*Dictyota* sp.	Tringali et al. 1984
12-hydroxygeranylgeraniol	Sargassaceae	*Bifurcaria bifurcata*	Gulioli et al. 2004
Isodictytriol monoacetate	Sargassaceae	*Cystoseira myrica*	Ayyad et al. 2003

Fraction / Compound	Organism		Reference(s)
Structure / Name	Family	Species	
Isozonarone	Dictyotaceae	*Dictyopteris zonarioides*	Fenical et al. 1973
Nordictyotalide	Dictyotaceae	*Dictyota dichotoma*	Ishitsuka et al. 1988
Sargol, Sargol I and II	Sargassaceae	*Sargassum tortile*	Numata et al. 1991
Terpenoid C	Dictyotaceae	*Stypopodium zonale*	Dorta et al. 2002

1 - This table intends to present examples of the chemical diversity of anti-proliferative compounds found in brown macroalgae, and not to be a comprehensive list of what has been published in the literature.

Several crude extracts have also demonstrated anti-proliferative activity. For example, the hexane fraction of *Sargassum swartzii* and *Cystoseira myrica* showed selective cytotoxicity against proliferation of Caco-2 (IC_{50}<100 μg/ml) and T47D (IC_{50}<100 μg/ml) cells, increasing apoptosis (Khanavi et al. 2010). Huang and collaborators (2005) demonstrated that the ethyl acetate extract from *Colpomenia sinuosa* inhibited the growth of human hepatoma HuH-7 cells and leukemia U937 and HL-60 cells in a time- and dose-dependent manner.

It is clear that marine brown algae are prolific producers of biologically active secondary metabolites, especially terpenoids, with cytotoxic activity. Global research aimed at the discovery of novel and clinically useful anti-tumor agents derived from marine sources continues nowadays at a remarkably active pace.

1.8 Bioactive secondary metabolites isolated from *Cystoseira* species

The genus *Cystoseira* is known to produce a range of relatively complex terpenoids. *Cystoseira usneoides* contains usneoidone E with antiviral and anti-proliferative activities. However, this compound also has a high level of cytotoxicity toward normal cells (Urones et al. 1992). Six tetraprenyltoluquinols, two triprenyltoluquinols and two tetraprenyltoluquinones were isolated from *C. crinita*, collected from the South Coast of Sardinia. All compounds were tested for antioxidant properties using the DPPH and TBARS assays. The six tetraprenyltoluquinols and the two triprenyltoluquinols exhibited potent radical scavenging effects, while the two tetraprenyltoluquinones were significantly less active, but still comparable to that of BHT (Fisch et al. 2003). *Cystoseira myrica*, collected in the Gulf of Suez, afforded four

hydroazulene diterpenes, namely dictyone acetate, dictyol F monoacetate, isodictytriol monoacetate and cystoseirol monoacetate. All compounds exhibited moderate cytotoxicity against the murine cancer cell line KA3IT, but reduced cytotoxicity against non-tumoral NIH3T3 cells (Ayyad et al. 2003).

Despite the progress done by authors such as Amico (1995) in the search of bioactive compounds from *Cystoseira* species, there are still numerous possibilities to explore.

1.9 Scope of research and justification of the thesis

Several studies have focused on the determination of the proximate composition and fatty acid profile of macroalgae. However, a complete study of these parameters in *Cystoseira* species was yet to be carried out. Research dealing with macroalgae natural products is still widely unexplored, especially for compounds with anti-tumoral potential. In addition, the study of natural products from macroalgae seldom considers the mechanisms and pathways involved in the observed bioactivities.

Thus, the main objective of the research conducted in this thesis was to investigate whether *Cystoseira* species had the potential to be used by the nutra- and/or pharmaceutical industries in an effort to identify their composition, bioactivities and underlying mechanisms of action.

To do so, several biological questions were addressed here for the first time:

- what is the proximate composition of *Cystoseira* species?
- what is their fatty acid profile?
- do they have the characteristics of a healthy food or food product?
- do they have antioxidant potential?
- do these species display anti-proliferative activity?
- which compounds are responsible for the bioactivies?
- what molecular pathways do these compounds modulate?

Collectively, with the results obtained in this thesis, we aim to improve the knowledge about *Cystoseira* composition in order to be used as a food, as part of a food product and/or as source of novel drug leads for the pharmaceutical industry.

1.10 References

Abatis, D., Vagias, C., Galanakis, D., Norris, J.N., Moreau, D., Roussakis, C. and Roussis, V. 2005. Atomarianones A and B: two cytotoxic meroditerpenes from the brown alga *Taonia atomaria*. Tetrahedron Lett. 46: 8525–8529.

Agardh, C. A. 1820. Species Algarum, Vol. 1, pt. 1, 168 pp. Lund.

Ale, M.T., H. Maruyama, H. Tamauchi, J.D. Mikkelsen and A.S. Meyer. 2011. Fucose-containing sulfated polysaccharides from brown seaweeds inhibit proliferation of melanoma cells and induce apoptosis by activation of caspase-3 *in vitro*. Mar Drugs 9: 2605-2621.

Airanthi, M. K., Hosokawa, M. and Miyashita, K. 2011. Comparative antioxidant activity of edible Japanese brown seaweeds. J Food Sci 76, C104-111.

Amaral, J. D., Xavier, J. M., Steer, C. J. and Rodrigues, C. M. 2010. The role of p53 in apoptosis. Discov Med 9, 145-152.

Amico, V. 1995. Marine brown algae of family Cystoseiraceae: Chemistry and chemotaxonomy. Phytochemistry 39, 1257-1279.

Amirkhiz, M.B., Rashtchizadeh, N., Nazemiyeh, H., Abdolalizadeh, J., Mohammadnejad, L. and Baradaran B. 2013. Investigating apoptotic effects of methanolic extract of *Dorema glabrum* seed on WEHI-164 Cells. ISRN Pharmacol, article ID 949871.

Andrade, P. B., Barbosa, M., Matos, R. P., Lopes, G., Vinholes, J., Mouga, T. and Valentao, P. 2013. Valuable compounds in macroalgae extracts. Food Chem 138, 1819-1828.

Andreeff, M., Goodrich, D.W. and Pardee, A.B. 2003. Cell Proliferation and Differentiation. *In*: Holland-Frei Cancer Medicine. Kufe D.W., Pollock R.E., Weichselbaum R.R., editors. 6th edition. Hamilton BC Decker.

Aronson, J. K. 2016. Defining "nutraceuticals": neither nutritious nor pharmaceutical. Br J Clin Pharmacol.

Asari, F., T. Kusumi and Kakisawa, H. 1989. Turbinaric acid, a cytotoxic secosqualene carboxylic acid from the brown alga *Turbinaria ornata*. J Nat Prod 52, 1167-1169.

Ayyad, S. E., Abdel-Halim, O. B., Shier, W. T. and Hoye, T. R. 2003. Cytotoxic hydroazulene diterpenes from the brown alga *Cystoseira myrica*. Z Naturforsch C 58, 33-38.

Badgley, C., Moghtader, J., Quintero, E., Zakem, E., Chappell, M.J., Avilés-Vázquez, K., Samulon, A. and Perfecto I. 2007. Organic agriculture and the global food supply. Renew Agr Food Syst 22, 86-108.

Balboa, E. M., Conde, E., Moure, A., Falque, E. and Dominguez, H. 2013. In vitro antioxidant properties of crude extracts and compounds from brown algae. Food Chem 138, 1764-1785.

Bermejo, R., de la Fuente, G., Vergara, J. J. and Hernandez, I. 2013. Application of the CARLIT index along a biogeographical gradient in the Alboran Sea (European Coast). Mar Pollut Bull 72, 107-118.

Besada, V., Andrade, J. M., Schultze, F. and Gonzalez, J. J. 2009. Heavy metals in edible seaweeds commercialised for human consumption. J Mar Syst 75, 305-313.

Bi, M. C., Rosen, R., Zha, R. Y., McCormick, S. A., Song, E. and Hu, D. N. 2013. Zeaxanthin Induces Apoptosis in Human Uveal Melanoma Cells through Bcl-2 Family Proteins and Intrinsic Apoptosis Pathway. Evid Based Complement Alternat Med 2013, 205082.

Blackman, A.J., Dragar, C. and Wells, R.J.. 1979. A new phenol from the brown alga *Perithalia caudata* containing a ‘‘reverse’’ isoprene unit at the 4-position. J Aust J Chem 32, 2783-2786.

Bocanegra, A., Bastida, S., Benedi, J., Rodenas, S. and Sanchez-Muniz, F. J. 2009. Characteristics and nutritional and cardiovascular-health properties of seaweeds. J Med Food 12, 236-258.

Boulares, A. H., Yakovlev, A. G., Ivanova, V., Stoica, B. A., Wang, G., Iyer, S. and Smulson, M. 1999. Role of poly(ADP-ribose) polymerase (PARP) cleavage in apoptosis. Caspase 3-resistant PARP mutant increases rates of apoptosis in transfected cells. J Biol Chem 274, 22932-22940.

Buono, S., Langellotti, A. L., Martello, A., Rinna, F. and Fogliano, V. 2014. Functional ingredients from microalgae. Food Funct 5, 1669-1685.

Burtin, P. 2003. Nutritional value of seaweeds. Electron J Environ Agric Food Chem 2, 498-503.

Calder, P. C. 2006. Polyunsaturated fatty acids and inflammation. Prostaglandins Leukot Essent Fatty Acids 75, 197-202.

Cardozo, K. H., Guaratini, T., Barros, M. P., Falcao, V. R., Tonon, A. P., Lopes, N. P., Campos, S., Torres, M. A., Souza, A. O., Colepicolo, P. and Pinto, E. 2007. Metabolites from algae with economical impact. Comp Biochem Physiol C Toxicol Pharmacol 146, 60-78.

Cavalier-Smith, T. and Chao, E. E. 2006. Phylogeny and megasystematics of phagotrophic heterokonts (kingdom Chromista). J Mol Evol 62, 388-420.

Cho, M., Lee, H. S., Kang, I. J., Won, M. H. and You, S. 2011. Antioxidant properties of extract and fractions from *Enteromorpha prolifera*, a type of green seaweed. Food Chem 127, 999-1006.

Colombo, M. L., Rise, P., Giavarini, F., L, D. E. A., Galli, C. and Bolis, C. L. 2006. Marine macroalgae as sources of polyunsaturated fatty acids. Plant Foods Hum Nutr 61, 67-72.

Czabotar, P. E., Lessene, G., Strasser, A. and Adams, J. M. 2014. Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. Nat Rev Mol Cell Biol 15, 49-63.

Dawczynski, C., Schubert, R. and Jahreis, G. 2007. Amino acids, fatty acids, and dietary fibre in edible seaweed products. Food Chem 103, 891-899.

Dewick, P.M. 2002. Medicinal natural products: A biosynthetic approach. 2nd ed., John Wiley and Sons, Chichester, UK. 507 pp.

Dias, D. A., Urban, S. and Roessner, U. 2012. A historical overview of natural products in drug discovery. Metabolites 2, 303-336.

Dorta, E., Cueto, M., Bito, I. and Darias, J. 2002. New terpenoids from the brown alga *Stypopodium zonale*. J Nat Prod 65, 1727-1730.

Draisma, S.G.A., Ballesteros, E., Rousseau, F. and Thibaut, T. 2010. DNA sequence data demonstrate the polyphyly of the genus *Cystoseira* and Sargassaceae genera (Phaeophyceae). J Phycol 46, 1329–1345.

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

Ercegovic, A. 1952. Sur les *Cystoseira* adriatiques, leur morphologie, ecologie et evolution. Fauna Flora Adriatic 2, 1-212.

Faulkner, D. J. 2002. Marine natural products. Nat Prod Rep 19, 1-48.

Fenical, W., Sims, J.J., Squatrito, D., Wing, R.M. and Radlick, P. 1973. Marine natural products. VII. Zonarol and isozonarol, fungitoxic hydroquinones from the brown seaweed *Dictyopteris zonarioides*. J Org Chem 38, 2383-2386.

Fisch, K. M., Bohm, V., Wright, A. D. and Konig, G. M. 2003. Antioxidative meroterpenoids from the brown alga *Cystoseira crinita*. J Nat Prod 66, 968-975.

Flora, S. J. 2009. Structural, chemical and biological aspects of antioxidants for strategies against metal and metalloid exposure. Oxid Med Cell Longev 2, 191-206.

Giam, M. and Rancati, G. 2015. Aneuploidy and chromosomal instability in cancer: a jackpot to chaos. Cell Div 10, 3.

Goodwin, T. 1974. Carotenoids and biliproteins. *In*: Algal physiology and biochemistry. Stewart W.D.P. (eds). Blackwell Scientific Publications, pp. 176-205.

Guardia, S.D., Valls, R., Mesguiche, V. Brunei, J.-M. and Gulioli, G. 1999. Enantioselective synthesis of (–)-bifuracadiol: a natural antitumor marine product. Tetrahedron Lett 40, 8359–8360.

Guiry, M.D. and Guiry, G.M. Algae Base. World-wide electronic publication, National University of Ireland, Galway, 2016; http://www.algaebase.org (searched on 14.01.16).

Gulioli, G., Oratalo-Magne, A., Daoudi, M., Guyon, H.T., Valls, R. and Piovetti, L. 2004. Trihydroxylated linear diterpenes from the brown alga *Bifurcaria bifurcata*. Phytochemistry 65, 2063–2069.

Haslett, C. 1992. Resolution of acute inflammation and the role of apoptosis in the tissue fate of granulocytes. Clin Sci (Lond) 83, 639-648.

Huang, H.L., Wu, S.L., Liao, H.F., Jiang, C.M., Huang, R.L., Chen, Y.Y., Yang, Y.C. and Chen, Y.J. 2005. Induction of apoptosis by three marine algae through generation of reactive oxygen species in human leukemic cell lines. J Agric Food Chem 9, 1776-1781.

Hussain, M. S., Fareed, S., Ansari, S., Rahman, M. A., Ahmad, I. Z. and Saeed, M. 2012. Current approaches toward production of secondary plant metabolites. J Pharm Bioallied Sci 4, 10-20.

Ishikawa, C., Tafuku, S., Kadekaru, T., Sawada, S., Tomita, M., Okudaira, T., Nakazato, T., Toda, T., Uchihara, J. N., Taira, N., Ohshiro, K., Yasumoto, T., Ohta, T. and Mori, N. 2008. Anti-adult T-cell leukemia effects of brown algae fucoxanthin and its deacetylated product, fucoxanthinol. Int J Cancer 123, 2702-2712.

Ishitsuka, O.M., Kusumi, T. and Kakisawa H. 1988. Antitumor xenicane and norxenicane lactones from the brown algae *Dictyota dichotoma*. J Org Chem 53, 5010-5013.

Jegou, C., Kervarec, N., Cerantola, S., Bihannic, I. and Stiger-Pouvreau, V. 2015. NMR use to quantify phlorotannins: the case of Cystoseira tamariscifolia, a phloroglucinol-producing brown macroalga in Brittany (France). Talanta 135, 1-6.

Kasibhatla, S. and Tseng, B. 2003. Why target apoptosis in cancer treatment? Mol Cancer Ther 2, 573-580.

Keeling, P. J. 2013. The number, speed, and impact of plastid endosymbioses in eukaryotic evolution. Annu Rev Plant Biol 64, 583-607.

Kerr, J.F., Wyllie, A.H. and Currie, A.R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. B J Cancer 26, 239-257.

Khanavi, M., Gheidarloo, R., Sadati, N., Ardekani, M.R., Nabavi, S.M., Tavajohi, S. and Ostad, S.N. 2012. Cytotoxicity of fucosterol containing fraction of marine algae against breast and colon carcinoma cell line. Pharmacogn Mag 8, 60-64.

Khanavi, M., Nabavi, M., Sadati, N., Shams Ardekani, M., Sohrabipour, J., Nabavi, S. M., Ghaeli, P. and Ostad, S. N. 2010. Cytotoxic activity of some marine brown algae against cancer cell lines. Biol Res 43, 31-37.

Kirkin, V., Joos, S. and Zornig, M. 2004. The role of Bcl-2 family members in tumorigenesis. Biochim Biophys Acta 1644, 229-249.

Kong, C. S., Kim, J. A., Yoon, N. Y. and Kim, S. K. 2009. Induction of apoptosis by phloroglucinol derivative from *Ecklonia cava* in MCF-7 human breast cancer cells. Food Chem Toxicol 47, 1653-1658.

Kumari, P., Reddy, C. R. and Jha, B. 2011. Comparative evaluation and selection of a method for lipid and fatty acid extraction from macroalgae. Anal Biochem 415, 134-144.

Lanigan, R. S. and Yamarik, T. A. 2002. Final report on the safety assessment of BHT(1). Int J Toxicol 21 Suppl 2, 19-94.

Lobo, V., Patil, A., Phatak, A. and Chandra, N. 2010. Free radicals, antioxidants and functional foods: Impact on human health. Pharmacogn Rev 4, 118-126.

Lourenço, S.O., Barbarino, E., De-Paula, J.C., da S. Pereira L.O. and Marquez, U.M.L. 2002. Amino acid composition, protein content and calculation of nitrogen-to- protein conversion factors for 19 tropical seaweeds. Phycol Res 50, 233-241.

Luning, K. and Pang, S. J. 2003. Mass cultivation of seaweeds: current aspects and approaches. Journal of Applied Phycology 15, 115-119.

Mabeau, S. and Fleurence, J. 1993. Seaweed in food products: biochemical and nutritional aspects. Trends Food Sci Tech 4, 103-107.

MacArtain, P., Gill, C. I., Brooks, M., Campbell, R. and Rowland, I. R. 2007. Nutritional value of edible seaweeds. Nutr Rev 65, 535-543.

Marinho-Soriano, E., Fonseca, P.C., Carneiro, M.A. and Moreira, W.S. 2006. Seasonal variation in the chemical composition of two tropical seaweeds. Bioresour Technol 97, 2402-2406.

Marsham, S., Scott, G.W. and Tobin, M.L. 2007. Comparison of nutritive chemistry of a range of temperate seaweeds. Food Chem 100, 1331-1336.

Martin, T. and Gutow, L. 2005. The Ecology of Rafting in the Marine Environment. I. the Floating Substrata. Oceanogr Mar Biol Ann Rev 43, 279-418.

Martínez, B. and Rico, M.J. 2002. Seasonal variation of P content and major N pools in *Palmaria palmata* (Rhodophyta). J Phycol 38, 1082–1089.

Megnin-Chanet, F., Bollet, M. A. and Hall, J. 2010. Targeting poly(ADP-ribose) polymerase activity for cancer therapy. Cell Mol Life Sci 67, 3649-3662.

Mhadhebi, L., Laroche-Clary, A., Robert, J. and Bouraoui, A. 2011. Antioxidant, anti-inflammatory, and antiproliferative activities of organic fractions from the Mediterranean brown seaweed *Cystoseira sedoides*. Can J Physiol Pharmacol 89, 911-921.

Misurcova, L., Machu, L. and Orsavova, J. 2011. Seaweed minerals as nutraceuticals. Adv Food Nutr Res 64, 371-390.

Mišurcová, L., Škrovánková, S., Samek, D., Ambrožová, J. and Machů, L. 2012. Health benefits of algal polysaccharides in human nutrition. Adv Food Nutr Res 66, 75-145.

Numata, A., Kambara, S., Takahashi, C., Fujiki, R., Yoneda, M., Fujita, E. and Nabeshima, Y. 1991. Cytotoxic activity of marine algae and a cytotoxic principle of the brown alga *Sargassum tortile*. Chem Pharm Bull 39, 2129-2131.

Patterson, E., Wall, R., Fitzgerald, G. F., Ross, R. P. and Stanton, C. 2012. Health implications of high dietary omega-6 polyunsaturated Fatty acids. J Nutr Metab 2012, 539426.

Pellegrini, L. 1980. Cytological studies on physodes in the vegetative cells of *Cystoseira stricta* Sauvageau (Phaeophyta, Fucales). J Cell Sci 41, 209-231.

Peng, J., Yuan, J. P., Wu, C. F. and Wang, J. H. 2011. Fucoxanthin, a marine carotenoid present in brown seaweeds and diatoms: metabolism and bioactivities relevant to human health. Mar Drugs 9, 1806-1828.

Pereira, H., Barreira, L., Figueiredo, F., Custodio, L., Vizetto-Duarte, C., Polo, C., Resek, E., Engelen, A. and Varela, J. 2012. Polyunsaturated Fatty acids of marine

macroalgae: potential for nutritional and pharmaceutical applications. Mar Drugs 10, 1920-1935.

Plaza, M., Amigo, M., Ibañez, E., Del Castillo, M.D. and Herrero, M. 2010. Neoformation of antioxidants in glycation model systems treated under subcritical water extraction conditions. Food Res Int 43, 1123-1129.

Pucci, B., Kasten, M. and Giordano, A. 2000. Cell cycle and apoptosis. Neoplasia 2, 291-299.

Ragan, M. A. and Craigie, J. S. 1976. Physodes and the phenolic compounds of brown algae. Isolation and characterization of phloroglucinol polymers from *Fucus vesiculosus* (L.). Can J Biochem 54, 66-73.

Rengarajan, T., Rajendran, P., Nandakumar, N., Balasubramanian, M. P. and Nishigaki, I. 2013. Cancer preventive efficacy of marine carotenoid fucoxanthin: cell cycle arrest and apoptosis. Nutrients 5, 4978-4989.

Riisberg, I., Orr, R. J., Kluge, R., Shalchian-Tabrizi, K., Bowers, H. A., Patil, V., Edvardsen, B. and Jakobsen, K. S. 2009. Seven gene phylogeny of heterokonts. Protist 160, 191-204.

Rivlin, N., Brosh, R., Oren, M. and Rotter, V. 2011. Mutations in the p53 Tumor Suppressor Gene: Important Milestones at the Various Steps of Tumorigenesis. Genes Cancer 2, 466-474.

Roberts, M. 1967. Studies on marine algae of the British Isles. 3. The genus *Cystoseira*, B Phycol Bull 3, 345-366.

Rocha-Santos, T. and Duarte, A.C. 2014. Introduction to the analysis of bioactive compounds in marine samples. *In*: Analysis of marine samples in search of bioactive compounds, vol. 65, T. Rocha-Santos and A. C. Duarte (eds). Elsevier, pp. 1–13.

Rodrigues, D., Freitas, A. C., Pereira, L., Rocha-Santos, T. A., Vasconcelos, M. W., Roriz, M., Rodriguez-Alcala, L. M., Gomes, A. M. and Duarte, A. C. 2015. Chemical composition of red, brown and green macroalgae from Buarcos bay in Central West Coast of Portugal. Food Chem 183, 197-207.

Russo, R., Gallelli, L., Cannataro, R., Perri, M., Calignano, A., Citraro, R., Russo, E., Gareri, P., Corsonello, A. and De Sarro, G. 2016. When Nutraceuticals Reinforce Drugs Side Effects: a case report. Curr Drug Saf.

Rwigemera, A., Mamelona, J. and Martin, L. J. 2015. Comparative effects between fucoxanthinol and its precursor fucoxanthin on viability and apoptosis of breast cancer cell lines MCF-7 and MDA-MB-231. Anticancer Res 35, 207-219.

Sarker, S. D., Latif, Z. and Nash, R. J. 2001. Application of gradient-enhanced nuclear Overhauser effect spectroscopy GOESY) in the structure elucidation of plant secondary metabolites. Phytochem Anal 12, 23-27.

Schreiber, V., Dantzer, F., Ame, J. C. and de Murcia, G. 2006. Poly(ADP-ribose): novel functions for an old molecule. Nat Rev Mol Cell Biol 7, 517-528.

Sheu, J.H., Wang, G.H., Sung, P.J. and Duh, C.Y. 1999. New cytotoxic oxygenated fucosterols from the brown alga *Turbinaria conoides*. J Nat Prod 62, 224–227.

Surova, O. and Zhivotovsky, B. 2013. Various modes of cell death induced by DNA damage. Oncogene 32, 3789-3797.

Tait, S. W. and Green, D. R. 2010. Mitochondria and cell death: outer membrane permeabilization and beyond. Nat Rev Mol Cell Biol 11, 621-632.

Tan, M. L., Ooi, J. P., Ismail, N., Moad, A. I. and Muhammad, T. S. 2009. Programmed cell death pathways and current antitumor targets. Pharm Res 26, 1547-1560.

Tao, J., Liu, Q., Wu, X., Xu, X., Zhang, Y., Wang, Q. and Luo, C. 2013. Identification of hypermethylation in hepatocyte cell adhesion molecule gene promoter region in bladder carcinoma. Int J Med Sci 10, 1860-1867.

Taylor, R. W. and Turnbull, D. M. 2005. Mitochondrial DNA mutations in human disease. Nat Rev Genet 6, 389-402.

Tringali, C., Prattellia, M. and Nicols, G. 1984. Structure and conformation of new diterpenes based on the dolabellane skeleton from *Dictyota* species. Tetrahedron 40, 703-799.

Urones, J.G., Basabe, P., Marcos, I.S., Pineda, J., Lithgow, A.M., Moro, R.F., Palma, F.M.S.B.,. Araújo, M.E.M. and Gravalos, M.D.G. 1992. Meroterpenes from *Cystoseira usneoides*. Phytochemistry 31, 179-182.

van Ginneken, V. J., Helsper, J. P., de Visser, W., van Keulen, H. and Brandenburg, W. A. 2011. Polyunsaturated fatty acids in various macroalgal species from North Atlantic and tropical seas. Lipids Health Dis 10, 104.

Wang, K. 2014. Molecular mechanisms of hepatic apoptosis. Cell Death Dis 5, e996.

Williams, G. T. 1991. Programmed cell death: apoptosis and oncogenesis. Cell 65, 1097-1098.

Zubia, M., Fabre, M. S., Kerjean, V., Le Lann, K., Stiger-Pouvreau, V., Fauchon, M. and Deslandes, E. 2009. Antioxidant and antitumoural activities of some Phaeophyta from Brittany coasts. Food Chem 116, 693-701.

CHAPTER 2

PROXIMATE BIOCHEMICAL COMPOSITION AND MINERAL CONTENT OF EDIBLE SPECIES FROM THE GENUS *CYSTOSEIRA* IN PORTUGAL

Vizetto-Duarte C, Custódio L, Barreira L, Moreira da Silva M, Rauter AP, Albericio F and Varela J (2016). Proximate biochemical composition and mineral content of edible species from the genus *Cystoseira* in Portugal. Accepted in Botanica Marina.
DOI: 10.1515/bot-2016-0014.

Proximate biochemical composition and mineral content of edible species from the genus *Cystoseira* in Portugal

Running title: Nutritional profile of *Cystoseira* algae

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2.1 Abstract

Macroalgae are valuable resources for human consumption in many countries. This work reports for the first time a comparative evaluation of the nutritional properties of five edible macroalgae from the genus *Cystoseira*, namely *C. humilis*, *C. tamariscifolia, C. nodicaulis, C. compressa* and *C. baccata*. For this purpose, their proximate composition was determined in terms of moisture, ash, and total contents of protein, lipids, carbohydrates and mineral profile. *Cystoseira tamariscifolia* and *C. baccata* were the species that in general had the higher ash, protein and lipid contents, while the highest levels of moisture and total carbohydrates were detected in *C. nodicaulis* and *C. compressa*. *Cystoseira* species had also high amounts of minerals, especially of potassium, calcium and iron, and a favorable Na/K ratio. The present study shows that *Cystoseira* has a balanced nutritional composition, suitable for human consumption, and that its intake can contribute to a healthy and well-balanced diet.

Keywords: brown algae; *Cystoseira*; minerals; nutritional profile; proximate composition.

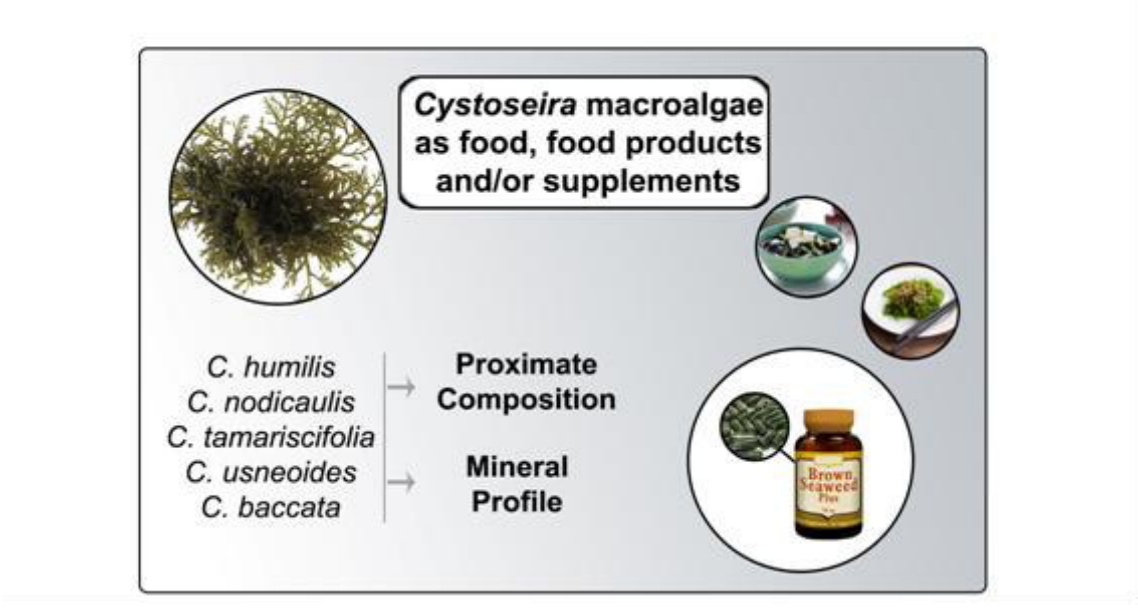

Fig. 2.1 – Graphical abstract for the work achieved in Chapter 2.

2.2 Introduction

It is estimated that the world's population will grow to 9 billion by mid-century, putting substantial demands on the planet's food supply. Macroalgae (also known as "seaweeds") are major coastal resources that are valuable for human consumption in many countries. Edible macroalgae are widely consumed in Asia, but the demand has grown worldwide especially in the United States of America and Europe. Seaweeds are a highly nutritive food that additionally can be eaten in raw salads, soups, cookies, meals, and condiments (Aguilera-Morales et al. 2005). The nutritional value ascribed to macroalgae along with their non-animal nature makes them particularly appropriate for use in the food and additive industries (Lordan et al. 2011). However, compared to their terrestrial counterparts, the chemical composition of macroalgae has been poorly investigated and most of the available information refers to species traditionally consumed in Japanese cuisine. There has also been a growing interest in the mineral content of macroalgae, which is higher than that of many land plant products (Tabarsa et al. 2012). *Cystoseira* is one of the most widely distributed genera of the class Phaeophyceae and is abundant not only on European coasts, but also in the Pacific and Indian Oceans (Valls and Piovetti 1995). Several species belonging to the Phaeophyceae, such as *Laminaria japonica* and *Sargassum naozhouense* (Patra et al. 2015; Peng et al. 2013), among many others, are traditionally used as food. Although *Cystoseira* species are not commonly used as food, they are defined as edible and are potential candidates for food products, nutraceutical and pharmaceutical preparations (Andrade et al. 2013) and thus could be considered as alternative sources of nutritional elements. Therefore, the focus of the present study was to evaluate the proximate composition and mineral content of five species of brown macroalgae belonging to the genus, namely *Cystoseira humilis* Schousboe ex Kützing, *C. tamariscifolia* (Hudson) Papenfuss, *C. nodicaulis* (Withering) M. Roberts, *C. compressa* (Esper) Gerloff & Nizamuddin and *C. baccata* (S.G. Gmelin) P.C. Silva, as well as to consider and discuss the use of these macroalgae as food and/or incorporated in supplements. To the best of the authors' knowledge this is the first report on the proximate composition and mineral contents of these *Cystoseira* species.

2.3 Materials and methods

2.3.1 Algal biomass sampling and preparation

Cystoseira humilis, *C. compressa*, *C. tamariscifolia*, *C. nodicaulis* and *C. baccata* were collected in the middle/lower intertidal areas of the Algarve (Albufeira and Odeceixe, Portugal) coast, in May 2010, during low tide (Table 2.1). Specimens were identified by Dr Aschwin Engelen (Centre of Marine Sciences, University of Algarve, Portugal) and Dr Javier Cremades Ugarte (Facultade de Ciencias, University of A Coruña). Voucher specimens of *C. compressa* (code number MB004), *C. humilis* (code number MB007), *C. tamariscifolia* (code number MB016), *C. nodicaulis* (code number MB014-2), and *C. baccata* (code number MB001) are deposited at the Centre of Marine Sciences, University of Algarve. Individual thalli were washed thoroughly with freshwater upon arrival in the laboratory to remove epiphytes and washed again with distilled water. In order to have enough biomass for the determination of the proximate composition and minerals, each replicate was prepared by combining two to three individual thalli of a given species. The replicates were assessed for moisture content while fresh and were then oven-dried, ground into a fine powder and stored in tightly closed plastic bags containing silica gel until analysis.

Table 2.1 Date and location of sample collection of different *Cystoseira* species from Portugal.

	Date	GPS coordinates	Location (Beach, County)	Voucher specimen code number
C. humilis	07-05-2010	37º4'36.19''N 8º18'36.49''W	Manuel Lourenço, Albufeira	MB007
C. compressa	08-05-2010	37º4'35.83''N 8º16'33.74''W	Arrifes, Albufeira	MB004
C. tamariscifolia	07-05-2010	37º5'24.31''N 8º11'9.72''W	Olhos de Água, Albufeira	MB016
C. nodicaulis	07-05-2010	37º4'36.19''N 8º18'36.49''W	Manuel Lourenço, Albufeira	MB014-2
C. baccata	11-05-2010	37º26'41.48''N 8º48'3.90''W	Odeceixe, Aljezur	MB001

2.3.2 Proximate biochemical analysis

For the determination of the proximate composition, 6 replicates were prepared as described in the previous section. Moisture was determined by drying the replicates at 95°C until constant weight as described by the AOAC method (AOAC 1995). Ash content was measured by weight difference before and after 5 hours of incineration in a muffle furnace at 525°C. Total nitrogen content of dried samples was determined using a Vario EL III elemental analyzer (Elementar), and the protein content was estimated by multiplying the total nitrogen content by a nitrogen conversion factor of 6.25 (Lourenço et al. 2002). Total lipid content was determined by a modified method of Bligh & Dyer (Pereira et al. 2013). Carbohydrate content was assumed to be the remaining biomass and was calculated from the difference between 100% and the summed contents of ash, protein and lipids (Marinho-Soriano et al. 2006).

2.3.3 Mineral content

Mineral elements were analyzed by atomic absorption spectrometry (AAS). Three replicates about approximately 300 mg of the dried powder from each species were digested by microwave (Milestone Ethos Touch) in high-pressure Teflon vessels. The digestion was made using 6 ml of Fluka supra-pure HNO_3 (65 %), 1 ml of Riedel-Dehaen p.a. $HClO_4$ (70 %) and 1 ml of Merck supra-pure H_2O_2 (30 %) (ETHOS PLUS, 2001). A procedural blank was prepared and included in each digestion batch of 10 samples. Calcium, magnesium, sodium, potassium, iron, manganese and zinc were analyzed by flame AAS with an air-acetylene flame in an atomic absorption spectrometer (GBC Avanta Sigma, Australia). The accuracy of the analytical procedure was assessed by the analysis of certified reference material (BCR-60 - aquatic plant *Lagarosiphon major*, from the Institute for Reference Materials and Measurements, JRC-IRMM, Belgium). Procedural blanks always accounted for less than 1% of the metal concentrations in samples. Values were expressed as g kg^{-1} dry weight (DW) for Ca, Mg, Na and K or mg kg^{-1} DW for Fe, Mn and Zn.

2.3.4 Statistical analysis

Biological replicates were collected on a given date and location (Table 2.1) and were used for the determination of the proximate (n = 6) and mineral composition (n = 3). Significant differences were assessed by analysis of variance (ANOVA) and significance between means was analyzed by the Tukey HSD test (p<0.05). SPSS statistical package for Windows (release 15.0, SPSS Inc.) was used.

2.4 Results

The present study aimed to evaluate the proximate composition of different *Cystoseira* species (namely *C. humilis*, *C. compressa*, *C. tamariscifolia*, *C. nodicaulis* and *C. baccata*) collected on the Algarve coast, Portugal. Results are summarized in Table 2.2. The moisture content ranged from 49% of wet weight (WW) in *C. tamariscifolia* to 63% in *C. compressa*, while the ash levels varied from 7% in *C. compressa* to 24% in *C. tamariscifolia*. *Cystoseira tamariscifolia* also presented the lowest total carbohydrate (54%) and the highest protein (13%) contents. Conversely, *C. nodicaulis*, along with *C. compressa*, had the highest total carbohydrate levels (73%) and low protein contents (9-10%). The highest level of total lipids was 11% in *C. baccata*, followed by *C. tamariscifolia* (10%) and the lowest value was observed in *C. nodicaulis* (4%, Table 2.2).

Table 2.2 Proximate composition of different *Cystoseira* species from Portugal, including moisture (% of wet weight), ash, total protein, total lipids, carbohydrates (% of dry weight). Values are mean (n = 6). Different superscript letters indicate that, for each variable, differences between species are significant at p = 0.05.

	C. humilis	***C. compressa***	***C. tamariscifolia***	***C. nodicaulis***	***C. baccata***
Moisture	57.06^{b}	63.05^{a}	48.99^{d}	58.95^{b}	51.43^{c}
Ash	$20.35^{a,b}$	7.30^{d}	23.85^{a}	13.45^{c}	19.10^{b}
Protein	10.34^{b}	10.16^{b}	12.52^{a}	9.20^{c}	12.46^{a}
Lipids	5.22^{c}	9.45^{b}	9.57^{b}	4.31^{d}	10.92^{a}
Carbohydrates	64.09	73.09	54.06	73.04	57.52

The mineral content of the samples was also evaluated, and results are given in Table 2.3. The total for all macrominerals, expressed as the sum of K, Na, Ca and Mg, ranged from 40 g kg^{-1} in *C. baccata* to 101 g kg^{-1} in *C. compressa*. However, in terms of

microminerals, *C. tamariscifolia* was the highest (1013 mg kg^{-1}) and *C. compressa* the lowest (173 mg kg^{-1}). Potassium was the most abundant macromineral in *C. compressa*, *C. humilis* and *C. baccata* (60, 30 and 22 g kg^{-1}, respectively). Sodium content ranged from 8 g kg^{-1} in *C. humilis* to 16 g kg^{-1} in *C. tamariscifolia*. The Na/K ratios varied from 0.15 in *C. compressa* and 0.97 in *C. tamariscifolia* (Table 2.3). In *Cystoseira tamariscifolia* and *C. nodicaulis*, calcium was the most abundant macromineral (both around 26 g kg^{-1}). Magnesium concentration varied from 5.7 g kg^{-1} in *C. nodicaulis* to 19 g kg^{-1} in *C. compressa*.

Table 2.3 Mineral content of different *Cystoseira* species. Values are mean (n = 3). Different superscript letters indicate that, for each variable, differences between species are significant at $p = 0.05$.

Mineral	Symbol	*C. humilis*	*C. compressa*	*C. tamariscifolia*	*C. nodicaulis*	*C. baccata*
Expressed as g kg^{-1} dry biomass						
Calcium	**Ca**	23.70^{b}	13.08^{c}	25.82^{a}	26.50^{a}	9.43^{d}
Magnesium	**Mg**	12.90^{b}	18.53^{a}	6.56^{c}	5.69^{d}	6.78^{c}
Sodium	**Na**	7.56^{e}	8.94^{d}	16.36^{a}	11.61^{b}	10.81^{c}
Potassium	**K**	29.78^{b}	60.02^{a}	16.87^{d}	15.98^{e}	22.10^{c}
Na/K ratio		0.25	0.15	0.97	0.73	0.49
Total		73.94	100.57	65.61	59.78	39.69
Expressed as mg kg^{-1} dry biomass						
Iron	**Fe**	171.88^{c}	149.74^{d}	508.06^{a}	413.14^{b}	109.59^{e}
Manganese	**Mn**	152.18^{c}	14.27^{e}	398.46^{a}	193.54^{b}	108.35^{d}
Zinc	**Zn**	41.16^{c}	9.43^{d}	105.99^{b}	113.82^{a}	106.73^{b}
Total		365.22	173.44	1012.51	720.50	324.67

Among the microminerals detected, iron was the most abundant ranging from 110 mg kg^{-1} in *C. baccata* to 508 mg kg^{-1} in *C. tamariscifolia*. *Cystoseira tamariscifolia* also had the highest content of manganese (398 mg kg^{-1}), while the lowest manganese content was observed in *C. compressa* (14 mg kg^{-1}), which also contained the lowest amount of zinc (9.4 mg kg^{-1}). The highest content of zinc was found in *C. nodicaulis* (114 mg kg^{-1}), though *C. tamariscifolia* and *C. baccata* also presented values above 100 mg kg^{-1} (Table 2.3).

2.5 Discussion

This study reports for the first time the proximate composition and mineral profiles of five species of *Cystoseira* found on the coast of Algarve (Portugal), namely *C. humilis*, *C. tamariscifolia*, *C. nodicaulis*, *C. compressa* and *C. baccata*. The first parameter assessed was the moisture of fresh specimens. As found in most brown macroalgae, the moisture values for fresh specimens were high. For example, the moisture determined for *C. compressa* was 63%, which was similar to the value reported for *Sargassum* (Holdt and Kraan 2011).

Most ash contents obtained for *Cystoseira* are in agreement with published values for other Phaeophyceae, such as those of *Ascophyllum* (18–27%), *Fucus* (19-30%) and *Sargassum* (14-44%). The only exception was the ash content for *C. compressa* (7%), which was lower than the usual range (Holdt and Kraan 2011).

The protein values obtained in this study (9-13% DW) are of the same order as those reported in previous studies on brown algae. In previous studies, the protein content of *Ascophyllum* and *Sargassum* ranged from 1.2-12% and 9-20%, respectively (Holdt and Kraan 2011). The protein content is often lower in brown macroalgae than in green and red macroalgae (Holdt and Kraan 2011).

Regarding total lipids, the *Cystoseira* species showed higher levels than its green and red counterparts. In general, macroalgae have low lipid contents, usually below 5% of dry matter (Burtin 2003). However, *C. compressa, C. tamariscifolia* and *C. baccata* showed total lipid contents higher than 9.4%. Gosch et al. (2012) claimed that brown macroalgae typically have the highest levels of total lipids amongst seaweeds. In fact, some species have total lipid content higher than 15%, particularly those from the Dictyotales order, which consistently display total lipid contents between 11 and 20% DW (McDermid and Stuercke 2003). Our results are in accordance with these data, showing that the closely related *Cystoseira* (Fucales) algae also contain relatively high lipid levels. The fatty acid profiles of *C. humilis*, *C. compressa*, *C. tamariscifolia*, *C. nodicaulis* and *C. baccata* have been reported previously (Vizetto-Duarte et al. 2015) and showed that polyunsaturated fatty acids (PUFA) in the studied *Cystoseira* species corresponded to 29 – 46% of the total fatty acids detected. In addition, *Cystoseira* species also previously presented high nutritional value due to their low PUFA *vs.* saturated fatty acids (SFA) ratio (PUFA/SFA), low n-6 PUFA/n-3 PUFA ratios as well

as favorable unsaturation, atherogenicity and thrombogenicity indices (Vizetto-Duarte et al. 2015).

In this study, the carbohydrate content varied from 42 to 73% DW in *Cystoseira* species. Interestingly, Hadj Ammar et al. (2015) showed lower total carbohydrate contents (13-45% DW) for *C. compressa*, *C. sedoides* and *C. crinita* collected in June in Tunisia. A possible explanation for the observed differences might be the location and season of the year when the thalli were sampled. Moreover, carbohydrate contents might also vary with the physiological state (e.g. developmental stage of the alga), and environmental factors (e.g. temperature and depth; Holdt and Kraan 2011). Previous studies have also suggested an inverse relationship between carbohydrates and proteins in macroalgae (Marinho-Soriano et al. 2006), which was also observed in our samples.

Macroalgae are known to have a high mineral content, usually higher than of most terrestrial plants (Ortega-Calvo et al. 1993). In fact, edible seaweeds are considered to be valuable nutritional resources due to their high content of several essential minerals (Bocanegra et al. 2009). Potassium and sodium values of the macroalgae here studied were higher than those reported for land vegetables, such as lettuce and spinach (USDA 2015). Nevertheless, the Na/K ratios were below 1.0 in all the species of *Cystoseira* studied (0.15–0.97, Table 2.3), which is interesting from a nutritional point of view, since diets with a high Na/K ratio have been related to the incidence of hypertension (Taboada et al. 2010). For instance, Na/K ratio in sausages is 4.89 (Ortega-Calvo et al. 1993). Calcium and magnesium also recorded high values compared to that of land vegetables (USDA 2015). Regarding microminerals, *C. tamariscifolia* and *C. nodicaulis* displayed remarkably high iron contents (> 400 mg kg^{-1}) as compared to those of other *Cystoseira* species (Table 2.3) and of commercial seaweeds such as *Fucus*, *Laminaria*, *Undaria*, *Chondrus* and *Porphyra* (33-103 mg kg^{-1}; Rupérez 2002). The same conclusion can be drawn with respect to the iron concentrations usually found in terrestrial vegetables (35-233 mg kg^{-1}; USDA 2015). Ortega-Calvo et al. (1993) reported that *Fucus* sp., a brown edible marine macroalga, had manganese contents ranging from 33–190 mg kg^{-1} and zinc levels from 42–37000 mg kg^{-1}. In a study by Rupérez (2002), *Fucus vesiculosus* contained 55 mg kg^{-1} of Mn and 71 mg kg^{-1} of Zn. The concentration of these trace elements detected by us (Table 2.3) also fall within the ranges observed in previous reports on macroalgae, except for *C. compressa* that exhibited lower values. Mineral composition is also known to vary according to

seasonal, environmental, geographical and physiological factors (Mabeau and Fleurence 1993).

2.6 Conclusions

Taken together, our results indicate that *Cystoseira* has a balanced nutritional composition suitable for human consumption, and that its intake could contribute to a healthy and well-balanced diet. This study showed that species of *Cystoseira* have high ash and carbohydrate contents, and relatively high total lipids compared to other macroalgae. Mineral analysis also showed that these species contain useful amounts of macrominerals and trace elements. Among the nutritional profiles described here for the first time, *C. tamariscifolia* stands out for its high content of ash (and therefore, minerals) and total protein, its high lipid content, and relatively low carbohydrate content. *Cystoseira tamariscifolia* also contains high Ca and was the species with highest micromineral levels, especially Fe and Mn. The Na/K ratio of all species was suitable for human diet. Together with recent studies on the edibility of *Cystoseira* and the need for alternative sources of non-animal food, our results show that these species could be used as alternative sources of valuable food products.

2.7 Acknowledgments

Financial support for this work was provided by the SEABIOMED (PTDC/MAR/103957/2008) and XTREMEBIO (PTDC/MAR-EST/4346/2012) projects and CCMAR/Multi/04326/2013 funded by the Foundation for Science and Technology (FCT) and the Portuguese National Budget. Vizetto-Duarte C. acknowledges an FCT doctoral research grant (SFRH/BD/81425/2011). Custódio L. is supported by FCT Investigator Programme (IF/00049/2012).

2.8 References

Aguilera-Morales, M., Casas-Valdez, M., Carrillo-Domìnguez, S., González-Acosta, B. and Perez-Gil, F. 2005. Chemical composition and microbiological assays of marine algae *Enteromorpha* spp. as a potential food source. J Food Compos Anal 18, 79-88.

Andrade, P.B., Barbosa, M., Matos, R.P., Lopes, G., Vinholes, J., Mouga, T. and Valentão, P. 2013. Valuable compounds in macroalgae extracts. Food Chem 138, 1819-1828.

AOAC. 1995. Official Methods of Analysis. Arlington, VA: Association of Official Analytical Chemists.

Bocanegra, A., Bastida, S., Benedí, J., Ródenas, S. and Sánchez-Muniz, F.J. 2009. Characteristics and nutritional and cardiovascular-health properties of seaweeds. J Med Food 12, 236-258.

Burtin, P. 2003. Nutritional value of seaweeds. Electron J Environ Agric Food Chem 2, 498-503.

Gosch, B.J., Magnusson, M., Paul, N. A. and de Nys, R. 2012. Total lipid and fatty acid composition of seaweeds for the selection of species for oil-based biofuel and bioproducts. GCB Bioenergy 4, 919-930.

Hadj Ammar, H., Lajili, S., Ben Said, R., Le Cerf, D., Bouraoui, A. and Majdoub, H. 2015. Physico-chemical characterization and pharmacological evaluation of sulfated polysaccharides from three species of Mediterranean brown algae of the genus *Cystoseira*. Daru 23, 1.

Holdt, S.L. and Kraan, S. 2011. Bioactive compounds in seaweed: functional food applications and legislation. J Appl Phycol 23, 543-597.

Lordan, S., Ross, R.P. and Stanton, C. 2011. Marine bioactives as functional food ingredients: potential to reduce the incidence of chronic diseases. Mar Drugs 9, 1056-1100.

Lourenço, S.O., Barbarino, E., De-Paula, J.C., Pereira, L.O.S. and Marquez, U.M.L. 2002. Amino acid composition, protein content and calculation of nitrogen-to-protein conversion factors for 19 tropical seaweeds. Phycol Res 50, 233-241.

Mabeau, S. and Fleurence, J. 1993. Seaweed in food products: biochemical and nutritional aspects. Trends Food Sci Tech 4, 103-107.

Marinho-Soriano, E., Fonseca, P.C., Carneiro, M.A. and Moreira, W.S. 2006. Seasonal variation in the chemical composition of two tropical seaweeds. Bioresour Technol 97, 2402-2406.

McDermid, K.J. and Stuercke, B. 2003. Nutritional composition of edible Hawaiian seaweeds. J Appl Phycol 15, 513-524.

Ortega-Calvo, J.J., Mazuelos, C., Hermosin, B. and Saiz-Jimenez, C. 1993. Chemical composition of *Spirulina* and eukaryotic algae food products marketed in Spain. J Appl Phycol 5, 425-435.

Patra, J.K., Das, G. and Baek, K.-H. 2015. Chemical Composition and Antioxidant and Antibacterial activities of an essential oil extracted from an edible seaweed, *Laminaria japonica* L. Molecules 20, 12093-12113.

Peng, Y., Xie, E., Zheng, K., Fredimoses, M., Yang, X., Zhou, X., Wang, Y., Yang, B., Lin, X., Liu, J. and Liu, Y. 2013. Nutritional and chemical composition and antiviral activity of cultivated seaweed *Sargassum naozhouense* Tseng et Lu. Mar Drugs 11, 20-32.

Pereira, H., Barreira, L., Custódio, L., Alrokayan, S., Mouffouk, F., Varela J., Abu-Salah, K.M. and Ben-Hamadou. R. 2013. Isolation and fatty acid profile of selected microalgae strains from the Red Sea for biofuel production. Energies 6, 2773-2783.

Rupérez, P. 2002. Mineral content of edible marine seaweeds. Food Chem 79, 23-26.

Tabarsa, M., Rezaei, M., Ramezanpour, Z., Waaland, J.R. and Rabiei, R. 2012. Fatty acids, amino acids, mineral contentes and proximate composition of some brown seaweeds. J Phycol 48, 285-292.

Taboada, C., Millán, R. and Míguez, I. 2010. Composition, nutritional aspects and effect on serum parameters of marine algae *Ulva rigida*. J Sci Food Agric 90, 445-449.

US Department of Agriculture, Agricultural research service. USDA National Database for Standard Reference, Release 28 (2015). Available at: http://ndb.nal.usda.gov/ndb/foods [accessed on 3 February, 2016].

Valls, R. and Piovetti, L. 1995. The chemistry of the cystoseiraceae (Fucales: Pheophyceae): Chemotaxonomic relationships. Biochem Syst Ecol 23, 723-745.

Vizetto-Duarte, C., Pereira, H., Bruno de Sousa, C., Rauter, A.P., Albericio, F., Custódio, L., Barreira, L. and Varela, J. 2015. Fatty acid profile of different species of algae of the *Cystoseira* genus: a nutraceutical perspective. Nat Prod Res 29, 1264-1270.

CHAPTER 3

FATTY ACID PROFILE OF DIFFERENT SPECIES OF ALGAE OF THE *CYSTOSEIRA* GENUS: A NUTRACEUTICAL PERSPECTIVE

Vizetto-Duarte C, Pereira H, Bruno de Sousa C, Rauter AP, Alberício F, Custódio L, Barreira L, Varela J (2015). Fatty acid profile of different species of algae of the *Cystoseira* genus: a nutraceutical analysis. Natural Products Research, 2: 1-7.
DOI: 10.1080/14786419.2014.992343.

Fatty acid profile of different species of algae of the *Cystoseira* genus: a nutraceutical perspective

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3.1 Abstract

The fatty acid (FA) composition of six macroalgae from the *Cystoseira* genus, namely *C. compressa*, *C. humilis*, *C. tamariscifolia, C. nodicaulis, C. baccata* and *C. barbata*, was determined. Polyunsaturated fatty acids (PUFA) corresponded to 29-46% of the total FA detected. *C. compressa*, *C. tamariscifolia* and *C. nodicaulis* stood out for their high PUFA/SFA, low n-6 PUFA/n-3 PUFA ratios as well as favourable unsaturation, atherogenicity and thrombogenicity indices, suggesting a high nutritional value with potential applications in the nutraceutical industry.

Keywords: brown algae; *Cystoseira*; fatty acids; nutrition; PUFA.

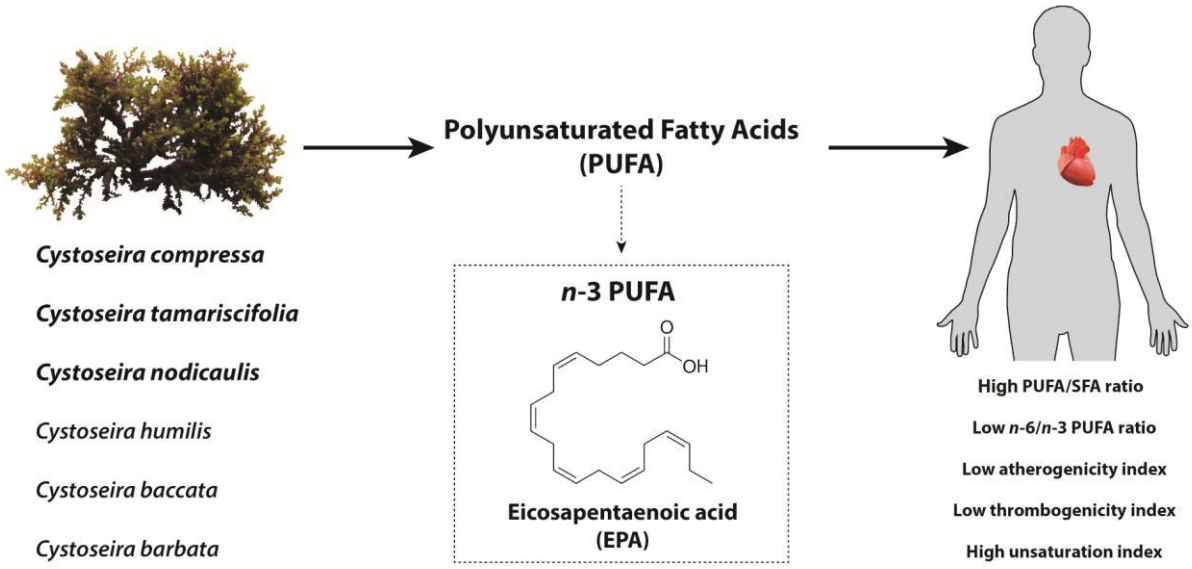

Fig. 3.1 – Graphical abstract of the study completed on Chapter 3.

3.2 Introduction

Marine macroalgae are being increasingly exploited as sources of polyunsaturated fatty acids (PUFA) for nutritional purposes (Pereira et al. 2012). The main dietary source of n-3 PUFA in humans is seafood, through the direct consumption of oily fish (e.g. salmon and mackerel). However, the sustainability of fish sources for the exploitation of PUFA is rather uncertain due to declining fish stocks and increasing market demand (FAO 2010). Hence, there is a need to find sustainable alternative sources of PUFA for food and feed applications. Ochrophyta (e.g. *Cystoseira*) is one of the most promising phyla of algae due to increased amounts of PUFA commonly detected in several species when compared to other algal phyla (Colombo et al. 2006; Silva et al. 2013).

In this sense, the objectives of this work are to report the fatty acid (FA) profile of six species of brown macroalgae belonging to the *Cystoseira* genus, namely *C. humilis*, *C. compressa*, *C. tamariscifolia*, *C. nodicaulis*, *C. baccata* and *C. barbata*, as well as consider and discuss the use of these seaweeds as sources of nutraceuticals . To the best of the authors' knowledge this is the first report on the FA profile of *C. compressa*, *C. humilis*, *C. baccata* and *C. barbata*.

3.3 Experimental procedures

3.3.1 Sampling and processing

C. compressa, *C. humilis*, *C. tamariscifolia*, *C. nodicaulis* and *C. baccata* were collected in May 2010 on the Algarve (Portugal) coast, namely in Albufeira and Odeceixe beaches. *C. barbata* was collected on the Mediterranean coast near Cádiz, Spain. Identification of specimens was made by Dr Aschwin Engelen (Centre of Marine Sciences, University of Algarve, Portugal) and Dr Javier Cremades Ugarte (Facultade de Ciencias, University of A Coruña). A voucher specimen of *C. compressa* (code number MB004), *C. humilis* (code number MB007), *C. tamariscifolia* (code number MB016), *C. nodicaulis* (code number MB014-2), *C. baccata* (code number MB001) and *C. barbata* (code number MB017) is deposited at the Centre of Marine Sciences, University of Algarve. Samples were kept cold and, upon arrival to the laboratory, washed thoroughly with freshwater to remove epiphytes. Biomass was then washed with distilled water, freeze dried, pulverized into powder and stored at -20 °C until further analysis.

3.3.2 FA composition

The extraction and analysis of FA were made according to a modified Lepage and Roy (1984) procedure as described by Pereira et al. (2012).

3.3.3 Determination of fatty acid methyl esters profile by GC-MS

Extracts were analyzed on an Agilent GC-MS (Agilent Technologies 6890 Network GC System, 5973 Inert Mass Selective Detector) as described in Pereira et al. (2012).

3.3.4 Determination of nutritional indices

The unsaturation index (UI) was determined by summing up the percentage of each FA multiplied by its number of double bonds (Kumari et al. 2013). The atherogenicity index (AI) and the thrombogenicity index (TI) were determined according to Ulbricht & Southgate (1991) equations:

$$\mathrm{AI} = \frac{\mathrm{C12{:}0} + 4 \times \mathrm{C14{:}0} + \mathrm{C16{:}0}}{\Sigma\mathrm{MUFA} + \Sigma(\mathrm{n-6\ PUFA}) + \Sigma(\mathrm{n-3\ PUFA})}$$

$$\mathrm{TI} = \frac{\mathrm{C14{:}0} + \mathrm{C16{:}0} + \mathrm{C18{:}0}}{0.5 \times \Sigma\mathrm{MUFA} + 0.5\ \times \Sigma(\mathrm{n-6\ PUFA}) + 3 \times \Sigma(\mathrm{n-3\ PUFA}) + \frac{\Sigma(\mathrm{n\text{-}3\ PUFA})}{\Sigma(\mathrm{n\text{-}6\ PUFA})}}$$

where ΣMUFA, Σ(n-3 PUFA) and Σ(n-6 PUFA) are the sum of MUFA, n-3 and n-6 PUFA in % of total fatty acids (TFA), respectively.

3.3.5 Statistical analysis

Results are represented as mean ± standard deviation (SD) of four replicates. Differences between species were assessed using analysis of variance (ANOVA). Post-hoc comparisons were determined using the Tukey HSD test. Significant differences were considered when $p < 0.05$ by means of the statistical program StatSoft STATISTICA (release 7.0).

3.4 Results and discussion

3.4.1 Total fatty acid methyl esters (FAME) concentration

Total FAME concentration (Fig.3.2) ranged from 6.7 in *C. humilis* to 9.4 mg/g of dry weight (DW) in *C. nodicaulis*. These data is in accordance with the values obtained for

other ochrophytes (Pereira et al. 2012). However, the total FAME concentration obtained for *C. nodicaulis* and *C. tamariscifolia* in this work (9.4 and 8.4, respectively) was higher than the ones reported by Silva et al. 2013 (1.9 and 0.8, respectively).

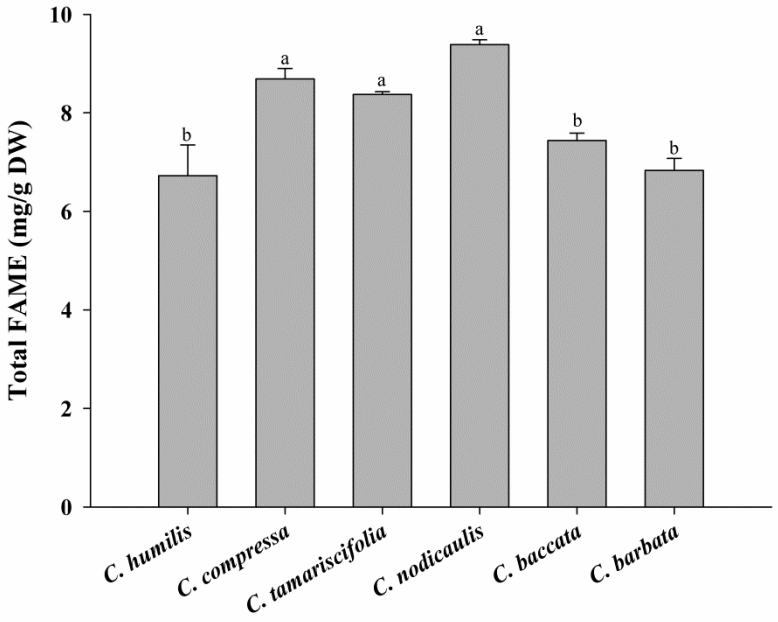

Fig. 3.2 – Total FAME concentration of different macroalgae from *Cystoseira* genus. Bars represent means ± SD ($n = 4$). Different letters (a-b) indicate significant differences by Tukey HSD test at $p < 0.05$.

3.4.2 Fatty acid profiles

Saturated fatty acid (SFA) varied from 34% in *C. compressa* to 54% in *C. barbata*. Palmitic acid (C16:0) was the most abundant SFA in all studied species, ranging from 26% in *C. compressa* and *C. nodicaulis* to 35% of total fatty acids (TFA) in *C. barbata* (Table 3.1). Our results are in accordance with the ones obtained in different *Cystoseira* species, namely *C. nodicaulis, C. tamariscifolia, C. usneoides, C. abies-marina, C. crinita* and *C. osmundacea* from other locations (Khotimchenko et al. 2002; Frikha et al. 2011; Ivanova et al. 2013; Patarra et al. 2013; Silva et al. 2013). It is known that a high intake of palmitic acid has a cholesterol-raising effect (Clandinin et al. 2000); however, this effect can be counterbalanced by high levels of linoleic acid (Clandinin et al. 2000; French et al. 2002). Monounsaturated fatty acids (MUFA) ranged from 13% of TFA in *C. humilis* to 23% of TFA in *C. baccata* (Table 3.1). Palmitoleic acid (C16:1) varied from 2% in *C. nodicaulis* to 13% in *C. baccata*. Oleic acid (C18:1) corresponded

to 9% and 17% of TFA in *C. tamariscifolia* and *C. compressa*, respectively. These MUFA are commonly reported as the majors MUFA in *C. barbata* (Frikha et al. 2011), *C. nodicaulis, C. tamariscifolia*, *C. usneoides* (Silva et al. 2013), *C. abies-marina* (Patarra et al. 2013), *C. crinita* (Ivanova et al. 2013) and *C. osmundacea* (Khotimchenko et al. 2002). Diets rich in MUFA (and PUFA) have showed to reduce the total cholesterol in plasma and low density lipoprotein cholesterol in clinical studies (Ginsberg et al. 1990). Regarding PUFA, *C. compressa*, *C. tamariscifolia* and *C. nodicaulis* were the species with the highest PUFA levels (Table 3.1). Total PUFA contents varied from 29% in *C. barbata* and 46% of TFA in *C. compressa.* Arachidonic acid (C20:4n-6, AA) was the most abundant PUFA detected in all studied species (12 - 27% of TFA) except for *C. humilis* which presented a higher abundance of linoleic acid (C18:2n-6, LA). These results are in accordance with previous studies, where AA was reported to be the main PUFA in *C. osmundacea* (Khotimchenko et al. 2002). Although AA may have a proinflammatory role, recent studies have shown that AA supplementation can contribute to lower coronary risk (Chowdhury et al. 2014) and might be beneficial for the development of the nervous central system (Uauy et al., 2001). Interestingly, n-3 PUFA, such as eicosapentaenoic acid (C20:5n-3, EPA), which was detected in most species at relatively high amounts (5% – 10%), has been shown to compete with the conversion of LA to AA, regulating in this way the relative amounts of n-3 and n-6 PUFA (Calder 2012). Moreover, EPA presents potential beneficial applications in asthma, psoriasis, rheumatoid arthritis, antibiotic, inflammatory bowel disease, depression, allergies, cardiovascular diseases and cancer treatment, among others (Calder 2010). Considering all these benefits there is increasing interest in the incorporation of EPA in diet in order to meet the European recommendations of n-3 PUFA, namely EPA + docosahexaenoic acid (DHA): 250 mg/day (EFSA, 2010). Although DHA was not detected in any of the samples evaluated in this work, these species can be interesting sources of EPA. DHA is generally absent or is present at low levels in different ochrophytes, including *C. nodicaulis*, *C. tamariscifolia* and *C. usneoides* (Pereira et al. 2012; Silva et al. 2013).

Table 3.1. Fatty acid methyl esters (FAME) profile of different *Cystoseira* species (*C. humilis*, *C. compressa*, *C. tamariscifolia*, *C. nodicaulis* and *C. baccata*).

Fatty acid (%)	Common name	*C. humilis*	*C. compressa*	*C. tamariscifolia*	*C. nodicaulis*	*C. baccata*	*C. barbata*
C12:0	Lauric acid	0.33 ± 0.04	nd	nd	nd	nd	nd
C14:0	Myristic acid	15.25 ± 1.06	4.34 ± 0.05	7.83 ± 0.05	7.15 ± 0.20	9.69 ± 0.31	8.59 ± 0.10
C15:0	Pentadecanoic acid	0.42 ± 0.01	0.32 ± 0.01	0.50 ± 0.01	0.48 ± 0.02	0.75 ± 0.10	1.11 ± 0.08
C16:0	Palmitic acid	33.86 ± 0.44	26.47 ± 0.08	27.47 ± 0.11	26.81 ± 0.12	28.09 ± 0.18	35.15 ± 0.61
C17:0	Margaric acid	nd	nd	0.17 ± 0.01	0.19 ± 0.01	1.14 ± 0.37	1.47 ± 0.02
C18:0	Stearic acid	1.08 ± 0.06	0.76 ± 0.01	1.22 ± 0.03	1.17 ± 0.11	1.48 ± 0.19	2.14 ± 0.15
C20:0	Arachidic acid	nd	0.72 ± 0.03	0.71 ± 0.01	0.98 ± 0.01	1.45 ± 0.05	1.78 ± 0.19
C22:0	Behenic acid	nd	0.93 ± 0.03	0.78 ± 0.01	0.67 ± 0.12	2.01 ± 0.09	2.17 ± 0.39
C24:0	Lignoceric acid	nd	0.74 ± 0.03	0.54 ± 0.02	nd	1.72 ± 0.07	1.98 ± 0.23
∑ SFA		**50.94 ± 1.15** [b]	**34.28 ± 0.11** [f]	**39.22 ± 0.13** [d]	**37.45 ± 0.28** [e]	**46.33 ± 0.57** [c]	**54.39 ± 0.81** [a]
C16:1	Palmitoleic acid	3.84 ± 0.12	2.74 ± 0.01	7.50 ± 0.08	2.31 ± 0.03	13.15 ± 0.35	4.87 ± 0.21
C18:1	Oleic acid	9.24 ± 0.22	17.07 ± 0.06	8.73 ± 0.16	16.44 ± 0.15	10.00 ± 0.21	11.68 ± 0.12
C20:1	Eicosenoic acid	nd	nd	0.21 ± 0.01	0.15 ± 0.01	nd	nd
∑ MUFA		**13.08 ± 0.20** [d]	**19.81 ± 0.06** [b]	**16.44 ± 0.09** [c]	**18.90 ± 0.15** [b]	**23.15 ± 0.41** [a]	**16.55 ± 0.24** [c]
C18:2 (n-6)	Linoleic acid	17.08 ± 0.41	10.45 ± 0.04	7.05 ± 0.06	8.93 ± 0.03	3.48 ± 0.23	5.31 ± 0.18
C20:2 (n-6)	Eicosadienoic acid	0.57 ± 0.01	0.60 ± 0.01	0.40 ± 0.01	0.62 ± 0.01	0.89 ± 0.02	1.26 ± 0.11
C16:3 (n-3)	Hexadecatrienoic acid	nd	nd	0.34 ± 0.01	nd	nd	nd
C18:3 (n-6)	γ-Linolenic acid	nd	2.28 ± 0.01	1.76 ± 0.02	nd	nd	nd
C20:3 (n-6)	Eicosatrienoic acid	1.04 ± 0.09	2.35 ± 0.01	2.13 ± 0.03	2.53 ± 0.05	0.99 ± 0.03	1.20 ± 0.12
C20:4 (n-6)	Arachidonic acid	11.71 ± 1.22	20.11 ± 0.05	22.82 ± 0.16	26.51 ± 0.20	19.93 ± 0.44	19.08 ± 0.82
C20:5 (n-3)	Eicosapentaenoic acid	5.58 ± 0.56	10.12 ± 0.05	9.84 ± 0.13	5.06 ± 0.02	5.23 ± 0.33	2.21 ± 0.21
∑ PUFA		**35.98 ± 0.40** [b]	**45.91 ± 0.09** [a]	**44.34 ± 0.22** [a]	**43.65 ± 0.21** [a]	**30.52 ± 0.60** [c]	**29.06 ± 0.88** [d]

Note: Results are expressed as means ± SD (% of total FAME). nd, not detected. [a-f] Different letters in the same row indicate significant differences between species ($p < 0.05$).

Table 3.2. Nutritional indices calculated for different *Cystoseira* species (*C. humilis*, *C. compressa*, *C. tamariscifolia*, *C. nodicaulis* and *C. baccata*).

	C. humilis	*C. compressa*	*C. tamariscifolia*	*C. nodicaulis*	*C. baccata*	*C. barbata*
PUFA/SFA	**0.71** [a]	**1.34** [a]	**1.13** [a]	**1.17** [a]	**0.66** [a]	**0.53** [a]
∑n-3	**5.58 ± 0.56** [b]	**10.12 ± 0.05** [a]	**10.18 ± 0.13** [a]	**5.06 ± 0.02** [b]	**5.23 ± 0.33** [b]	**2.21 ± 0.21** [b]
∑n-6	**30.40 ± 1.29** [c]	**35.79 ± 0.07** [a,b]	**34.16 ± 0.18** [b,c]	**38.59 ± 0.21** [a]	**25.29 ± 0.50** [d]	**26.85 ± 0.86** [d]
∑n-6/∑n-3	**5.45** [b, c]	**3.54** [b, c]	**3.36** [c]	**7.63** [b]	**4.84** [b, c]	**12.15** [a]
UI	**126.17** [d]	**186.83** [a]	**184.52** [a]	**176.95** [b]	**140.72** [c]	**120.67** [e]
AI	**1.94** [a]	**0.67** [e]	**0.97** [d]	**0.89** [d]	**1.25** [c]	**1.52** [b]
TI	**1.30** [b]	**0.54** [e]	**0.65** [e]	**0.80** [d]	**0.98** [c]	**1.61** [a]

Note: SFAs: Saturated fatty acids, MUFAs: Monounsaturated fatty acids, PUFA: Polyunsaturated fatty acids, UI: Unsaturation index, AI: Atherogenic index, TI: Thrombogenic index. [a-e] Different letters in the same row indicate significant differences between species by Tukey HSD test at $p < 0.05$.

3.4.3 Nutritional assessment

The PUFA/SFA ratio was found to be between 1.34 for *C. compressa* and 0.53 for *C. barbata* (Table 3.2). Since the mean ratio of PUFA/SFA recommended by the British Department of Health is 0.45 or higher (HMSO 1994) all examined species exhibit a favourable PUFA/SFA ratio. A growing attention has been given to the n-6 PUFA/n-3 PUFA ratio. In fact, WHO currently recommends a ratio lower than 10 in order to prevent inflammatory, cardiovascular and neurological disorders (Kumari et al. 2013). The n-6 PUFA/n-3 PUFA ratios determined in this study ranged from 3.36 in *C. tamariscifolia* to 7.63 in *C. nodicaulis* (Table 3.2), which are within the values recommended by WHO. The only exception was obtained with *C. barbata* (12.15). The present Western diet is considered to be deficient in n-3 PUFA, with estimated n-6 to n-3 ratios of 15-20 (Simopoulos 2008). Hence, brown macroalgae, and *Cystoseira* in particular, could decrease n-6 PUFA/n-3 PUFA ratios if used in nutraceuticals applications or in food products. Furthermore, the high unsaturation indices (UI) determined for *Cystoseira* algae (Table 3.2) suggests that these macroalgae may also be beneficial for the prevention of type-2 diabetes mellitus, as higher flexibility of biological membranes has been linked to improved glucose transport effectiveness (Weijers 2012). Lastly, in this study, the atherogenicity (AI) and thrombogenic (TI) indices ranged from 0.67 to 1.94 and 0.54 to 1.61, respectively (Table 3.2). These low AI and TI are similar to those reported by Kumari et al. (2013) for other *Cystoseira* species, such as *C. indica* (AI = 0.8 and TI = 0.8) and *C. trinodis* (AI = 0.6 and TI = 0.5). These results are significant, as it has been shown that the introduction of brown algae in the diet of hyperlipidemic-induced rats decreases the AI of their serum lipid profile (Yoon et al. 2008). Moreover, López-López et al. (2009) have shown that the addition of macroalgae to meat products improved their TI and AI, thereby illustrating the potential of macroalgae in development of healthier lipid formulations.

3.5 Conclusions

Among the FAME profiles described for the first time *C. compressa* stands out for its high content of unsaturated fatty acids (especially PUFA), its low n-6 PUFA/n-3 PUFA ratios and AI and TI indices as well as high PUFA/SFA and UI indices. Overall, this species, together with *C. tamariscifolia* and *C. nodicaulis*, presents a FA profile that

would be considered as beneficial if incorporated in the formulation of low fat food and feed and PUFA–rich nutraceuticals.

3.6 Acknowledgments

Financial support for this work was provided by the SEABIOMED (PTDC/MAR/103957/2008) project funded by the Foundation for Science and Technology (FCT) and the Portuguese National Budget. Vizetto-Duarte C. (SFRH/BD/81425/2011) and Bruno de Sousa C. (SFRH/BD/78062/2011) are FCT doctoral research fellows. The authors would also like to acknowledge Dr Ricardo Bermejo for kindly providing samples of *C. barbata*.

3.7 References

Calder, P.C. 2010. Omega-3 fatty acids and inflammatory processes. Nutrients 2, 355-374.

Calder, P.C. 2012. Mechanisms of action of (n-3) fatty acids. J Nutr 142, 592S-599S.

Chowdhury, R., Warnakula, S., Kunutsor, S., Crowe, F., Ward, H.A., Johnson, L., Franco, O.H., Butterworth, A.S., Forouhi, N.G., Thompson, S.G., Khaw, K.T., Mozaffarian, D., Danesh, J., Di Angelantonio, E. 2014. Association of dietary, circulating, and supplement fatty acids with coronary risk: a systematic review and meta-analysis. Ann Intern Med 160, 398-406.

Clandinin, M.T., Cook, S.L., Konard, S.D., French, M.A. 2000. The effect of palmitic acid on lipoprotein cholesterol levels. Int J Food Sci Nutr 51 Suppl, S61-71.

Colombo, M.L., Risé, P., Giavarini, F., de Angelis, L., Galli, C., Bolis, C.L. 2006. Marine macroalgae as source of polyunsaturated fatty acids. Plant Food Hum Nutr 61, 67-72.

EFSA. 2010. Scientific opinion on dietary reference values for fats, including saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, trans fatty acids, and cholesterol. EFSA Journal 8, 1461.

FAO Fisheries and Aquaculture Department. 2010. The State of World Fisheries and Aquaculture 2010; Food and Agriculture Organization of the United Nations: Rome, Italy, pp. 197.

French, M.A., Sundram, K., Clandinin, M.T. 2002. Cholesterolaemic effect of palmitic acid in relation to other dietary fatty acids. Asia Pac J Clin Nutr 11 Suppl 7, S401-7.

Frikha, F., Kammoun, M., Hammami, N., Mchirgui, R.A., Belbahri, L., Gargouri, Y., Miled, N., Ben-Rebah, F. 2011. Chemical composition and some biological activities of marine algae collected in Tunisia. Cienc Mar 37, 113–124.

Ginsberg, H.N., Barr, S.L., Gilbert, A., Karmally, W., Deckelbaum, R., Kaplan, K., Ramakrishnan, R., Holleran, S., Dell, R.B. 1990. Reduction of plasma cholesterol levels in normal men on an American Heart Association Step 1 diet or a Step 1 diet with added monounsaturated fat. N Engl J Med 322, 574-579.

Harris, W.S., Mozaffarian, D., Rimm, E., Kris-Etherton, P., Rudel, L.L., Appel, L.J., Engler, M.M., Engler, M.B., Sacks, F. 2009. Omega-6 fatty acids and risk for cardiovascular disease: a science advisory from the American Heart Association Nutrition Subcommittee of the Council on Nutrition, Physical Activity, and Metabolism; Council on Cardiovascular Nursing; and Council on Epidemiology and Prevention. Circulation 119, 902-907.

HMSO, UK. 1994. Nutritional aspects of cardiovascular disease (report on health and social subjects No. 46). London: HMSO.

Ivanova, V., Stancheva, M., Petrova, D. 2013. Fatty acid composition of Black sea *Ulva rigida* and *Cystoseira crinita*. Bulg J Agric Sci 19 S1, 42–47.

Khotimchenko, S.V., Vaskovsky, V.E., Titlyanova, T.V. 2002. Fatty acids of marine algae from the Pacific coast of North California. Bot Mar 45, 17-22.

Kumari, P., Bijo, A.J., Mantri, V.A., Reddy, C.R., Jha, B. 2013. Fatty acid profiling of tropical marine macroalgae: an analysis from chemotaxonomic and nutritional perspectives. Phytochemistry 86, 44-56.

Lepage, G., Roy, C.C. 1984. Improved recovery of fatty acid through direct transesterification without prior extraction or purification. J Lipid Res 25, 1391–1396.

López-López, I., Cofrades, S., Jiménez-Colmenero, F. 2009. Low fat frankfurters enriched with n-3 PUFA and edible seaweed: effects of olive oil and chilled storage on physicochemical, sensory and microbial characteristics. Meat Sci 83, 148–154.

Patarra, R.F., Leite, J., Pereira, R., Baptista, J., Neto, A.I. 2013. Fatty acid composition of selected macrophytes. Nat Prod Res 27, 665-669.

Pereira, H., Barreira, L., Figueiredo, F., Custódio, L., Vizetto-Duarte, C., Polo, C., Rešek, E., Engelen, A., Varela, J. 2012. Polyunsaturated fatty acids of marine macroalgae: potential for nutritional and pharmaceutical applications. Mar Drugs 10, 1920-1935.

Silva, G., Pereira, R.B., Valentão, P., Andrade, P.B., Sousa, C. 2013. Distinct fatty acid profile of ten brown macroalgae. Braz J Pharmacog 23, 608-613.

Simopoulos, A.P. 2008. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. Exp Biol Med 233, 674-688.

Uauy, R., Hoffman, D.R., Peirano, P., Birch, D.G., Birch, E.E. 2001. Essential fatty acids in visual and brain development. Lipids 36, 885-895.

Ulbricht, T.L.V., Southgate, D.A.T. 1991. Coronary heart disease: seven dietary factors. Lancet 338, 985-992.

Weijers, R.N. 2012. Lipid composition of cell membranes and its relevance in type 2 diabetes mellitus. Curr Diabetes Rev 8, 390-400.

Yoon, N.Y., Kim, H.R., Chung, H.Y., Choi, J.S. 2008. Anti-hyperlipidemic effect of an edible brown algae, *Ecklonia stolonifera*, and its constituents on poloxamer 407-induced hyperlipidemic and cholesterol-fed rats. Arch Pharm Res 31, 1564-1571.

CHAPTER 4

CAN MACROALGAE PROVIDE PROMISING ANTI-TUMORAL COMPOUNDS? A CLOSER LOOK AT *CYSTOSEIRA TAMARISCIFOLIA* AS A SOURCE FOR ANTIOXIDANT AND ANTI-HEPATOCARCINOMA COMPOUNDS

Vizetto-Duarte C, Custódio L, Acosta G, Lago JHG, Morais TR, Bruno de Sousa C, Gangadhar KN, Rodrigues MJ, Pereira H, Lima RT, Vasconcelos MH, Barreira L, Rauter AP, Albericio F and Varela J (2016). Can macroalgae provide promising anti-tumoral compounds? A closer look at *Cystoseira tamariscifolia* as a source for antioxidant and anti-hepatocarcinoma compounds. PeerJ 4:e1704.
DOI: 10.7717/peerj.1704.

Can macroalgae provide bioactive compounds with pharmaceutical potential? A closer look at *Cystoseira tamariscifolia* as a source for antioxidant and anti-hepatocarcinoma compounds

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4.1 Abstract

Marine organisms are a prolific source of drug leads in a variety of therapeutic areas. In the last few years, biomedical, pharmaceutical and nutraceutical industries have shown growing interest in novel compounds from marine organisms, including macroalgae. *Cystoseira* is a genus of Phaeophyceae (Fucales) macroalgae known to contain bioactive compounds. Organic extracts (hexane, diethyl ether, ethyl acetate and methanol extracts) from three *Cystoseira* species (*C. humilis*, *C. tamariscifolia* and *C. usneoides*) were evaluated for their total phenolic content, radical scavenging activity against 1,1-diphenyl-2picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radicals, and antiproliferative activity against a human hepatocarcinoma cell line (HepG2 cells). *C. tamariscifolia* had the highest TPC and RSA. The hexane extract of *C. tamariscifolia* (CTH) had the highest cytotoxic activity (IC50=2.31 µg/mL), and was further tested in four human tumor (cervical adenocarcinoma HeLa; gastric adenocarcinoma AGS; colorectal adenocarcinoma HCT-15; neuroblastoma SH-SY5Y), and two non-tumor (murine bone marrow stroma S17 and human umbilical vein endothelial HUVEC) cell lines in order to determine its selectivity. CTH strongly reduced viability of all tumor cell lines, especially of HepG2 cells. Cytotoxicity was particularly selective for the latter cells with a selectivity index = 12.6 as compared to non-tumor cells. Incubation with CTH led to a 2-fold decrease of HepG2 cell proliferation as shown by the bromodeoxyuridine (BrdU) incorporation assay. CTH-treated HepG2 cells presented also pro-apoptotic features, such as increased Annexin V/propidium iodide (PI) binding and dose-dependent morphological alterations in DAPI-stained cells. Moreover, it had a noticeable disaggregating effect on 3D multicellular tumor spheroids. Demethoxy cystoketal chromane, a derivative of the meroditerpenoid cystoketal, was identified as the active compound in CTH and was shown to display selective *in vitro* cytotoxicity towards HepG2 cells.

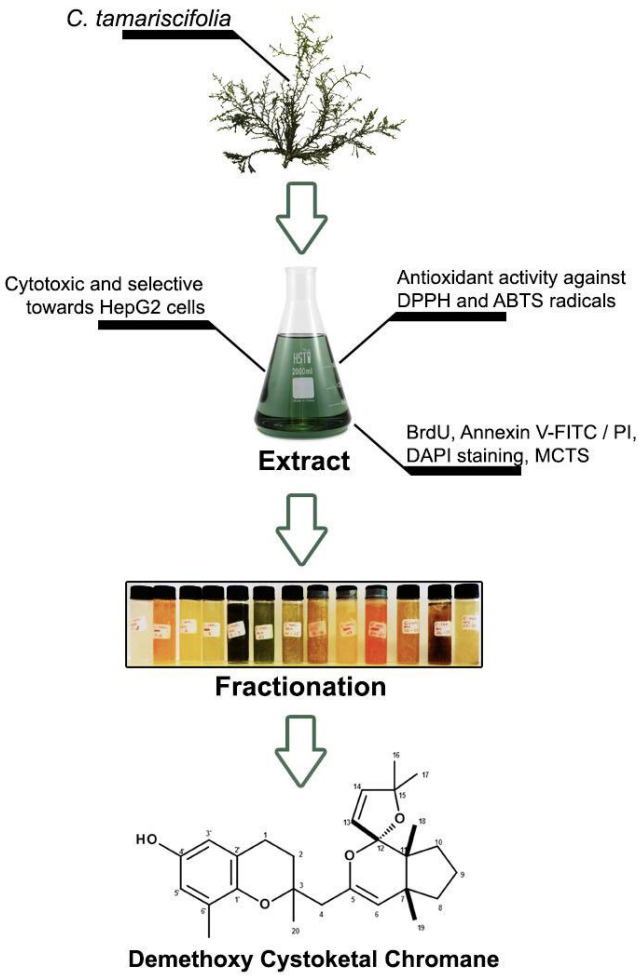

Fig. 4.1 – Graphical abstract demonstrating the main results accomplished in Chapter 4.

4.2 Introduction

Macroalgae are used as food and feed, and also as sources of bioactive metabolites. In particular, Phaeophyceae algae have high contents of polysaccharides, minerals, polyunsaturated fatty acids and vitamins (Balboa et al., 2013). Furthermore, these organisms contain high levels of secondary metabolites with pharmacological interest, such as terpenoids, phenolic compounds and alkaloids, which have been linked to interesting biomedical activities, including antitumoral and neuroprotective (Smit, 2004; Blunt et al., 2014). Among Phaeophyceae, the *Cystoseira* genus comprises a large number of species widely distributed in the Atlantic and Mediterranean Sea (Guiry and Guiry, 2015). Phytochemical studies have revealed that species belonging to this genus are rich in phlorotannins, sterols, meroditerpenoids and sesquiterpenoids (Amico, 1995; Moreno et al., 1998; Khanavi et al., 2012; Sathya et al., 2013; Montero et al., 2014), some of which exhibiting antioxidant, antitumoral, antifouling and/or antimicrobial activities with potential applications in the pharmaceutical industry (Amico, 1995; Gouveia et al., 2013; Valls and Piovetti, 1995).

Phaeophyceae algae have already shown interesting biomedical properties such as *Dictyota ciliolata*, *Padina sanctae-crucis*, *Turbinaria tricostata* and *Petalonia fascia* with antiproliferative activity in cancer cell lines (Caamal-Fuentes et al., 2014; Kurt et al., 2014). *Cystoseira* and *Fucus* genus are also known to contain molecules with antioxidant properties (Mhadhebi et al., 2011; Heffernan et al., 2015; Hadj Ammar et al., 2015). Bearing in mind the high biotechnological potential of brown algae, in this work we evaluated the total phenolic contents and antioxidant activity of organic extracts of *C. tamariscifolia*, *C. humilis* and *C. usneoides*. The anti-proliferative potential was screened on human hepatocellular carcinoma HepG2 cells, a cell line known to be recalcitrant to cytotoxic drugs (Liu et al., 2010). The most bioactive extract (*C. tamariscifolia* hexane extract; named CTH) was also evaluated in several other human tumor cell lines and compared to non-tumor cells used as selectivity controls. Cytotoxicity was then further studied in terms of its action on cell proliferation inhibition and apoptosis induction, important features for potential anti-cancer therapies. It was also evaluated its effect on multicellular tumor spheroids (MCTS). This extract was then subjected to a bioactivity-guided fractionation to afford the meroditerpene demethoxy cystoketal chromane, which was bioactive against HepG2 cell line.

4.3 Material and Methods

4.3.1 General

Hexane, ethyl acetate (EtOAc) and diethyl ether were from Prolabo (VWR International, Leuven, Belgium). Roswell Park Memorial Institute medium (RPMI), Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine and penicillin/streptomycin were obtained from Lonza Ibérica (Barcelona, Spain). 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from AppliChem and Calbiochem, respectively. 1,1-diphenyl-2-picrylhydrazyl (DPPH), potassium persulfate, sodium carbonate and bromodeoxyuridine (BrdU) were purchased from Sigma-Aldrich (Steinheim, Germany). Mouse anti-BrdU antibody was acquired from Dako (Glostrup, Denmark). Vectashield mounting medium for fluorescence with 4',6-diamidino-2-phenylindole (DAPI) was acquired from Vector Laboratories Inc., Peterborough, UK. Merck (Darmstadt, Germany) supplied dimethyl sulphoxide (DMSO), trichloroacetic acid (TCA) and Folin-Ciocalteu (F-C) reagent, whereas methanol was from Fisher Scientific (Loughborough, UK). FITC-conjugated Annexin V/ propidium iodide (PI) assay kit was acquired from Cayman Chemical Company, USA. Silica gel (Merck, 40-63 µm mesh) was used for column chromatographic separation, while silica gel 60 PF_{254} (Merck) was used for analytical (0.25 mm) TLC. DMSO-d_6 (Aldrich) was used as solvent for ^{1}H and ^{13}C NMR spectra acquisition and TMS (Aldrich) was used as internal standard. 1D and 2D NMR spectra were recorded at Bruker Digital Avance 800 MHz spectrometer. Additional reagents and necessary solvents were purchased from VWR International (Leuven, Belgium).

4.3.2 Sampling

Samples of *C. tamariscifolia, C. humilis* and *C. usneoides* were collected in the middle/lower intertidal areas, during the low tide, between May and September 2012 on the Portuguese coast. Identification of specimens was made by Dr. Aschwin Engelen (Centre of Marine Sciences, University of Algarve, Portugal) and Dr. Javier Cremades Ugarte (Facultade de Ciencias, University of A Coruña). Voucher specimens of *C. humilis* (code number MB007), *C. tamariscifolia* (code number MB016) and *C. usneoides* (code number MB013) are deposited at the Centre of Marine Sciences, University of Algarve. Biomass was cleaned with distilled water (dH_2O) and

macroscopic epiphytes and extraneous matter were carefully removed. Samples were freeze-dried and stored at -20 °C until the extraction procedure.

4.3.3 Preparation of the Extracts

The extracts were prepared by sequential extraction with solvents of increasing polarities index (PI), namely hexane (PI = 0.1), diethyl ether (PI = 2.8), ethyl acetate (PI = 4.4) and methanol (PI = 5.1). Biomass was mixed with hexane (1:10, w/v) and homogenized for 2 min using a disperser IKA T10B Ultra-Turrax at room temperature (RT). The tubes were then vortexed for 1 min, centrifuged (5000 *g*, 10 min, RT) and the supernatants recovered. The extraction procedure was repeated 3 times and the supernatants combined and filtered. The residue was then sequentially extracted with diethyl ether, ethyl acetate and methanol as described above. The organic extracts were dried at 40ºC under vacuum. All extracts were dissolved in DMSO for biological activities screening or in the adequate solvent for chemical characterization, aliquoted and stored (-20°C).

4.3.4 Total Phenolic Content (TPC)

TPC was determined using the F-C colorimetric method (Velioglu et al., 1998). Briefly, 5 µL of the extracts at the concentration of 10 mg/mL were mixed with 100 µL of a 10-fold diluted F–C reagent, incubated at RT for 5 min and mixed with 100 µL of sodium carbonate (75 g/L, w/v). After a 90 min incubation period at RT, absorbance was measured at 725 nm on a microplate reader (Biotek Synergy 4). The amount of TPC was calculated as gallic acid equivalents (GAE) using a calibration curve prepared with gallic acid standard solutions, and expressed as GAE in milligrams per gram of dried extract.

4.3.5 Antioxidant activity

4.3.5.1 Radical-scavenging activity (RSA) against DPPH

RSA against the DPPH radical was determined according to the method described by Brand-Williams et al. (1995) adapted to 96-well microplates (Moreno et al., 2006). Samples (22 µL) at concentrations ranging from 0.125 to 10 mg/mL were mixed with 200 µL of DPPH solution (120 µM) in methanol and incubated in darkness at RT for 30 min. The absorbance was measured at 515 nm (Biotek Synergy 4) and results expressed as antioxidant activity (%) relative to a control containing DMSO and

as half maximal inhibitory concentration (IC_{50}, mg/mL). Butylated hydroxytoluene (BHT, E320) was used as a positive control at the same concentrations of the extracts.

4.3.5.2 RSA against ABTS

RSA against ABTS was evaluated according to Re et al. (1999). A stock solution of $ABTS^{\bullet+}$ (7.4 mM) was prepared in potassium persulfate (2.6 mM) as the oxidizing agent, and placed in darkness for 12-16h at RT. The $ABTS^{\bullet+}$ solution was diluted with ethanol down to an absorbance of 0.7 units at 734 nm on a Biotek Synergy 4 microplate reader. Samples (10 µL) at concentrations ranging from 0.125 to 10 mg/mL were mixed with 190 µL of $ABTS^{\bullet+}$ solution in 96-well flat bottom microtitration plates, and 6 min upon mixing absorbance was read at 734 nm. Results were expressed as antioxidant activity (%) relative to a DMSO-containing control and as IC_{50} values (mg/mL). BHT was used as a positive control at the same concentrations of the extracts.

4.3.6 Cell Lines and Culture Conditions

Human hepatocellular carcinoma HepG2 (ATCC® HB-8065™), human cervix adenocarcinoma HeLa (ATCC® CCL-2™), human gastric adenocarcinoma AGS (ATCC® CRL-1739™) and human colorectal adenocarcinoma HCT-15 (ATCC® CCL-225™) cell lines were maintained in RPMI-1640 culture media supplemented with 10% FBS (v/v), L-glutamine (2 mM), penicillin (50 U/mL) and streptomycin (50 µg/mL). Murine bone marrow stromal S17 cell line was kindly provided by D. Rawlings, UCLA, Los Angeles, CA. The latter cell line as well as human umbilical vein endothelial HUVEC (ATCC® CRL-1730™) and human neuroblastoma SH-SY5Y (ATCC® CRL-2266™) cell lines were grown in DMEM culture media supplemented with 10% FBS (v/v), L-glutamine (2 mM), penicillin (50 U/mL) and streptomycin (50 µg/mL). All cells were grown in an incubator at 37 ºC and 5.0% CO_2 in humidified atmosphere.

4.3.7 *In vitro* cytotoxic activity and selectivity

In vitro cytotoxic activity of the extracts was assessed by the MTT colorimetric assay (Mosmann, 1983). Briefly, exponentially growing cells were seeded at a density of 5×10^3 cells/well on 96-well plates and incubated for 24h at 37°C in 5.0% CO_2. The extracts were then applied at concentrations ranging from 125 to 3.9 µg/mL for 72h and cytotoxicity was evaluated. Positive and negative control cells were treated for 72h with etoposide at the same concentrations of the extracts and DMSO at the highest

concentration used in the test wells (0.5%, v/v), respectively. Two hours before the end of the incubation period, 20 µL of MTT (5 mg/mL in PBS, w/v) were added to each well and further incubated for 2h at 37ºC. The optical density (OD) was measured on a Biotek Synergy 4 spectrophotometer at 590 nm. Results were expressed in terms of cell viability (%) and as half maximal inhibitory concentration (IC_{50}, µg/mL). The selectivity index (SI) of the extracts was determined using the equation $SI = C_T/C_{NT}$, where C_T and C_{NT} correspond to the extract-induced cytotoxicity on tumor (e.g. HepG2) and non-tumor cells (e.g. S17), respectively (Oh et al., 2011).

4.3.8 Cellular proliferation analysis by the BrdU incorporation assay

The effect of the extracts on HepG2 cells proliferation was evaluated by the BrdU incorporation assay. HepG2 cells were incubated for 72h with complete medium, DMSO (0.5%, v/v), or with CTH at the concentrations of 2.31 or 4.62 µg/mL, which were the IC_{50} or $2\times IC_{50}$ concentration previously determined by the MTT assay. After a 1h pulse with 10 µM BrdU, cells were washed with phosphate buffer saline (PBS), fixed in 4% paraformaldehyde in PBS, and cytospins prepared. After incubation in 2M HCl for 20 min, cells were incubated with mouse anti-BrdU (1:10, v/v) and further incubated with fluorescein-labeled rabbit anti-mouse antibody (1:100, v/v). For nuclear staining, Vectashield mounting medium for fluorescence with DAPI was used. Cells were observed in a LEICA DM2000 microscope using a 200 × magnification, and a semi-quantitative evaluation was performed by counting a minimum of 500 cells per slide.

4.3.9 Detection of apoptosis

4.3.9.1 Flow cytometry apoptosis detection through Annexin V-FITC staining

Apoptotic cells were identified and quantified by flow cytometry using the FITC-conjugated Annexin V/PI assay kit, according to the manufacturer's instructions. Briefly, cells were treated for 72h with complete medium, DMSO (0.5%, v/v), or with CTH at the concentrations of 3.9, 7.8 and 15.6 µg/mL. Etoposide treated-cells at IC_{50} concentration (1.85 µg/mL) were used as positive control. HepG2 cells were washed with ice-cold PBS, resuspended in 100 µL binding buffer, and stained with 5 µL of FITC-conjugated Annexin V (10 mg/mL) and 10 µL of propidium iodide PI (50 mg/mL). The cells were incubated for 15 min at RT in the dark and then 500 µL of binding buffer was added. Flow cytometry was performed using a FACS Calibur Flow

Cytometer (Becton-Dickinson, USA) and data acquisition and analysis were done with CellQuest Pro software. At least 1×10^4 events were recorded for each sample and represented as dot plots. For analysis, HepG2 cells were gated separately according to their size and granularity on forward scatter *vs.* side scatter plots. Apoptosis was evaluated on fluorescence channel 2 (for PI) *vs.* fluorescence channel 1 (for Annexin) plots (Zhang et al., 1997; Abu Bakar et al. 2010).

4.3.9.2 DAPI staining

HepG2 cells were grown in 6-well plates at seeding densities of 5×10^5 cells/well and treated for 72h with CTH at 3.9, 7.8 and 15.6 µg/mL. Cells incubated with culture medium or with DMSO at the concentration of 0.5% (v/v) were used as blank or negative control, respectively. Etoposide treated-cells at IC_{50} concentration (1.85 µg/mL) were used as positive control. Cells were then washed with PBS and incubated with DAPI (5 µg/mL in PBS) for 2 min at RT. Fluorescence was visualized using a Leica DM LB (Leica Microsystems DI, Cambridge) microscope, magnification 400 ×. Images were acquired using a Leica DC 300 FX digital camera. Cells under apoptosis were identified by marked condensation of chromatin and cytoplasm (apoptotic cells), plasma membrane blebbing (apoptotic bodies), and intra- and extracellular chromatin fragments (Murugan et al., 2010).

4.3.10 Determination of cytotoxic activity in a 3D multicellular tumor spheroids model (MCTS)

4.3.10.1 Generation of MCTS

HepG2 cells were used to produce spheroids by modification of the hanging drop method (Keller, 1995). Single-cell suspensions ($1x10^4$ cells/mL) were generated from trypsinized monolayers. Aggregate culture of HepG2 cells were generated by growth on non-adherent, bacterial-grade polystyrene Petri dishes. Cell suspension (30 mL) was then dispensed into 6 drops into the lid of a Petri dish. Upon inversion of the lid, the hanging drops were held in place by surface tension and cells accumulated at the free liquid–air interface. The Petri lids were placed in the dishes with PBS and incubated for four days under standard conditions.

4.3.10.2. MCTS treatment with bioactive extract

After four days, in each Petri dish, three of the six multicellular tumor spheroids (MCTS) were incubated with CTH at 20, 40 and 80 µg/mL for 24 and 48 h. Incubation was carried out by replacing the medium with 30 µL of fresh culture medium containing the extract. The remaining three MCTS were used as control; the cultured medium was replaced by fresh medium containing the same volume of DMSO. Images were captured at incubation time 0, 24, 48 and 72 hours by means of an Olympus SZX7 microscope (using a 20 × magnification) with a digital camera (Optica B3). Each experiment was done in triplicate.

4.3.11 Compound isolation and elucidation

CTH (9 g) was fractionated by column chromatography (2.5×18 cm) over silica gel (SiO_2) using increasing amounts of ethyl acetate in hexane (9:1; 85:15; 4:1; 75:25; 7:3; 3:2; 1:1) and increasing amounts of methanol in ethyl acetate (9:1; 8:1; 5:1; 2:1; 1:1), methanol (100%) and water (100%) as eluents to give 57 fractions. Each fraction was analyzed by TLC and pooled together to afford 21 samples. These samples were tested for cytotoxic activity and selectivity and the active fraction **7** (21.6 mg) was chosen for characterization. Fraction **7** was re-fractionated over SiO_2 eluted with hexane; hexane/EtOAc (8:2); hexane/EtOAc (7:3); hexane/EtOAc (6:4); hexane/EtOAc (5:5); hexane/EtOAc (4:6); EtOAc and MeOH to afford compound **1** (1.1 mg).

Compound **1**. Oil. ^{1}H NMR (DMSO-d_6, 500 MHz) δ 8.50 (1H, s, 4'-O<u>H</u>), 6.34 1H, br s, H-5'), 6.25 (1H, d, *J* = 3.0 Hz, H-3'), 6.20 (1H, d, *J* = 5.0 Hz, H-14), 5.57 (1H, d, *J* = 5.0 Hz, H-13), 4.29 (1H, s, H-6), 2.71 (2H, t, *J* = 7.5 Hz, H-1), 2.16 (2H, s, H-4), 2.07 (3H, s, 6'-C<u>H</u>$_3$), 1.90 – 1.20 (6H, m, H-8, H-9 and H-10), 1.79 (2H, m H-2), 1.31/1.28 (3H, s, H-20), 1.25 (3H, s, H-17), 1.24 (3H, s, H-16), 1.23 (3H, s, H-19), 0.83 (3H, s, H-18); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 149.4 (C-4´), 146.3 (C-1'), 143.9 (C-5), 140.1 (C-13), 126.2 (C-6'), 125.7 (C-14), 120.7 (C-2'), 115.5 (C-12), 114.6 (C-3'), 112.4 (C-5'), 110.4 (C-6), 87.8 (C-15), 74.8 (C-3), 45.6 (C-4), 43.6 (C-11), 43.2 (C-7), 42.5 (C-8), 35.6 (C-10), 30.8 (C-2), 28.5 (C-17), 26.3 (C-18), 24.9 (C-20), 22.6 (C-1), 21.9 (C-16), 19.9 (C-9), 19.6 (C-19), 15.9 (6'-<u>C</u>H$_3$); LRESIMS *m/z* 425 $[M+H]^+$. Chemical shifts are reported in δ units (parts per million) and coupling constants (*J*) in Hertz.

4.3.12 Statistical Analysis

Results were expressed as mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) was assessed using the SPSS statistical package for Windows (release 15.0, SPSS Inc.), and significance between means was analysed by the Tukey HSD test ($p<0.05$). The IC_{50} values were calculated by sigmoidal fitting of the data by means of GraphPad Prism v. 5.0 (GraphPad Software, Inc., La Jolla, CA). Pearson correlation coefficient (r) was also calculated ($p < 0.01$) to assess the strength of the linear relationship between two variables.

4.4 Results and discussion

4.4.1 TPC and antioxidant activity

The results of total phenolic content and antioxidant activity are summarized in Table 4.1. *C. tamariscifolia* was the species with the highest TPC, mainly in the hexane, diethyl ether and ethyl acetate extracts, which presented TPC values higher than 100 mg GAE/g DW. *C. usneoides* diethyl ether extract also had a high TPC (122 mg GAE/g DW), whereas *C. humilis* methanol extract had the lowest levels of phenolic content (4.78 mg GAE/g DW). The highest RSA were observed with *C. tamariscifolia* ethyl acetate, diethyl ether and hexane extracts which IC_{50} for DPPH ($IC_{50\text{-}DPPH}$) and for $ABTS^{\bullet+}$ ($IC_{50\text{-}ABTS}$) ranged from 0.17 to 0.63 mg/mL and from 0.26 to 0.52 mg/mL, respectively. Similar results were obtained with *C. usneoides* diethyl ether extract ($IC_{50\text{-}DPPH}$ = 0.65 mg/mL; $IC_{50\text{-}ABTS}$ = 0.60 mg/mL). The hexane and methanol extracts of *C. humilis* had the lowest scavenging activity (IC_{50} > 10 mg/mL for both radicals).

Taken together, our results indicate that *C. tamariscifolia* contains phenolic compounds of different polarities, which occur mainly in the less polar extracts. Distribution of phenolic compounds through different solvents may vary greatly usually due to their amphipathic properties and wide range of structures (Ivanova et al., 2005; Demiray et al., 2009). Though commonly found in polar extracts such as methanol and water, phenolic compounds can also be present in less polar extracts including hexane, diethyl ether and ethyl acetate (Li et al., 2007; Maimoona et al., 2011). This may in fact explain the high levels of phenolic compounds in less polar extracts of *Cystoseira*, since the sequential extraction procedure used began with solvents of lower polarity (Li et al., 2007).

Phenolic compounds are described as strong antioxidants (Dai and Mumper, 2010). In this work, a significant correlation was observed between TPC and RSA on

DPPH (r^2=0.868, p<0.01) as well as TPC and RSA on ABTS (r^2=0.921, p<0.01), suggesting that the antioxidant activity observed might be due to the activity of phenolic compounds. Data on TPC and antioxidant activity in macroalgae are scarce, but the *Cystoseira* genus generally has one of the highest total phenolic levels and antioxidant activities among Phaeophyceae macroalgae, such as *Fucus serratus*, *Dictyota dichotoma*, *Bifurcaria bifurcata*, *Sargassum horneri* and *Alaria crassifolia* among others (Zubia et al., 2009; Airanthi et al., 2011). A few authors were able to relate the elevated antioxidant activity with tocopherol-like compounds, such as tetraprenyltoluquinol derivatives (Foti et al., 1994; Fisch et al. 2003).

Table 4.1 Total phenolic content (TPC, mg GAE/g DW), and radical scavenging activity (RSA) on DDPH and ABTS radicals (IC_{50}, mg/mL) of organic extracts of different species of *Cystoseira*.

Species /compound	Extract	TPC (mg GAE/g DW)	$IC_{50\text{-DPPH}}$ (mg/mL)	$IC_{50\text{-ABTS}}$ (mg/mL)
C. humilis	Hexane	24.42 ± 0.46^{e}	> 10	> 10
	Diethyl ether	20.34 ± 0.68^{e}	8.28 ± 0.13^{d}	8.85 ± 0.23^{d}
	Ethyl acetate	32.06 ± 0.72^{d}	5.04 ± 0.13^{c}	9.25 ± 0.43^{d}
	Methanol	4.78 ± 0.80^{f}	> 10	> 10
C. tamariscifolia	Hexane	113.13 ± 2.31^{b}	0.63 ± 0.01^{a}	0.52 ± 0.02^{a}
	Diethyl ether	116.61 ± 2.44^{b}	0.30 ± 0.00^{a}	0.47 ± 0.02^{a}
	Ethyl acetate	165.28 ± 1.92^{a}	0.17 ± 0.00^{a}	0.25 ± 0.01^{a}
	Methanol	45.04 ± 2.28^{d}	1.08 ± 0.06^{b}	2.93 ± 0.67^{b}
C. usneoides	Hexane	75.56 ± 0.21^{c}	4.37 ± 0.03^{c}	5.54 ± 0.06^{c}
	Diethyl ether	122.30 ± 0.81^{b}	0.65 ± 0.01^{a}	0.60 ± 0.01^{a}
	Ethyl acetate	17.76 ± 0.78^{e}	7.37 ± 0.76^{d}	> 10
	Methanol	17.03 ± 0.70^{e}	7.16 ± 0.01^{d}	> 10
BHT*		n.a.	0.07 ± 0.01	0.11 ± 0.00

Results are expressed as mean ± SEM of data obtained from six independent experiments. $^{a\text{-}f}$ Different letters in the same row indicate significant differences by Duncan's New Multiple Range Test at $p < 0.05$. *positive control, 1 mg/mL; n.a. not applicable.

In addition, the high RSA obtained for *Cystoseira* extracts suggests that these macroalgae are potential sources of novel antioxidants that may help prevent oxidative stress and also an alternative to BHT and butyl-4-hydroxyanisole (BHA), two synthetic antioxidants found to be toxic and carcinogenic in animal models (Ito et al., 1986; Safer and Al-Nughamish, 1999).

Oxidative stress is considered to be one of the underlying causes of several chronic diseases, including cancer, and is implicated in both cytotoxic and apoptotic mechanisms (Goswami and Singh, 2006). The link between oxidative stress and cell death has been associated, for example, with lipid peroxidation, a process of oxidative degradation of lipids in which free radicals 'remove' electrons from membrane lipids. These events damage lipid bilayers, and impair several intra- and extra mitochondrial membrane transport systems, thus contributing to apoptosis. As a result, antioxidant compounds from natural sources have attracted much attention due to their ability to diminish oxidative stress. In fact, antioxidant compounds play an important role in regulation of gene expression and protection of DNA, lipids and proteins from oxidative stress-induced injury (Saura-Calixto, 2011). Because of this protective effect, it has been proposed that antioxidants may inhibit apoptosis when cancer cells should undergo cell death (Zeisel 2004). However, the opposite has also been shown, i.e. molecules with known antioxidant properties have been described to also promote apoptosis (Moustapha et al., 2015). Therefore the chemical structure of the antioxidant and its biological properties seemed to be essential to define the outcome of a given therapy with compounds with antioxidant properties.

4.4.2 Cytotoxic activity and selectivity

Natural extracts are considered as promising sources of antitumoral compounds when they exhibit IC_{50} values lower than 30 µg/mL (Dos Santos et al., 2010). This was the case for the hexane (CTH) and diethyl ether extracts of *C. tamariscifolia,* with IC_{50} values of 2.31 and 6.83 µg/mL, respectively (Table 4.2). In fact, in the literature, *C. tamariscifolia* also stood out as a potential source of antiproliferative compounds among other Phaeophyceae species (Zubia et al., 2009; Khanavi et al. 2010).

Table 4.2 *In vitro* cytotoxic activity, expressed as IC_{50} values (µg/mL) of organic extracts of different species of *Cystoseira* and etoposide on a human hepatocarcinoma cell line (HepG2).

Extracts	*C. humilis*	*C. tamariscifolia*	*C. usneoides*	Etoposide
Hexane	> 125	2.31 ± 0.08^{a}	31.4 ± 3.22^{b}	1.85 ± 0.12^{a}
Diethyl ether	> 125	6.83 ± 0.01^{a}	52.0 ± 3.19^{b}	
Ethyl acetate	> 125	44.2 ± 1.41^{b}	> 125	
Methanol	> 125	> 125	> 125	

Results are expressed as mean ± SEM of data obtained from six independent experiments. [a,b] Different letters in the same row indicate significant differences by Duncan's New Multiple Range Test at $p < 0.05$.

Interestingly, CTH had an IC_{50} statistically similar to that of the pure chemotherapeutic drug etoposide (IC_{50}=1.85 µg/mL). This result indicates that CTH has cell growth inhibitory activity *in vitro* comparable with etoposide, a potent anti-cancer compound that acts as a topoisomerase II inhibitor (Scott and William, 2000). In fact, etoposide is one of the most potent drugs used in the treatment of several types of tumors, including testicular and ovarian cancer (Hande, 1998). However, different success rates are described for the treatment of different types of cancer with that compound. For example, Miao et al. (2003) reported the occurrence of resistant cell lines to this compound. It is also noteworthy to mention that HepG2 cells are known to display greater resistance to drugs and toxins comparing to other cells lines (Liu et al., 2010).

Since CTH had the highest cytotoxic activity towards HepG2 cells, this extract was further evaluated in other human tumor cell lines, namely cervical (HeLa), neuroblastoma (SH-SY5Y), gastric (AGS) and colorectal (HCT-15) carcinoma cells. Furthermore, CHT treatment was also carried out in murine stromal S17 and human umbilical HUVEC cell lines, both non-tumor cell lines, to determine the selectivity index (SI). As shown in Fig. 4.2, CTH had a strong cytotoxic activity in all tumor cell lines tested, except HeLa cells. This effect was, however, more pronounced towards HepG2 cells (IC_{50}=2.31 µg/mL, p<0.01 *vs*. S17 and HUVEC cells). Samples with SI values higher than 3 are deemed as highly selective (Mahavorasirikul et al., 2010). CTH

was therefore considered highly selective when comparing HepG2 and S17 cells (SI=5.5, Fig.4.2) and especially against HUVEC cells (SI=12.6). Based on these results, CTH was further used to study the mode of action associated with the cytotoxicity observed on HepG2 cells.

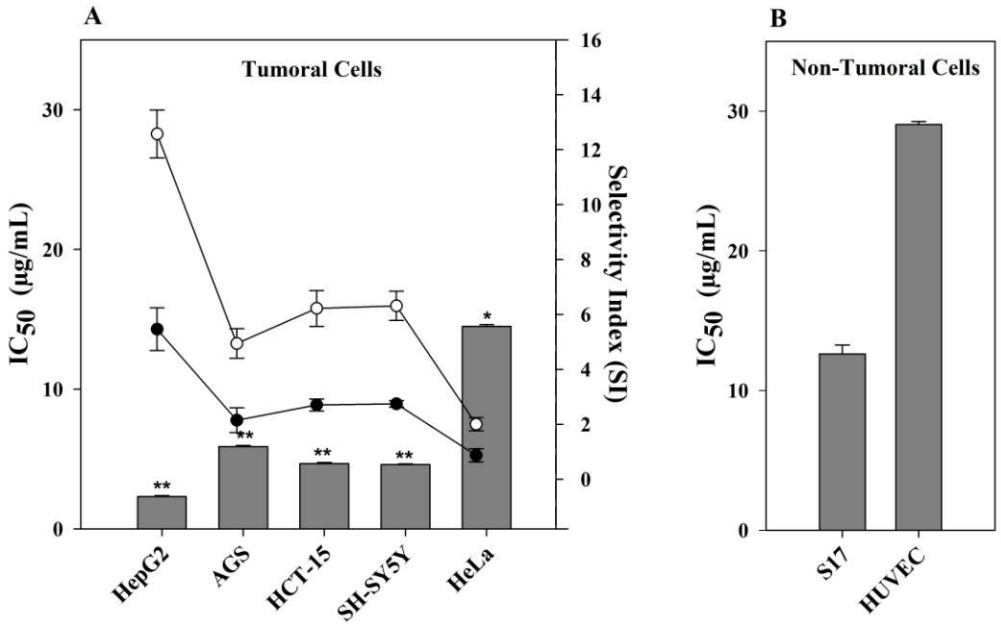

Fig. 4.2 – Effect of CTH on the viability of different cell lines. (A) IC_{50} values of CTH on tumor and non-tumor cells (bars). Selectivity (scatter lines) was calculated using IC_{50} values of the non-tumor cell line S17 (●) or HUVEC (○) *vs*. the tumor cell lines. (B) IC_{50} value of CTH on non-tumor cell lines. Results are expressed as mean ± SEM of data obtained from six independent experiments, *p<0.05, **p<0.01 *vs*. HUVEC cells.

4.4.3 Cytotoxicity mechanisms

4.4.3.1 Cellular proliferation analysis by the BrdU incorporation assay

The BrdU incorporation assay was used in order to assess the effect of CTH on HepG2 cells proliferation. Results show that cells treated with CTH at concentrations of 2.31 or 4.62 µg/mL incorporated less BrdU than control cells (treated with medium only or with 0.5% DMSO). In fact, the proliferation levels, expressed as the percentage of proliferating cells, underwent an almost 2-fold reduction, decreasing from 25.8% to 17.5% or 13.6% respectively after treatment with CHT at the concentration of 2.31 µg/mL or 4.62 µg/mL for 72h ($p < 0.01$, Fig. 4.3).

Although previous data on the inhibition of cell proliferation with macroalgae extracts is very limited, it is interesting to observe that these results are consistent with studies from Funahashi et al. (1999). According to those authors, rats fed with commercial feed supplemented with wakame, an edible brown macroalga (*Undaria pinnatifida*) also belonging to the Phaeophyceae, showed significantly lower BrdU indices in tumor mammary cells as compared to a control group eating commercial feed alone. In fact, the authors showed that this phaeophyta had a strong suppressive effect on rat mammary carcinogenesis without toxicity, possibly via apoptosis induction.

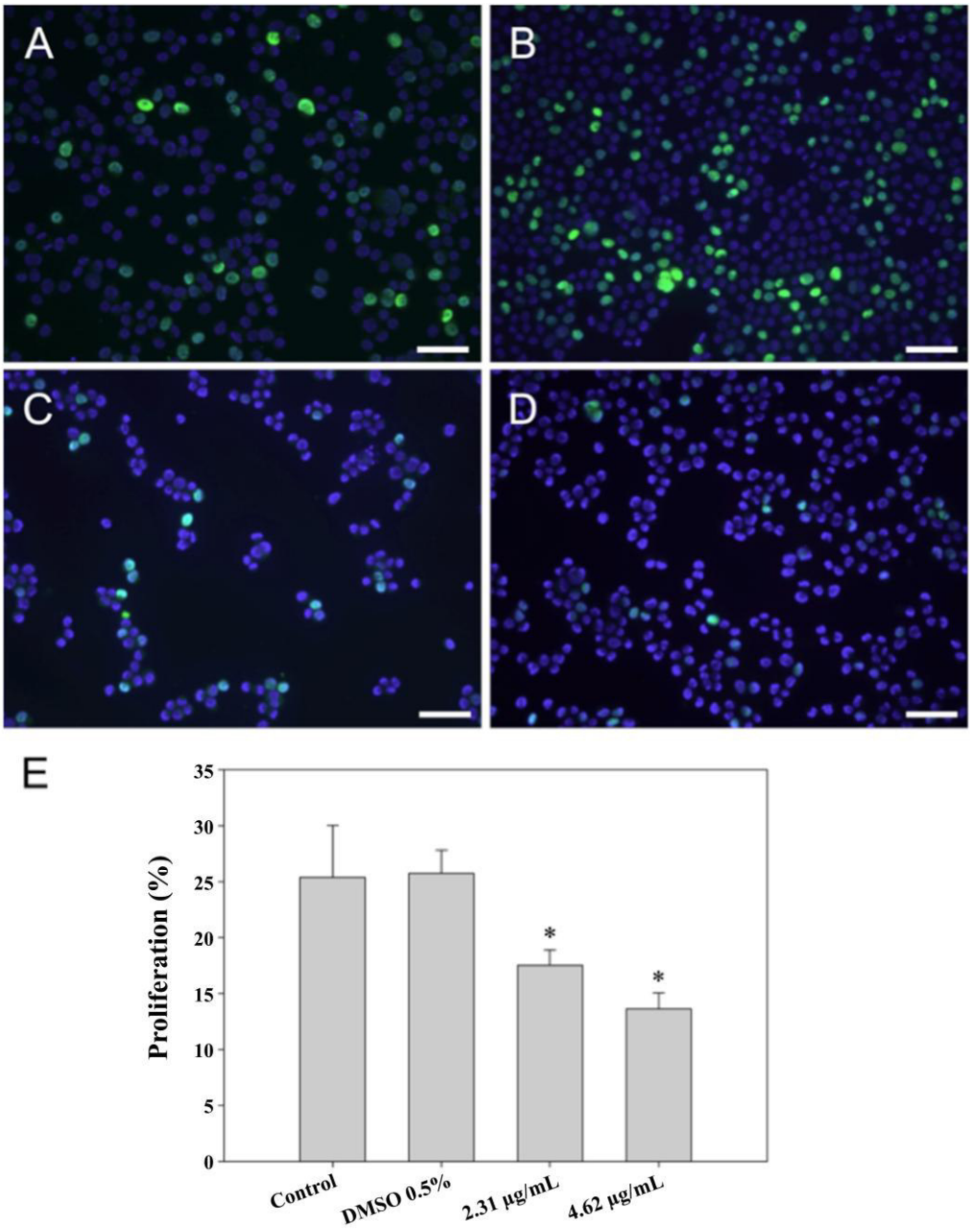

Fig. 4.3 – BrdU incorporation (stained in green) with nuclei labelled with DAPI (stained in blue). HepG2 cells (200 × magnification) were treated for 72h with complete medium alone (A), 0.5% DMSO (B), or CTH at concentrations of 2.31 (C) or 4.62 µg/ml (D). Semi-quantitative analysis of BrdU incorporation was carried out by counting a minimum of 500 cells per treatment in each independent experiment (E). Results are expressed as the mean ± SEM of three independent experiments, *p<0.05 *vs.* DMSO 0.5%. Scale bar = 100 µm.

4.4.3.2 Apoptosis-inducing activity

In order to verify whether CTH had apoptotic-inducing effect on HepG2 cells, two methodologies were applied: (i) analysis of the externalization of phosphatidylserine using flow cytometry (FITC-conjugated Annexin V/PI assay) and (ii) visualization of morphological alterations following DAPI staining.

During apoptosis there is a loss of membrane asymmetry due to the translocation of phosphatidylserine from the inner to the outer layer of the cell membrane (Koopman et al., 1994). This translocation occurs before nuclear breakdown and DNA fragmentation (Koopman et al., 1994; Wu et al., 2005). Since Annexin V strongly binds to phosphatidylserine, Annexin V binding to cells is considered to be a major marker of apoptosis (Zhang et al., 1997). The FITC-conjugated annexin V/PI assay is a well-established method for the detection of living cells in early and late apoptosis. The four different quadrants of flow cytometric data represent four different states of cells. The lower left (LL) quadrant shows annexin-/PI-normal healthy cells. The lower right (LR) and upper right (UR) represent early (annexin+/PI-) and late apoptotic (annexin+/PI+) cells, respectively. On the upper left quadrant (UL), necrotic (annexin-/PI+) cells are displayed.

In this study, treatment of HepG2 cells with CTH resulted in a 2, 4 and 5-fold increase in the number of apoptotic cells, at the concentrations of 3.9, 7.8 and 15.6 µg/mL respectively (Fig. 4.4). Necrotic cells were also observed, but mostly after incubation with the highest concentration tested (15.6 µg/mL; 7.12 %). In fact, it has been described that treatment with cytotoxic drugs might stimulate apoptosis at lower doses and necrosis at higher doses (Zong and Thompson, 2006). Etoposide treated-cells (positive control) demonstrated 36.07 % of apoptotic cells after 72h. These results indicate that apoptosis contributed significantly to the reduction in HepG2 viability when exposed to CTH. Moreover, the morphological alterations observed upon DAPI staining confirmed the results of the FITC-conjugated Annexin V/PI assay. In fact, treated cells exhibited noticeable morphological alterations typical of apoptosis, such as nuclear fragmentation and chromatin condensation (Fig. 4.5). These morphological modifications were dose-dependent and already visible after treatment with the lowest concentration tested (3.9 µg/mL).

Failure of apoptosis is a characteristic of the tumorigenic process. Thus, one strategy underlying anticancer drug development is the induction of the apoptotic machinery in cancer cells. In fact, most cytotoxic compounds used for cancer treatment

are apoptotic inducers (Vecchione and Croce, 2010). On that note, recent research has shown strong evidence for anti-proliferative, pro-apoptotic and growth-inhibiting properties of Phaeophyceae extracts in a number of tumor models, including melanoma, lymphoma and lung cancer (Aisa et al., 2005; Culioli et al., 2004; Dias et al., 2005). Taken together, these results strongly indicate that *C. tamariscifolia* contains compounds that are able to induce apoptosis in a human hepatocarcinoma cell line.

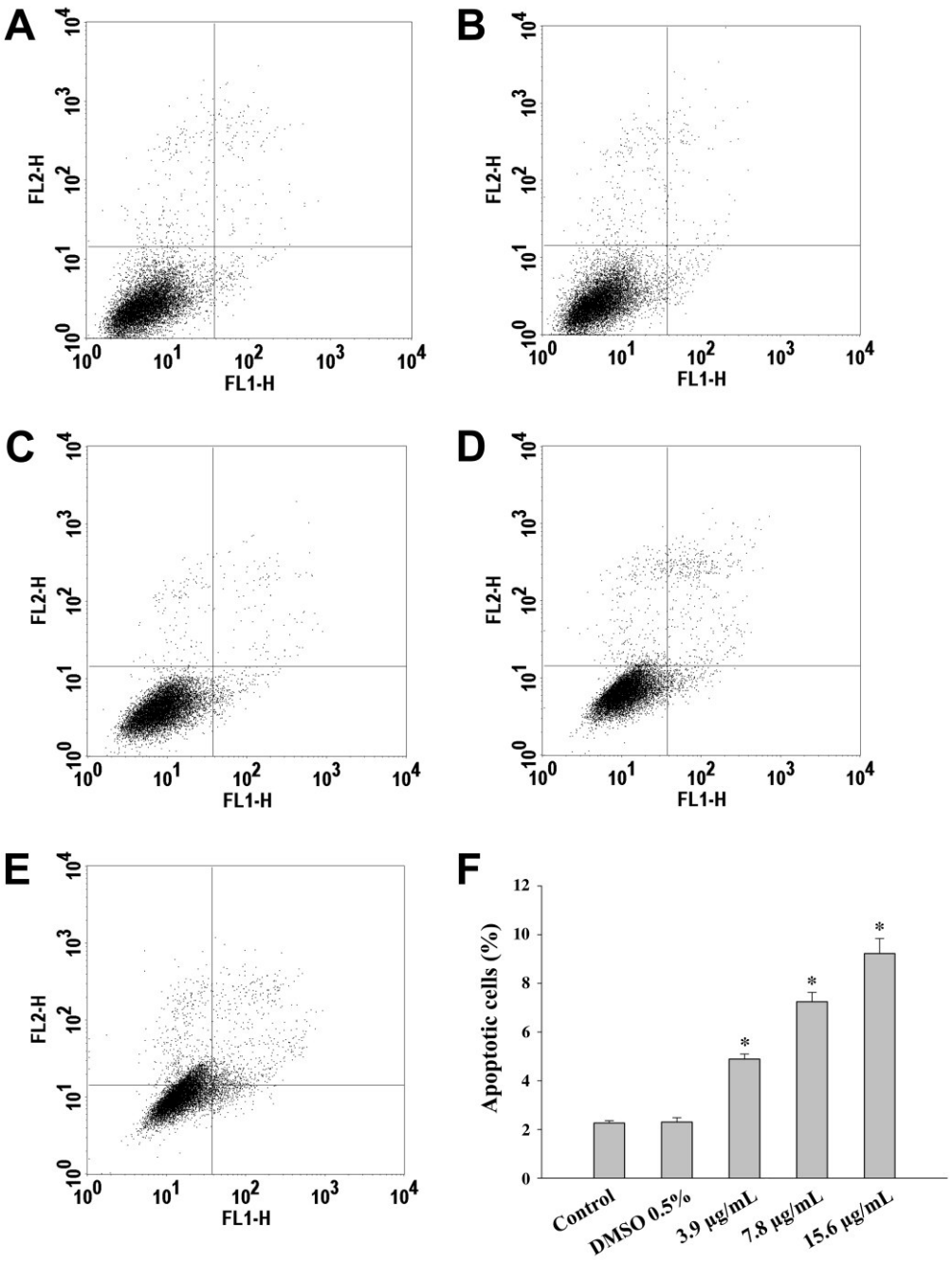

Fig. 4.4 – Incubation of HepG2 cells with CTH promotes apoptosis. Hepatocytes were treated with medium alone as blank (A), DMSO 0.5% (B, control), or CTH at concentrations of 3.9 (C), 7.8 (D) or 15.6 µg/mL (E) for 72h. Hepatocytes were then stained with PI/Annexin V-FITC and analyzed by flow cytometry. (F) Quantitative analysis of apoptotic cells. Solid bars and errors represent the mean ± SEM, respectively ($n = 6$), *$p < 0.01$ *vs.* DMSO 0.5%.

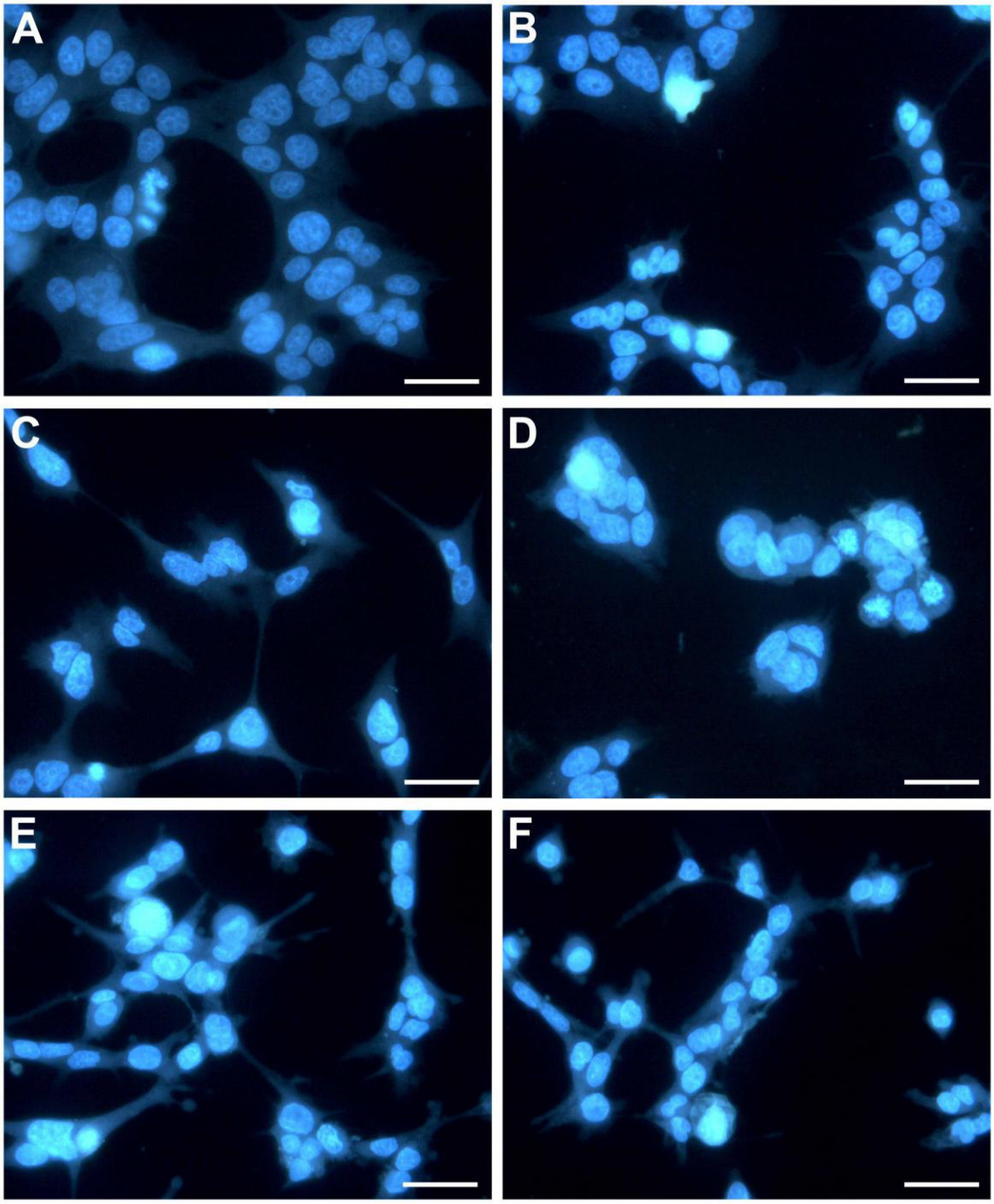

Fig. 4.5 – HepG2 cells exposed to CTH showing apoptotic features. Representative images (400 × magnification) in which hepatocyte nuclei are stained with DAPI (in blue) are shown. Hepatocytes were treated with medium alone as blank (A), DMSO 0.5% (B, control), or CTH at concentrations of 3.9 (C), 7.8 (D), 15.6 µg/mL (E) or 1.85 µg/mL etoposide (F) as a positive control for 72h. Scale bar = 50 µm.

4.4.4 Effect on MCTS

Anticancer drugs must penetrate into tumor cell masses to reach all cells at adequate concentrations. According to the vast majority of literature reports, many treatments are expected to lose efficacy in a three-dimensional (3D) pathophysiological environment, and testing on *in vitro* spheroid tumors is often considered a useful tool for negative selection to reduce animal testing or to evaluate drug candidates with enhanced tissue distribution and efficacy (Hirschhaeuser et al., 2010).

The effect of the application of CTH on 3D MCTS was examined in detail by optical microscopy. As shown in Fig. 4.6, MCTS presented a homogeneous size distribution in the controls. Loss of spheroid integrity was observed after 24 and 48 h following application of the extract at a concentration of 20 µg/mL. After incubation with 40 µg/mL of extract, this outcome was more evident and incubation with the hexane extract at 80 µg/mL, total disaggregation of MCTS occurred.

Generally, tumor cell lines are more resistant to antineoplastic agents when the cells are grown as spheroids rather than as monolayer cultures. The resistance of MCTS to anticancer drugs appears to reflect both limited drug penetration into the inner regions of the 3D cell masses as well as acquired resistance at the multicellular level (Gong et al. 2015). Although *in vivo*, tumors are affected by other cell types such as fibroblasts monocultures of multicellular spheroids from human tumor cell lines have proven to be a prevailing tool in the study of the micro-environmental regulation of tumor cell physiology and therapeutic problems associated with metabolic and proliferative gradients in a 3D cellular context (Rodriguez-Enriquez et al., 2008). The fact that the whole mass of tumor cells completely lost their adherence demonstrates that the compounds present in CTH have penetrated and may be effective in a multicellular tumor stage. The observed results combined with the anti-proliferative data confirmed the potential of CTH as a promising source of anticancer compounds.

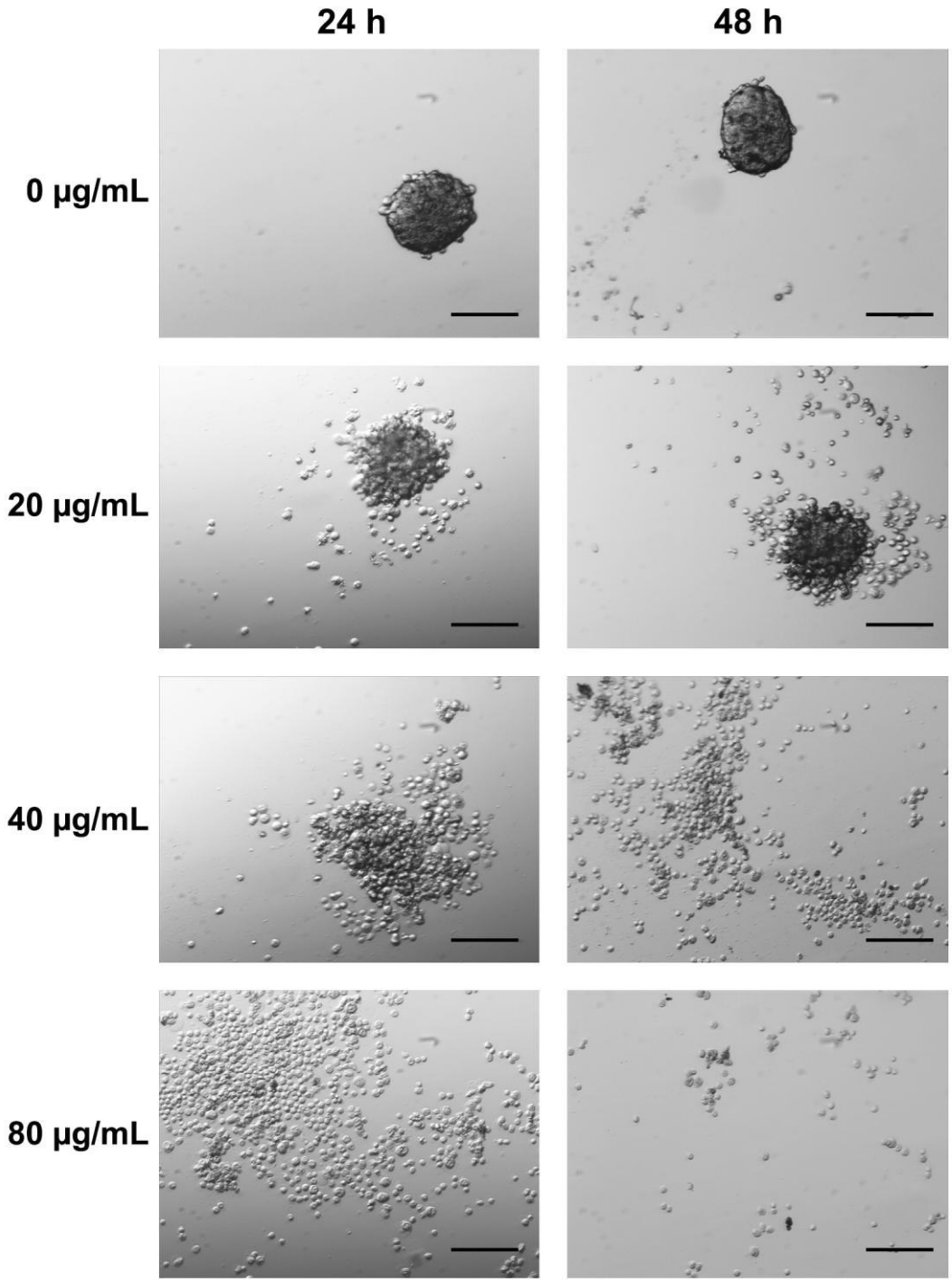

Fig. 4.6 – MCTS aggregation and morphology was influenced by CTH (20 × magnification). Control MCTS, growing as a suspension of multicellular aggregates, are shown with no incubation. The multicellular aggregates dissociated with 20, 40 and 80 µg/ml incubations for 24 and 48 h. Scale bar = 200 µm.

4.4.5 Compound isolation, structural elucidation and bioactivities

CTH was subjected to a bio-guided fractionation, affording 21 fractions, which were tested for cytotoxicity at 20 µg/mL against HepG2 using the MTT assay (Fig. 4.7). Fractions 7, 9, 13 and 14 were those that strongly reduced the viability of HepG2 cells (Fig. 4.7, $p < 0.001$). Among these, fraction 7 was the one combining a high effect on HepG2 cell viability and highest selectivity index comparing to S17 cells (SI = 5.6). Thus, fraction 7 was further purified in order to isolate and identify its major compound.

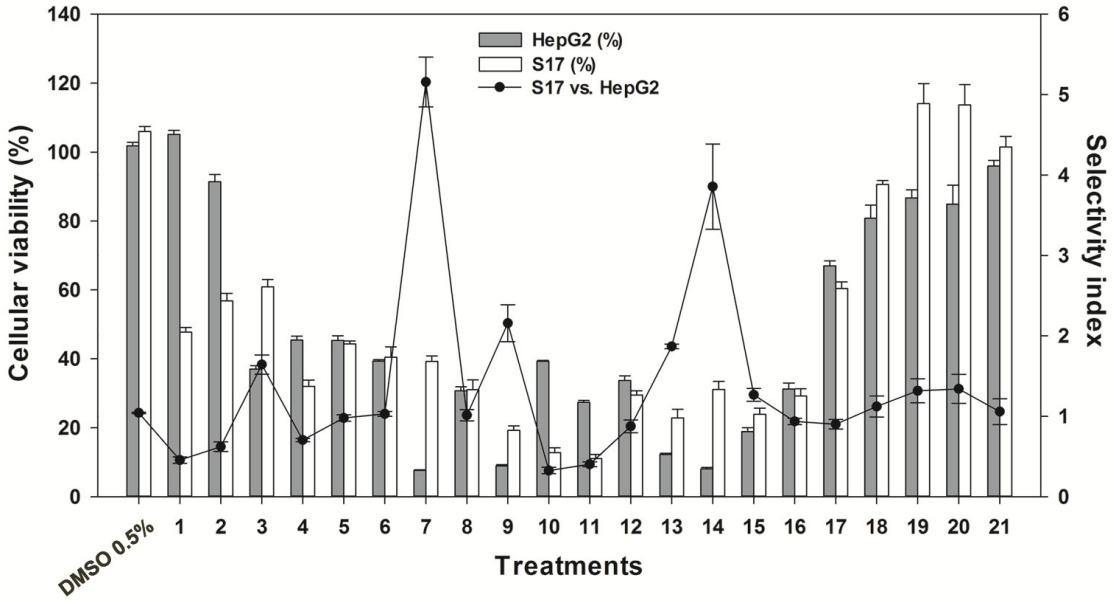

Fig. 4.7 – Effect of different fractions obtained from CTH, at a concentration of 20µg/mL, on HepG2 and S17 cellular viability. Results are expressed as % of viability relative to a control containing DMSO (0.5%, v/v). Solid bars and errors represent the average and SEM, respectively (n = 12). Selectivity (scatter lines) was calculated using IC_{50} values of the non-tumor cell line S17 *vs*. the tumor cell lines.

Compound **1** (Fig. 4.8) was obtained as an epimeric mixture at C-3 and 4. The ^{1}H NMR spectrum (DMSO-d_6) showed two coupled aromatic hydrogen atoms at δ 6.34 (br, s) and 6.25 (d, J = 3.0 Hz), assigned to H-5' and H-3', respectively. Two chemical shifts attributed to hydrogens linked to sp^2 carbons were also observed at δ 4.29 (s, H-6), 5.57 (d, J = 5.0 Hz, H-13) and 6.20 (d, J = 5.0 Hz, H-14) while six methyl groups were detected at δ 2.07 (6'-C$\underline{H}_3$), 1.31/1.28 (H-20), 1.25 (H-17), 1.24 (H-16), 1.23 (H-19), and 0.83 (H-18). The occurrence of a chromane moiety in the molecule was proposed due the cross peaks at d 2.58 (t, J = 7.5 Hz) and 1.79 (m), assigned to H-1 and H-2,

respectively, as observed in the COSY spectrum. The ^{13}C NMR data confirmed the structural similarity with a cystoketal derivative (Amico et al., 1984) mainly due to the signals attributed to carbons of aromatic ring C-1' to C-6' (δ 112.4 – 149.4), to carbons of double bonds C-5 (δ 143.9), C-6 (δ 110.4), C-13 (δ 140.1), C-14 (δ 125.7) as well as to carbinolic carbons C-12 (δ 115.5), C-15 (δ 87.8), and C-3 (δ 75.8). LRESIMS spectrum showed the protonated molecular ion $[M+H]^+$ at *m/z* 425, establishing the molecular formula $C_{27}H_{36}O_4$. Data of the isolated compound was consistent with demethoxy cystoketal chromane (compound **1**, Fig. 4.8), a meroditerpene previously isolated from *C. amentacea* (Valls et al., 1996), a species closely related to *C. tamariscifolia*.

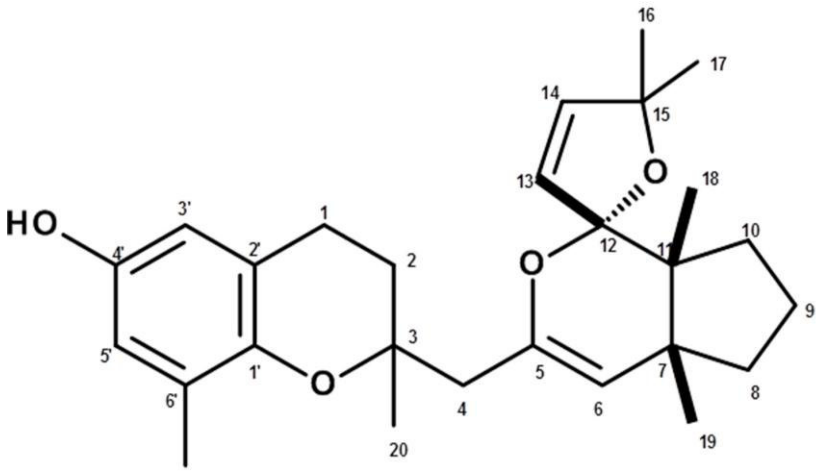

Fig. 4.8 – Structure of compound **1** (demethoxy cystoketal chromane).

Compound **1** was evaluated for antioxidant activity at the concentration of 1 mg/mL, and had an activity of 18.21% and 13.73% towards DPPH and ABTS radicals, respectively. These results indicate that compound **1** was not responsible for the antioxidant activity detected in the crude extract. In fact, the antioxidant activity is most likely a result of a synergistic effect between different constituents of the crude extract as described by Palafox-Carlos et al. (2012).

Finally, compound **1** was tested towards HepG2 and S17 cells and was able to significantly reduce the viability of HepG2 cells (IC_{50} = 14.77 µg/ml) while maintaining a high selectivity towards S17 (IC_{50} = 48.46 µg/ml, SI = 3.28). Meroditerpenoids consist of a polyprenyl chain attached to hydroquinone ring moiety. In those, plastoquinones, chromanols and chromenes are included and they are found in animals,

plants, and microorganisms (Luckner, 1984). In the marine environment, these compounds are especially abundant in brown algae such as species belonging to the *Cystoseira* and *Sargassum* genera (Blunt et al. 2014). In addition, various diterpenes have been identified as bioactive in *C. crinita* (Fisch et al., 2003), *C. myrica* (Ayyad et al., 2003) and *C. usneoides* (Urones et al., 1992). Furthermore, brown algal-derived chromene metabolites have shown to exhibit anticancer and antimutagenic activities as well as inhibitory activities against various enzymes (Stonik, Makarieva, and Dimitrenok 1992; Yamamoto et al. 1999).

To date, no bioactivities of this compound have been reported previously. To the authors' knowledge, this is the first time that demethoxy cystoketal chromane has been isolated from *C. tamariscifolia* and described as antiproliferative in HepG2 cells. In the future this molecule could be structurally optimized in order to increase pharmacokinetic and pharmacodynamic parameters among others.

4.5 Conclusions

In this work, three *Cystoseira* species were evaluated for their potential as sources of antioxidant and cytotoxic compounds. *C. tamariscifolia* had a strong antioxidant potential and a high content of phenolic compounds as well as a potent selective cytotoxic effect against hepatocellular carcinoma cells, especially its hexane extract (CTH). Moreover, CTH reduced cell proliferation and inhibited cell growth through apoptosis induction. This extract also had promising results in a 3D MCTS model, promoting the disaggregation of the mass of tumor cells after 24 h. Using bioactivity-guided fractionation procedures, it was possible to isolate and identify demethoxy cystoketal chromane as the major compound of CTH, and its selective cytotoxicity towards the recalcitrant HepG2 cell line was confirmed. It is also important to mention that this is the first description of demethoxy cystoketal chromane (**1**) in *C. tamariscifolia*, which was fully characterized as demethoxy cystoketal by analysis of MS and NMR spectral data. Overall, *Cystoseira* can be considered a valuable source of bioactive secondary metabolites and a promising source of health products.

4.6 Acknowledgements

The authors would like to thank Jesus Garcia (IRB – Institute for Research in Biomedicine) for the realization of the spectra.

4.7 References

Abu Bakar, M.F., Mohamad, M., Rahmat, A., Burr, S.A. and Fry, J.R. 2010. Cytotoxicity, cell cycle arrest, and apoptosis in breast cancer cell lines exposed to an extract of the seed kernel of *Mangifera pajang* (bambangan). Food Chem Toxicol 48, 1688-1697.

Airanthi, M.K., Hosokawa, M. and Miyashita, K. 2011. Comparative antioxidant activity of edible Japanese brown seaweeds. J Food Sci 76, C104-111.

Aisa, Y., Miyakawa, Y., Nakazato, T., Shibata, H., Saito, K., Ikeda, Y. and Kizaki, M. 2005. Fucoidan induces apoptosis of human HS-sultan cells accompanied by activation of caspase-3 and down-regulation of ERK pathways. Am J Hematol 78, 7-14.

Amico, V. 1995. Marine brown algae of family Cystoseiraceae: Chemistry and chemotaxonomy. Phytochemistry 39, 1257-1279.

Amico V., Cunsolo F., Oriente G., Piatelli M. and Ruberto, G. 1984. Cystoketal, a new metabolite from the Brown alga *Cystoseira balearica*. J Nat Prod 47, 947-952.

Ayyad, S.E., Abdel-Halim, O.B., Shier, W.T. and Hoye, T.R. 2003. Cytotoxic hydroazulene diterpenes from the brown alga *Cystoseira myrica*. Z Naturforsch C Bio Sci 58, 33 – 38.

Balboa, E.M., Conde, E., Moure, A., Falqué, E. and Domínguez, H. 2013. *In vitro* antioxidant properties of crude extracts and compounds from brown algae. Food Chem 138, 1764-85.

Blunt, J.W., Copp, B.R., Keyzers, R.A., Munro, M.H.G. and Prinsep, M.R. 2014. Marine natural products. Nat Prod Rep 31, 160-258.

Brand-Williams, W., Cuvelier, M. and Berset, C. 1995. Use of a free radical method to evaluate antioxidant activity. LWT - Food Sci Tech 28, 25–30.

Caamal-Fuentes, E., Moo-Puc, R., Freile-Pelegrín, Y. and Robledo D. 2014. Cytotoxic and antiproliferative constituents from *Dictyota ciliolata*, *Padina sanctae-crucis* and *Turbinaria tricostata*. Pharm Biol 27, 1-5.

Culioli, G., Ortalo-Magne, A., Daoudi, M., Thomas-Guyon, H., Valls, R. and Piovetti, L. 2004. Trihydroxylated linear diterpenes from the brown alga *Bifurcaria bifurcata*. Phytochemistry 65, 2063-2069.

Custódio, L., Soares, F., Pereira, H., Barreira, L., Vizetto-Duarte, C., Rodrigues, M.J., Rauter, A.P., Alberício, F. and Varela, J. 2013. Fatty acid composition and biological activities of *Isochrysis galbana* T-ISO, *Tetraselmis* sp. and *Scenedesmus* sp.: possible application in the pharmaceutical and functional food industries. J Appl Phycol 26, 151-161.

Dai, J. and Mumper, R.J. 2010. Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. Molecules 15, 7313-7352.

Demiray, S., Pintado, M.E. and Castro, P.M.L. 2009. Evaluation of phenolic profiles and antioxidant activities of Turkish medicinal plants: *Tilia argentea, Crataegi folium* leaves and *Polygonum bistorta* roots. World Acad Sci Eng Technol 3, 270-275.

Dias, P.F., Siqueira, J.M. Jr, Vendruscolo, L.F., de Jesus Neiva, T., Gagliardi, A.R., Maraschin, M. and Ribeiro-do-Valle, R.M. 2005. Antiangiogenic and antitumoral properties of a polysaccharide isolated from the seaweed *Sargassum stenophyllum*. Cancer Chemother Pharmacol 56, 436-446.

Dos Santos, J.H., Oliveira, D., De, C.D., Pinto, J., Campos, V., Mourão, A., Pessoa, C., De M.M. and Costa L.L. 2010. Evaluation of native and exotic Brazilian plants for anticancer activity. J Nat Med 64, 231-238.

Fisch, K.M., Böhm, V., Wright, A.D. and König, G.M. 2003. Antioxidative meroterpenoids from the brown alga *Cystoseira crinita*. J Nat Prod 66, 968-975.

Foti, M., Piattelli, M., Amico, V. and Ruberto, G. 1994. Antioxidant activity of phenolic meroditerpenoids from marine algae. J Photochem Photobiol B 26, 159-164.

Funahashi, H., Imai, T., Tanaka, Y., Tsukamura, K., Hayakawa, Y., Kikumori, T., Mase, T., Itoh, T., Nishikawa, M., Hayashi, H., Shibata, A., Hibi, Y., Takahashi, M. and Narita, T. 1999. Wakame seaweed suppresses the proliferation of 7,12-

dimethylbenz(a)-anthracene-induced mammary tumors in rats. Jpn J Cancer Res 90, 922-927.

Gong, X., Lin, C., Cheng, J., Su, J., Zhao, H., Liu, T., Wen, X. and Zhao, P. 2015. Generation of multicellular tumor spheroids with microwell-based agarose scaffolds for drug testing. PLoS One 10, e0130348.

Goswami, P.C. and Singh, K.K. 2006. Oxidative stress and multistage carcinogenesis. *In*: Oxidative stress, disease and cancer. Singh K. editor. Imperial College Press, London, pp 705-731.

Gouveia, V.L.M., Seca, A.M.L., Barreto, M.C., Neto, A.I., Kijjoa, A. and Silva, A.M.S. 2013. Cytotoxic meroterpenoids from the macroalga *Cystoseira abies-marina*. Phytochem Lett 6, 593-597.

Guiry, M.D. and Guiry, G.M. 2015. Algae Base. World-wide electronic publication, National University of Ireland, Galway,; http://www.algaebase.org (searched on 14.01.15).

Hadj Ammar, H., Lajili, S., Ben Said, R., Le Cerf, D., Bouraoui, A. and Majdoub, H. 2015. Physico-chemical characterization and pharmacological evaluation of sulfated polysaccharides from three species of Mediterranean brown algae of the genus *Cystoseira*. Daru 23, 1.

Hande, K.R. 1998. Etoposide: four decades of development of a topoisomerase II inhibitor. Eur J Cancer 34, 1514-1521.

Heffernan, N., Brunton, N.P., FitzGerald, R.J. and Smyth, T.J. 2015. Profiling of the molecular weight and structural isomer abundance of macroalgae-derived phlorotannins. Mar Drugs 13, 509-528.

Hirschhaeuser, F., Menne, H., Dittfeld, C., West, J., Mueller-Klieser, W. and Kunz-Schughart, L.A. 2010. Multicellular tumor spheroids: an underestimated tool is catching up again. J Biotechnol 148, 3-15.

Ito, N., Hirose, M., Fukushima, H., Tsuda, T., Shirai, T. and Tatenatsu, M. 1986. Studies on antioxidants: their carcinogenic and modifying effects on chemical carcinogens. Food Chem Toxycol 24, 1071-1092.

Ivanova, D., Gerova, D., Chervenkov, T. and Yankova, T. 2005. Polyphenols and antioxidant capacity of Bulgarian medicinal plants. J Ethnopharmacol 97, 145-150.

Khanavi, M., Gheidarloo, R., Sadati, N., Ardekani, M.R.S., Nabavi, S.M.B., Tavajohi, S. and Ostad, S.N. 2012. Cytotoxicity of fucosterol containing fraction of marine algae against breast and colon carcinoma cell line. Pharmacogn Mag 8, 60-64.

Koopman, G., Reutelingsperger, C.P., Kuijten, G.A., Keehnen, R.M., Pals, S.T. and van Oers, M.H. 1994. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood 84, 1415-1420.

Kurt, O., Ozdal-Kurt, F., Tuğlu, M. and Akçora, C. 2014. The cytotoxic, neurotoxic, apoptotic and antiproliferative activities of extracts of some marine algae on the MCF-7 cell line. Biotech Histochem 28, 1-9.

Li, H.-B., Cheng, K.-W., Wong, C.-C., Fan, K.-W., Chen, F.and Jiang, Y. 2007. Evaluation of antioxidant capacity and total phenolic content of different fractions of selected microalgae. Food Chem 102, 771-776.

Liu, Y., Zhao, C., Li, H., Yu, M., Gao, J., Wang, L. and Zhai, Y. 2010. Cytotoxicity and apoptosis induced by a new podophyllotoxin glucoside in human hepatoma (HepG2) cells. Can J Physiol Pharmacol 88, 472-9.

Mahavorasirikul, W., Viyanant, V., Chaijoroenkul, W., Itharat, A.and Na-Bangchang, K. 2010. Cytotoxic activity of Thai medicinal plants against human cholangiocarcinoma, laryngeal and hepatocarcinoma cells *in vitro*. BMC Complement Altern Med 10, 1-8.

Maimoona, A., Naeem, I., Saddiqe, Z. and Ali, N. 2011. Analysis of total flavonoids and phenolics in different fractions of bark and needle extracts of *Pinus roxburghiiand Pinus wallichiana*. J Med Plants Res 5, 5216-5220.

Mhadhebi, L., Laroche-Clary, A., Robert, J. and Bouraoui, A. 2011. Antioxidant, anti-inflammatory, and antiproliferative activities of organic fractions from the Mediterranean brown seaweed *Cystoseira sedoides*. Can J Physiol Pharmacol 89, 911-921.

Miao, Z.H., Tang, T., Zhang, Y.X., Zhang, J.S. and Ding, J. 2003. Cytotoxicity, apoptosis induction and downregulation of MDR-1 expression by the anti-topoisomerase II agent, salvicine, in multidrug-resistant tumor cells. Int J Cancer 106, 108-115.

Montero, L., Herrero, M., Ibáñez, E. and Cifuentes, A. 2014. Separation and characterization of phlorotannins from brown algae *Cystoseira abies-marina* by comprehensive two-dimensional liquid chromatography. Electrophoresis 35, 1644-1651.

Moreno, P., Petkov, G., Ramazanov, Z. and Garsia, G. 1998. Lipids, fatty-acids and sterols of *Cystoseira abies-marina*. Bot Mar 41, 375-378.

Moreno, S., Scheyer, T., Romano, C.S. and Vojnov, A.A. 2006. Antioxidant and antimicrobial activities of rosemary extracts linked to their polyphenol composition. Free Radical Res 40, 223-231.

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 65, 55-63.

Murugan, R.S., Priyadarsini, R.V., Ramalingam, K., Hara, Y., Karunagaran, D. and Nagini, S. 2010. Intrinsic apoptosis and NF-κB signaling are potential molecular targets for chemoprevention by black tea polyphenols in HepG2 cells *in vitro* and in a rat hepatocarcinogenesis model *in vivo*. Food Chem Toxicol 48, 3281-3287.

Oh, S.H., Ahn, J., Kang, D.H. and Lee, H.Y. 2011. The effect of ultrasonificated extracts of *Spirulina maxima* on the anticancer activity. Mar Biotechnol 13, 205-214.

Palafox-Carlos, H., Gil-Chávez, J., Sotelo-Mundo, R.R., Namiesnik, J., Gorinstein, S. and González-Aguilar, G.A. 2012. Antioxidant interactions between major phenolic compounds found in 'Ataulfo'Mango pulp: chlorogenic, gallic, protocatechuic and vanillic acids. Molecules 17, 12657–12664.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and Rice-Evans, C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 26, 1231-1237.

Rodriguez-Enriquez, S., Gallardo-Perez, J.C., Aviles-Salas, A., Marin-Hernandez, A., Carreno-Fuentes, L., Maldonado-Lagunas, V. and Moreno-Sanchez, R. 2008. Energy metabolism transition in multi-cellular human tumor spheroids. J Cell Physio 216, 189–197.

Safer, A.M. and Al-Nughamish, A.J. 1999. Hepatotoxicity induced by the antioxidant food additive butylated hydroxytoluene (BHT) in rats: an electron microscopical study. Histol Histopathol 14, 391-406.

Sathya, R., Kanaga, N., Sankar, P. and Jeeva, S. 2013. Antioxidant properties of phlorotannins from brown seaweed *Cystoseira trinodis* (Forsskål) C. Agardh. Arabian J Chem doi:10.1016/j.arabjc.2013.09.039.

Saura-Calixto, F. 2011. Dietary fiber as a carrier of dietary antioxidants: an essential physiological function. J Agric Food Chem 59, 43–49.

Scott, H.K. and William, C.E. 2000. Induction of apoptosis by cancer chemotherapy. Exp Cell Res 256, 42-49.

Smit, A.J. 2004. Medicinal and pharmaceutical uses of seaweed natural products: A review. J Appl Phycol 16, 245-262.

Urones, J.G., Basabe, P., Marcos, I.S., Pineda, J., Lithgow, A.M., Moro, R.F., Palma, F.M.S.B., Araújo, M.E.M. and Gravalos, M.D.G. 1992. Meroterpenes from *Cystoseira usneoides*. Phytochemistry 31, 179-182.

Valls, R., Mesguiche, V., Piovetti, L., Prost, M. and Peiffer, G. 1996. Meroditerpenes from the brown alga *Cystoseira amentacea* var. *stricta* collected off the French mediterranean coast. Phytochemistry 41, 1367-1371.

Valls, R. and Piovetti, L. 1995. The chemistry of the Cystoseiraceae (Fucales, Pheophyceae): chemotaxonomic relationships. Biochem Syst Ecol 23, 723-745.

Vecchione, A. and Croce, C.M. 2010. Apoptomirs: small molecules have gained the license to kill. Endocrine-Related Cancer 17, F37-50.

Velioglu, Y.S., Mazza, G., Gao, L. and Oomah, B.D. 1998. Antioxidant activity and total phenolics in selected fruits, vegetables and grain products. J Agric Food Chem 46, 4113-4117.

Wong, R.S. 2011. Apoptosis in cancer: from pathogenesis to treatment. J Exp Clin Cancer Res 30, 87.

Wu, S.J., Ng, L.T. and Lin, C.C. 2005. Cinnamaldehyde-induced apoptosis in human PLC/PRF/5 cells through activation of the proapoptotic Bcl-2 family proteins and MAPK pathway. Life Sci 77, 938-951.

Yu, B.P. 1994. Cellular defenses against damage from reactive oxygen species. Physiol Rev 74, 139-162.

Zhang, G., Gurtu, V., Kain, S.R. and Yan, G. 1997. Early detection of apoptosis using a fluorescent conjugate of Annexin V. Biotechniques 23, 525-531.

Zong, W.X. and Thompson, C.B. 2006. Necrotic death as a cell fate. Genes Dev 20, 1-15.

Zubia, M., Fabre, M.S., Kerjean, V., Lann, K.L., Stiger-Pouvreau, V., Fauchon, M. and Deslandes, E. 2009. Antioxidant and antitumoural activities of some Phaeophyta from Brittany coasts. Food Chem 116, 693-701.

CHAPTER 5

ISOLOLIOLIDE, A CAROTENOID METABOLITE ISOLATED FROM THE BROWN ALGAE *CYSTOSEIRA TAMARISCIFOLIA*, IS CYTOTOXIC AND ABLE TO INDUCE APOPTOSIS IN HEPATOCARCINOMA CELLS THROUGH CASPASE-3 ACTIVATION, DECREASED BCL-2 LEVELS, INCREASED P53 EXPRESSION AND PARP CLEAVAGE

Vizetto-Duarte C, Custódio L, Gangadhar KN, Lago JHG, Dias C, Matos AM, Neng N, Nogueira JMF, Barreira L, Albericio F, Rauter AP and Varela J (2016). Isololiolide, a carotenoid metabolite isolated from the brown alga *Cystoseira tamariscifolia*, is cytotoxic and able to induce apoptosis in hepatocarcinoma cells through caspase-3 activation, decreased Bcl-2 levels, increased p53 expression and PARP cleavage. Phytomedicine, 23: 550 – 557. DOI:10.1016/j.phymed.2016.02.008.

Isololiolide, a carotenoid metabolite isolated from the brown alga *Cystoseira tamariscifolia*, is cytotoxic and able to induce apoptosis in hepatocarcinoma cells through caspase-3 activation, decreased Bcl-2 levels, increased p53 expression and PARP cleavage

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5.1 Abstract

Background: Brown macroalgae have attracted attention because they display a wide range of biological activities, including antitumoral properties. In a previous screen we isolated isololiolide from *Cystoseira tamariscifolia* for the first time.

Purpose: To examine the therapeutical potential of isololiolide against tumor cell lines.

Methods/Study design: The structure of the compound was established and confirmed by 1D and 2D NMR as well as HRMS spectral analysis. The *in vitro* cytotoxicity was analyzed by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay in tumoral as well as in non-tumoral cell lines. Cell cycle arrest and induction of apoptosis were assessed by flow cytometry. Alteration of expression levels in proteins important in the apoptotic cascade was analyzed by western blotting.

Results: Isololiolide was isolated for the first time from the brown macroalga *Cystoseira tamariscifolia*. Isololiolide exhibited significant cytotoxic activity against three human tumoral cell lines, namely hepatocarcinoma HepG2 cells, whereas no cytotoxicity was found in non-malignant MRC-5 and HFF-1 human fibroblasts. Isololiolide completely disrupted the HepG2 normal cell cycle and induced significant apoptosis. Moreover, Western blot analysis showed that isololiolide altered the expression of proteins that are important in the apoptotic cascade, namely increasing PARP cleavage and p53 expression while decreasing procaspase-3 and Bcl-2 levels.

Conclusion: Isololiolide isolated from *C. tamariscifolia* is able to exert a selective cytotoxic activity on hepatocarcinoma HepG2 cells as well as induce apoptosis through the modulation of apoptosis-related proteins.

Keywords: Marine natural product; *Cystoseira*; Isololiolide; Carotenoid metabolite; Cell cycle; Apoptosis

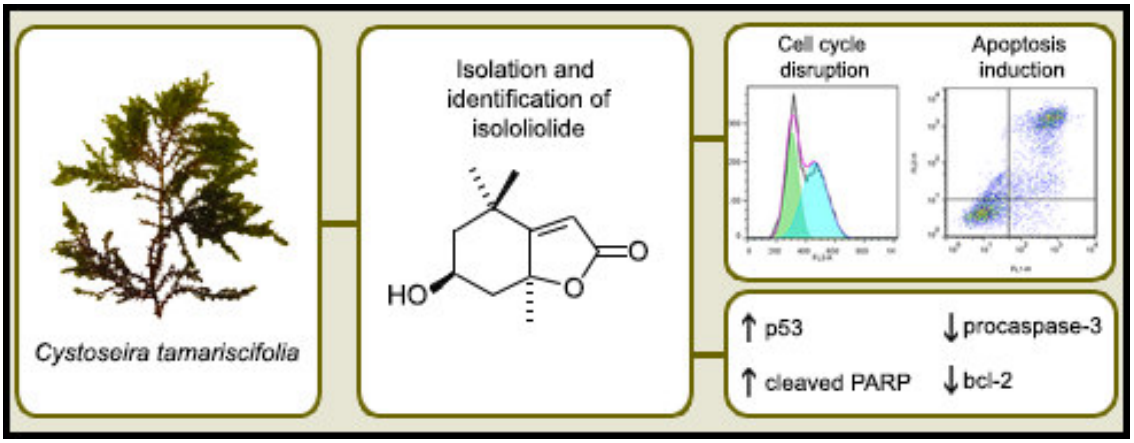

Fig. 5.1 – Graphical abstract for the work accomplished in Chapter 5.

5.2 Introduction

Cancer is a major public health problem with an estimated prevalence of about 3% in Europe, increasing to 15% at old age. Moreover, cancer related deaths are estimated to increase to over 11 million in 2030 (WHO, 2010). Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide, after lung and stomach cancer (Ferenci et al. 2010). The current therapeutics used for HCC treatment involves surgical resection, transplantation and/or systemic chemotherapy; however, surgery and transplantation may not be appropriate for many patients and chemotherapy often fails (Liu et al. 2014). Chemotherapy is also constrained by its toxicity, significant resistance to available chemotherapeutic agents and side effects, including neutropenia and myelosuppression (Chau et al. 2006). Current studies involved in developing effective cancer prevention approaches have focused on the use of bioactive natural agents that may have less adverse effects and can exert selective cytoxicity against cancer cells (Ghate et al. 2014).

The chemical and biological diversity of the marine environment is immeasurable and therefore is an extraordinary resource for the discovery of novel anticancer drugs. Brown algae are a rich source of secondary metabolites displaying a wide variety of bioactivities with important features for pharmaceutical purposes. *Cystoseira tamariscifolia* has demonstrated interesting biological activities such as antibacterial, antifungal, antiprotozoal, cell division inhibition, anti-inflammatory, antioxidant and cytotoxic properties (Bennamara et al. 1999, Spavieri et al. 2010, Lopes et al. 2012, Andrade et al. 2013). These properties have been ascribed to the presence of different classes of molecules that were identified in *C. tamariscifolia*, such as phlorotannins (fucophloroethol, fucodiphloroethol, fucotriphloroethol, 7-phloroeckol, phlorofucofuroeckol and bieckol/dieckol), phloroglucinol, proline, β-sitosterol, fucosterol, and diverse fatty acids (Ferreres et al. 2012, Andrade et al. 2013, Vizetto-Duarte et al. 2015). As *C. tamariscifolia* extracts have previously demonstrated cytotoxic potential, in this study we describe the identification of isololiolide, a known carotenoid metabolite, as a selective cytotoxic compound that was isolated from the brown macroalga *Cystoseira tamariscifolia* for the first time. Here we show evidence that exposure of hepatocarcinoma HepG2 cells to isololiolide is associated with changes in the expression of p53, PARP, Bcl-2 and procaspase-3. These results might explain the dramatic suppression of the S phase as well as the induction of apoptosis caused by this monoterpene.

5.3 Material and methods

5.3.1 Chemicals and reagents

Hexane and ethyl acetate were purchased from Prolabo (VWR International, Leuven, Belgium). Merck (Darmstadt, Germany) supplied dimethyl sulfoxide (DMSO). Roswell Park Memorial Institute medium (RPMI), Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine and penicillin/streptomycin were obtained from Lonza Ibérica (Barcelona, Spain). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Calbiochem. Primary antibodies for poly (ADP-ribose) polymerase (PARP), p53, Bcl-2, actin and respective secondary antibodies were from Santa Cruz Biotechnology Inc., Heidelberg, Germany. FITC-conjugated annexin V/ propidium iodide (PI) assay kit was acquired from Cayman Chemical Company, USA. Silica gel (Merck, 40-63 µm mesh) was used for column chromatographic separation, while silica gel 60 PF_{254} (Merck) was used for analytical (0.25 mm) TLC. $CDCl_3$ (Aldrich) was used as solvent for ^{1}H and ^{13}C NMR spectra acquisition and TMS (Aldrich) was used as internal standard. 1D and 2D NMR spectra were recorded at Bruker Digital Avance 800 MHz spectrometer. Additional reagents and necessary solvents were purchased from VWR International (Leuven, Belgium).

5.3.2 Sampling

Cystoseira tamariscifolia was collected in the middle/lower intertidal areas, during the low tide, between May and September 2012 on the Portuguese coast. Biomass was rinsed with distilled water and macroscopic epiphytes and extraneous matter were carefully removed. Identification of specimens was made by Dr Aschwin Engelen (Centre of Marine Sciences, University of Algarve, Portugal) and Dr Javier Cremades Ugarte (Facultade de Ciencias, University of A Coruña) and a voucher specimen of *C. tamariscifolia* (code number MB016) was deposited at the Centre of Marine Sciences (CCMAR), University of Algarve. Samples were freeze-dried and stored at -20 °C until the extraction procedure.

5.3.3 Extraction

Biomass was mixed with hexane (1:10, w/v) and homogenized for 2 minutes using a disperser IKA T10B Ultra-Turrax at room temperature (RT). The tubes were then vortexed for 1 minute, centrifuged (5000 *g*, 10 minutes, RT) and the supernatants recovered. The extraction procedure was repeated 3 times and the supernatants combined and filtered. The extract was dried at 40 ºC under vacuum and dissolved in DMSO for biological activities screening or in the adequate solvent for chemical characterization, aliquoted and stored (-20 °C).

5.3.4 Isolation and elucidation of isololiolide

C. tamariscifolia hexane extract (9 g) was fractionated by column chromatography (2.5 cm × 18 cm) over silica gel (SiO_2) using increasing amounts of EtOAc in hexane (9:1; 85:15; 4:1; 75:25; 7:3; 3:2; 1:1) and increasing amounts of MeOH in EtOAc (9:1; 8:1; 5:1; 2:1; 1:1), MeOH (100%) and H_2O (100%) as eluents. This procedure afforded 57 fractions, which were analyzed by TLC and pooled together in 21 groups (A – U). Fraction 14 (70 mg) was re-fractionated over SiO_2 eluted with hexane (100 %); hexane/EtOAc (9:1, 8:2, 7.5:2.5, 7:3, 6.5:3.5, 6:4, 5.5:4.5, 1:1, 4:6), EtOAc (100 %) and MeOH (100 %) to afford 151 fractions which were pooled together in 9 groups after TLC analysis. Group 6 – 8, obtained from the hexane/ EtOAc elution (6:4 through 1:1), was purified by reverse phase preparative HPLC to afford 3 mg of isololiolide.

Isololiolide. Pale yellow oil;^{1}H NMR (800 MHz, $CDCl_3$, TMS, ppm) δ 5.71 (1H, s, H-7), 4.21 (1H, m, H-3), 2.55 (2H, br d, J = 2.4 Hz, H-4), 2.03 (1H, br d, J = 2.4 Hz, H-2), 1.59 (3H, s, H-11), 1.23 (3H, s, H-10), 1.21 (3H, s, H-9). ^{13}C-NMR δ (200 MHz, $CDCl_3$, TMS, ppm): 181.2 (C-6), 171.5 (C-8), 113.3 (C-7), 86.4 (C-5), 65.1 (C-3), 49.8 (C-2), 47.9 (C-4), 35.0 (C-1), 29.9 (C-9), 25.6 (C-11), 25.1 (C-10); HRESIMS *m/z* 219.0993 $[M + Na]^+$ (calc to $C_{11}H_{16}O_3Na$ 219.0997).

5.3.5 Cell culture

HepG2 cells (human hepatocellular carcinoma) were maintained in RPMI-1640 culture media supplemented with glucose (1000 mg/ml), 10% FBS, L-glutamine (2 mM), penicillin (50 U/ml) and streptomycin (50 µg/ml). MRC-5 and HFF-1 human fibroblasts, AGS human gastric cancer, HCT-15 human colon cancer cells were grown

in DMEM culture media supplemented with glucose (1000 mg/ml), 10% FBS, L-glutamine (2 mM), penicillin (50 U/ml) and streptomycin (50 µg/ml). Cell lines were grown in an incubator at 37 ºC and 5.0% CO_2 in humidified atmosphere.

5.3.6 Anti-proliferative assay

In vitro cytotoxic activity of isololiolide was assessed by the MTT colorimetric assay. Hepatocarcinoma HepG2, gastric cancer AGS and colon cancer HCT-15, and also non-tumoral cells (MRC-5 and HFF-1 human fibroblasts) were seeded at a density of 5×10^3 cells/well on 96-well plates and incubated for 24 h at 37 °C in 5.0% CO_2. The effect of isololiolide was evaluated on the viability of these cells and the half maximal inhibitory concentration (IC_{50}) was calculated upon a 72 h incubation period. Positive control cells were treated with etoposide, while negative control cells were treated with DMSO at the highest concentration used in test wells (0.5%, v/v). The selectivity of the compound was estimated using the following equation: Selectivity = CT/CNT, where CT and CNT indicate the compound-induced cytotoxicity on tumoral cells and on non-tumoral cells, respectively (Oh et al., 2010).

5.3.7 Cell cycle distribution analysis

HepG2 cells were plated at a density of 5×10^4 cells/ml in 6-well plates and incubated with complete medium only (blank), medium with the solvent DMSO (control, 0.5% v/v) or with isololiolide at IC_{50} concentration (13.15 µM), which was previously determined by the MTT assay. Cells were harvested following 72 h incubation and further processed for cell cycle analysis. Cellular DNA content for cell cycle distribution analysis was evaluated using an Epics XL-MCL Coulter flow cytometer plotting at least 10000 events per sample. Cell cycle distribution data analysis was subsequently performed using the FlowJo 7.2 software (Tree Star, Ashland, USA).

5.3.8 Apoptosis detection

HepG2 cells were plated at a concentration of 5×10^4 cells/ml in 6-well plates and incubated with complete medium only (blank), medium with the solvent DMSO (control, 0.5% v/v), or with isololiolide at IC_{50} concentration (13.15 µM) for 72 h. Induction of apoptosis was evaluated by the annexin V-FITC/PI apoptosis Kit (Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions. Measurement of annexin V binding due to phosphatidylserine externalization was

analyzed using an Epics XL-MCL Coulter flow cytometer plotting at least 20 000 events per sample. Apoptotic data analysis was subsequently performed using the FlowJo 7.2 software (Tree Star, Ashland, USA).

5.3.9 Protein expression analysis

For the analysis of protein expression, HepG2 cells were treated with complete medium (blank), medium with the solvent (DMSO) or with loliolide at the IC_{50} concentration (13.15 µM), and incubated for 24, 48 and 72 h. After each incubation period cells were lysed in Winman's buffer (1% NP-40, 0.1 M Tris–HCl pH 8.0, 0.15 M NaCl and 5 mM EDTA) with EDTA-free protease inhibitor cocktail (Boehringer, Mannheim, Germany). Proteins were quantified using the DC Protein Assay Kit (BioRad, Hercules, CA, USA) and separated in 12% tris-glycine sodium dodecyl sulphate (SDS)–polyacrylamide gel. Proteins were then transferred to a nitro-cellulose membrane (GE Healthcare, Madrid, Spain). The membranes were incubated with the following primary antibodies for PARP (1:4000), actin (1:2000), p53 (1:250), Bcl-2 (1:200) and procaspase-3 (1:2000), and further incubated with the appropriate secondary antibodies conjugated with horseradish peroxidase (HRP) diluted 1:2000 in 5% non-fat dried milk in T-TBS. The signal was detected with the Amersham ECL kit (GE Healthcare). Hyperfilm ECL (GE Healthcare) and Kodak GBX developer and fixer twin pack (Sigma).

5.3.10 Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) was assessed using the SPSS statistical package for Windows (release 15.0, SPSS Inc.), and significance between means was analyzed by the Tukey HSD test ($p < 0.05$). IC_{50} values were calculated by sigmoidal fitting of the data using GraphPad Prism v. 5.0 (GraphPad Software, Inc., La Jolla, CA). Statistical analysis was performed by the non-parametric Friedman's test followed by Dunn's Post-test using GraphPad Prism 5 software. P values < 0.05 were considered as statistically significant.

5.4 Results and discussion

5.4.1 Characterization of isololiolide

HRESIMS of the isolated compound showed a $[M + Na]^+$ *quasi*-molecular ion peak at *m/z* 219.0993, indicating the molecular formula $C_{11}H_{16}O_3$, with four unsaturations. Its ^1H NMR spectrum displayed, despite other signals, peaks assigned to hydrogens of three methyl groups at δ_H 1.21 (s, 3H), 1.23 (s, 3H) and 1.59 (s, 3H), one olefinic hydrogen at δ_H 5.71 (s, 1H) and one oxymethine hydrogen at δ_H 4.13 (m, 1H). The ^{13}C and DEPT 135 showed eleven peaks assigned to three methyl, two methylene, two methine and four quaternary carbons, including one α,β-unsaturated carbonyl group at δ_C 171.5 (C-8), 113.3 (C-7) and 181.2 (C-8) and one carbinolic carbon at δ_C 65.1 (C-3). HMBC spectrum showed cross peaks between the signals at H-11 with C-4/C-5/C-6, H-9 with C-1/C-6/C-10, H-10 with C-2/C-6/C-9 and H-7 with C-5/C-6/C-8. Isololiolide (Fig. 5.2) was identified comparing the obtained data with that reported in the literature (Kimura and Maki 2002).

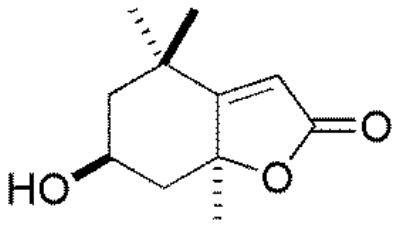

Fig. 5.2 – Chemical structure of isololiolide.

5.4.2 Anti-proliferative activity of isololiolide in tumoral and non-tumoral cell lines

Isololiolide obtained from the *C. tamariscifolia* hexane extract was tested on human hepatocellular carcinoma cells (HepG2), gastric cancer cells (AGS) and colon cancer cell line (HCT-15). Additionally, the anti-proliferative activity of the compounds was evaluated in human fibroblasts (MRC-5 and HFF-1). The compound proved to be cytotoxic against the different tumoral cell lines, namely AGS (IC_{50} = 32.36 μM), HCT-15 (IC_{50} = 23.59 μM) and especially HepG2 cells (IC_{50} = 13.15 μM; Table 5.1), showing selectivity indices (SI) of up to 86 and 47 against MRC-5 and HFF-1 fibroblasts, respectively (Table 5.1).

Table 5.1 – IC_{50} (μM) of isololiode obtained from the hexane fraction from *C. tamariscifolia* in different cell lines at 72h.

Cell lines	IC_{50}	SI: MRC-5	SI: HFF-1
HepG2	13.15 ± 0.56^{a}	86.07^{A}	47.04^{B}
AGS	32.36 ± 0.20^{c}	34.97^{C}	19.12^{D}
HCT-15	23.59 ± 0.15^{b}	47.98^{B}	26.22^{D}
MRC-5	1131.76 ± 1.22^{e}	-	-
HFF-1	618.62 ± 1.12^{d}	-	-

The values presented correspond to IC_{50} values and are the mean ± S.E; of at least 3 independent experiments in triplicate. In the same column, values followed by different letters (a-e for IC_{50} values and A-D for SI) are significantly different (Tukey HSD test, $p < 0.05$). HepG2: human hepatocarcinoma; AGS: human gastric adenocarcinoma; HCT-15: human colorectal adenocarcinoma; MRC-5: human lung fibroblast derived from healthy tissue; HFF-1: human foreskin fibroblast derived from healthy tissue.

The cytotoxic effect of this molecule towards HepG2 cells was particularly evident, whereas no significant toxic effect was observed in MRC-5 or HFF-1 human fibroblasts. Interestingly, extracts from *C. tamariscifolia* had previously demonstrated antiproliferative potential against Daudi (human Burkitt's lymphoma), Jurkat (human leukemic T cell lymphoblast) and K562 (human chronic myelogenous leukemia) cells (Zubia et al. 2009). Isololiolide has been described as a carotenoid metabolite whose precursor is zeaxanthin (Repeta 1989). Carotenoids, such as zeaxanthin, lycopene and astaxanthin have been previously described as modulators of growth factors that play important roles in cell cycle regulation and carcinogenesis (Bi et al. 2013, Alvarez et al. 2014). Moreover, it has been previously demonstrated that breakdown products of carotenoids (e.g. 3-OH-β-apo-10'-carotenal and apo-10'-lycopenal) might act as chemotherapeutic agents against breast and hepatic cancer (Tibaduiza et al. 2002, Ip et al. 2014). Loliolide, an isololiolide isomer, is also a well-known carotenoid metabolite derived from the breakdown from fucoxanthin able to inhibit algal growth (Taylor and Burden 1970). On the other hand, isololiolide has been previously isolated from brown algae namely from *Undaria pinnatifida* (Kimura and Maki 2002), *Dictyopteris divaricata* (Song et al. 2004) and *Homoeostrichus formosana* (Fang et al. 2015). However, this is the first report describing the occurrence of isololiolide in *C. tamariscifolia*.

5.4.3 Effects on cell cycle profile

Because of the potential application of carotenoid breakdown products in cancer therapeutics and the observed cytoxicity in HepG2 cells, we researched the effectiveness of isololiolide in arresting the cell cycle in the latter hepatocarcinoma cell line. For this purpose, HepG2 cells were incubated with isololiolide at 13.15 µM (IC_{50}) for 72 h and its effect on cell cycle distribution was studied. Analysis of the cell cycle was performed by flow cytometry and the results showed that this monoterpenoid completely disrupted the normal HepG2 cell cycle. In fact, isololiolide induced G2/M cell cycle arrest along with a concomitant decrease in the percentage of cells in the S phase (Fig. 5.3A) and this effect was sustained throughout the 72 h treatment. In fact, the percentage of cells in G2/M phase was 15.09 % and 14.91 % for the control and DMSO 0.5 %, respectively, increasing to 57.95 % upon treatment with isololiolide at 13.15 µM for 72 h (Fig. 5.3B). In addition, there were virtually no cells in the S phase after the same incubation. Taken together, these results suggest that isololiolide affects the molecular pathways monitoring and controlling cell cycle progression by arresting the cells at the G2/M checkpoint. The cell cycle checkpoints play an important role in the control system by sensing defects occurring during essential processes, such as DNA replication or chromosome segregation, inducing a cell cycle arrest until the defects detected are repaired (Malumbres 2012).

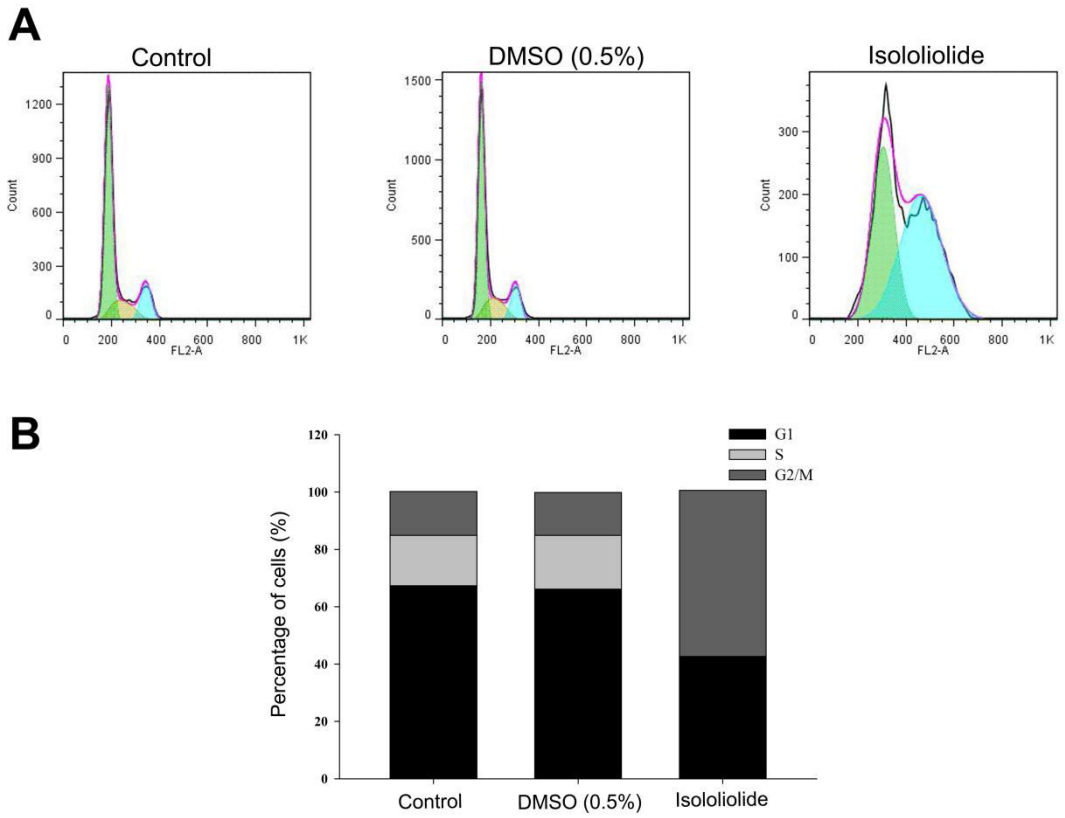

Fig. 5.3 – HepG2 cell cycle analysis (A) from control (incubation with complete medium only), DMSO (0.5% v/v) or isololiolide (13.15 µM) treatment for 72 h. Percentage of cells in G1, S and G2/M phases upon the treatments described above (B).

5.4.4 Apoptosis induction by isololiolide treatment

The annexin V-FITC/PI flow cytometry assay was used in order to determine if isololiolide was inducing apoptosis in HepG2 cells. Bivariate staining using annexin V-FITC/PI further demonstrated that isololiolide induced apoptosis at the IC_{50} concentration (Fig. 5.4A). HepG2 cells were treated with isololiolide for 72 h and a significant increase ($P < 0.01$) in the percentage of apoptotic cells was observed, from 6.9 % in untreated cells, to 29.1 % in cells treated with isololiolide (Fig. 5.4B). Apoptosis is described as an active process of programmed cellular death that avoids an exacerbated inflammatory response (Fink and Cookson 2005) and is associated with responses to cancer therapy. In fact, it is widely described that resistance to apoptosis is one of the hallmarks of cancer cells (Hanahan and Weinberg 2011). This resistance enables cancerous cells to survive and divide even in the presence of endogenous proapoptotic stimuli. Therefore, induction of apoptosis is an important mechanism in selecting novel molecules with anti-cancer potential.

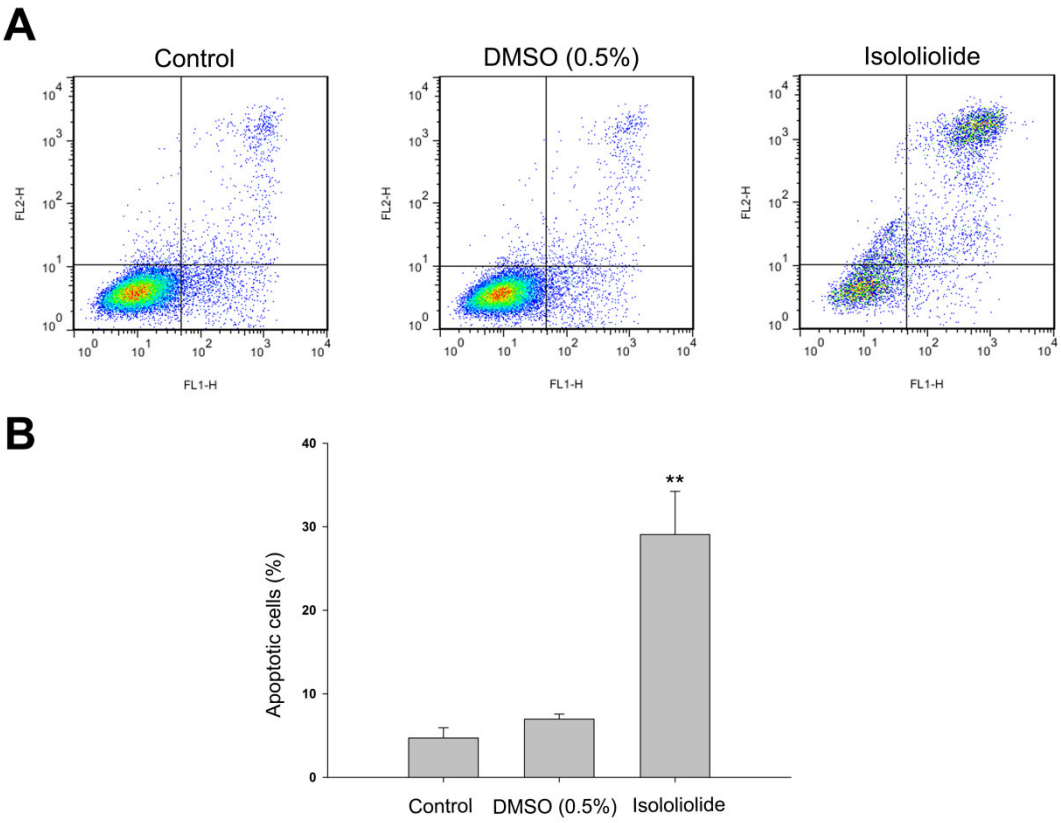

Fig. 5.4 – Flow cytometric analysis (A) and the proportion of apoptotic (B) HepG2 cells treated for 72 h with control (incubation with complete medium only), DMSO (0.5% v/v) or isololiolide (13.15 µM) for 72 h stained for annexin V-FITC/PI. $^{**}P < 0.01$ *vs.* DMSO 0.5 %.

5.4.5 Western blot analysis of apoptosis-related proteins

Based on the results obtained with annexin V-FITC demonstrating that apoptosis is occurring, the expression of apoptosis-related proteins was evaluated by assessing procaspase-3, PARP, Bcl-2 and p53 protein levels expression in HepG2 cells incubated with complete medium (control), vehicle (DMSO 0.5% v/v) or isololiolide (13.15 µM). Concerning caspase-3, a decrease in procaspase-3 expression upon isololiolide treatment at 24 h was measured (Fig. 5.5, $P < 0.05$). In human cells, apoptosis takes place through a cascade of events involving two main pathways: the intrinsic and the extrinsic pathways (Kroemer et al. 2007). Both pathways ultimately converge on the activation of procaspases (primarily procaspase-3, but also procaspase-7 and procaspase-6) to caspases, which are the cysteine proteases that cleave their protein substrates within the cell. Effector caspase-3 zymogen (or procaspase-3) exists within

the cytosol as an inactive dimer (Boatright and Salvesen 2003). It is activated by limited proteolysis within the interdomain linker, which is carried out by an initiator caspase or occasionally by other proteases under specific circumstances. At cytosolic concentrations in human cells, the caspase-3 zymogens are already dimers, but cleavage within their respective linker segments is required for activation (Boatright and Salvesen 2003). Therefore, a decrease in procaspase-3 levels is due to its proteolysis, leading to caspase-3 activation. Our results showed that incubation of HepG2 cells with isololiolide resulted in a 2-fold decrease of procaspase-3 levels, strongly suggesting that procaspase-3 was processed to caspase-3. In addition, concentrations of procaspase-3 in certain cancerous cells are significantly higher than those in non-cancerous controls (Putt et al. 2006).

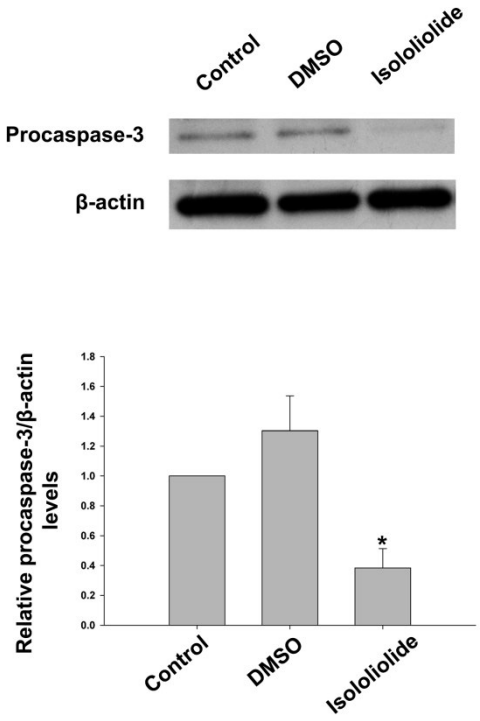

Fig. 5.5 – Procaspase-3 expression levels upon incubation with complete medium (control), vehicle (DMSO 0.5 %) or isololiolide (13.15 µM) at 24 h. **P* < 0.05 *vs.* DMSO 0.5 %.

PARP cleavage increased about 4-fold upon isololiolide incubation at 13.15 µM for 24 h (Fig. 5.6, *P* < 0.01). Interestingly, this increment seems to be time-dependent as shown in Fig. 5.6. PARP plays an active role in key biological processes, such as transcription and cell cycle regulation, response to DNA damage, apoptosis and maintenance of genome integrity. The presence of cleaved PARP is one of the most

used biomarkers for the detection of apoptosis (Duriez and Shah 1997). Moreover, PARP is a substrate of caspase-3 and its cleavage into two fragments has been considered to be indicative of functional caspase activation (Bressenot et al. 2009). Cleaved PARP was observed after treatment with isololiolide, in a time-dependent manner. In fact, (Soldani et al. 2001) reported that PARP proteolysis by caspase is a very early response to the apoptotic stimulus.

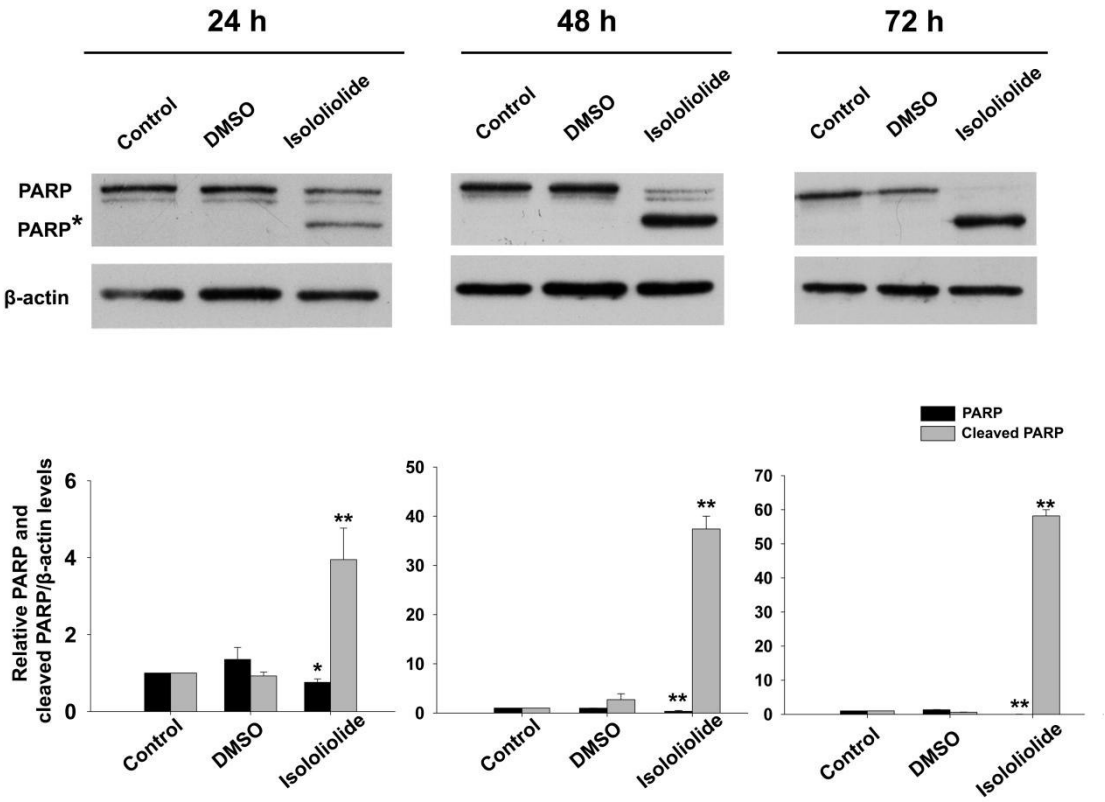

Fig. 5.6 – Full length (PARP) and cleaved PARP (PARP*) expression levels upon incubation with complete medium (control), vehicle (DMSO 0.5 %) or isololiolide (13.15 µM) at 24, 48 and 72 h. *$P < 0.05$, **$P < 0.01$ *vs.* DMSO 0.5 %.

Western blot performed in the cell lysates obtained from isololiolide-treated cells showed increased expression of p53 at 24 h (Fig. 5.7, $P < 0.05$ *vs.* DMSO 0.5 %). The tumor suppressor protein p53 acts as a key player in tumor suppression, as it induces apoptosis and cell cycle arrest as well as suppress angiogenesis (Amaral et al. 2010). p53 is usually responsible for activating DNA repair proteins when DNA has extensive damage, arresting the cell cycle at regulation points or initiating apoptosis if DNA damage shows to be irreparable. Interestingly, p53 not only induces G1 cell cycle

arrest, but it is also described to act at the G2/M checkpoint, preventing cells from entering mitosis if DNA damage is found (Taylor and Stark 2001). Furthermore, p53 has the ability to activate the transcription of various pro-apoptotic genes, including those encoding members of the Bcl-2 family (Roos and Kaina 2006).

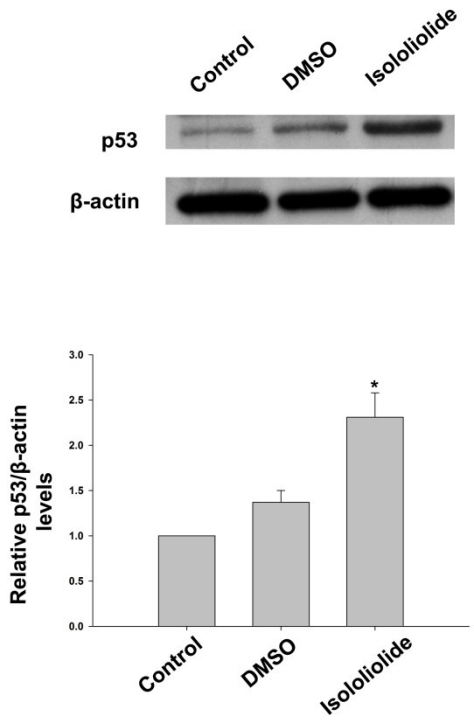

Fig. 5.7 – p53 expression levels upon incubation with complete medium (control), vehicle (DMSO 0.5 %) or isololiolide (13.15 µM) at 24 h.*$P < 0.05$ *vs.* DMSO 0.5 %.

Our results showed that anti-apoptotic Bcl-2 protein expression remained unchanged after 24 h of incubation with isololiolide, decreasing after 48 h with the same treatment (Fig. 5.8, $P < 0.05$ *vs* DMSO 0.5 %). Overexpression of anti-apoptotic Bcl-2 family members have been associated with chemotherapy resistance in various human cancers, and targeting the anti-apoptotic Bcl-2 family members have shown promising results in preclinical studies (Kang and Reynolds 2009). Bcl-2 suppresses apoptosis by binding to Bax or Bak. It is described that inhibiting the anti-apoptotic Bcl-2 could sensitize tumor cells to chemo- and radiotherapy. Therefore, decrease of Bcl-2 levels may be a plan of choice to increase treatment efficacy. Furthermore, it was suggested that p53 also modulates Bcl-2 by downregulation (Kirkin et al. 2004).

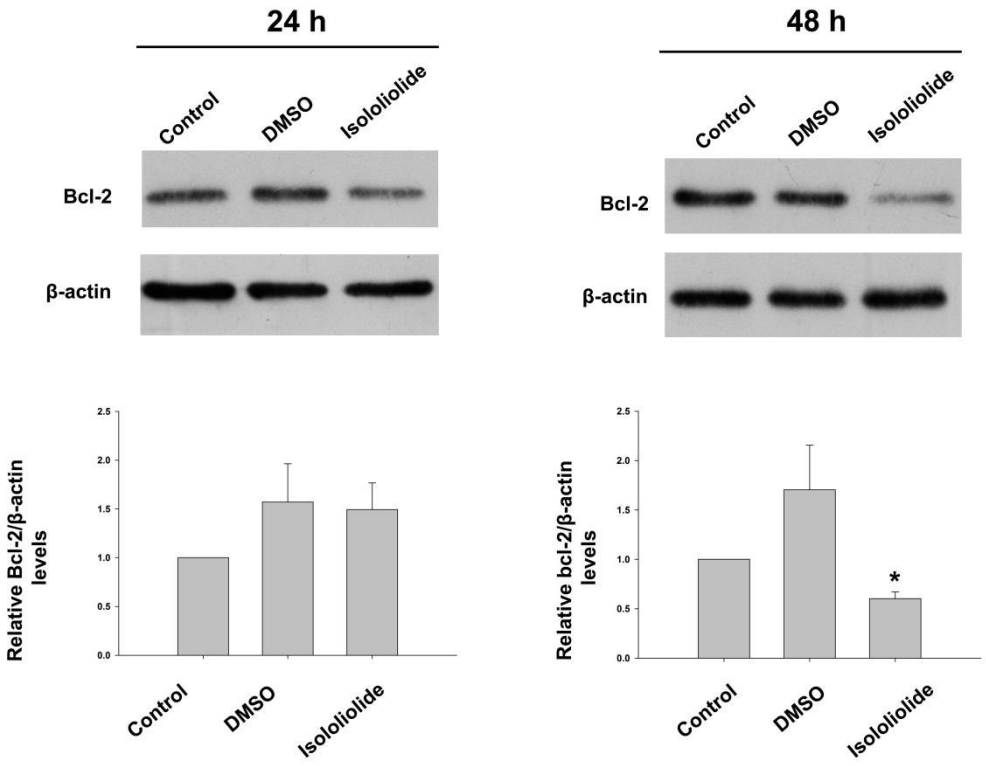

Fig. 5.8 – Bcl-2 expression levels upon incubation with complete medium (control), vehicle (DMSO 0.5 %) or isololiolide (13.15 µM) at 24 and 48 h. **P* < 0.05 *vs.* DMSO 0.5 %.

In summary, application of isololiolide resulted in the increase on caspase-3 expression, concomitant with increase in PARP cleavage and p53 expression. Corresponding down-regulation of anti-apoptotic/pro-survival Bcl-2 protein was also detected. Indeed, molecules that activate caspase-3 and p53, cleave PARP or bind to Bcl-2 have shown potential in cell culture and preclinical models of cancer (Peterson et al. 2009).

Taken together, our results strongly suggest that isololiolide is able to exert potent anti-proliferative properties, significantly promoting cell cycle arrest in S phase and inducing cellular apoptosis in a human-derived hepatocarcinoma cell line.

5.5 Conclusions

This report demonstrates for the first time the *in vitro* anti-tumoral activity of isololiolide obtained from *C. tamariscifolia* hexane extract against hepatocarcinoma through the induction of apoptosis by altering the expression of proteins important to the apoptotic cascade. As isololiolide exhibited no cytotoxicity on non-tumoral human fibroblasts under the same conditions, it would be important in the future to perform structure-activity relationships (SARs) analysis for further studies. In addition, it may provide novel clues as to how carotenoids and their metabolites play a role in preventing and/or slowing down cancer progression.

5.6 Acknowledgments

Financial support for this work was provided by the SEABIOMED (PTDC/MAR/103957/2008), XtremeBio (PTDC/MAR-EST/4346/2012) and CCMAR/Multi/04326/2013 funded by the Foundation for Science and Technology (FCT) and the Portuguese National Budget. Vizetto-Duarte C. is an FCT doctoral research fellow (SFRH/BD/81425/2011) as well as Dias C. (SFRH/BDE/51998/2012) and Matos A.M. (SFRH/BD/93170/2013). Custódio L. was supported by FCT Investigator Programme (IF/00049/2012). Lago J.H.G. acknowledges CAPES and CNPq.

5.7 References

Alvarez, R., Vaz, B., Gronemeyer, H. and de Lera, A. R. 2014. Functions, therapeutic applications, and synthesis of retinoids and carotenoids. Chem Rev 114, 1-125.

Amaral, J. D., Xavier, J. M., Steer, C. J. and Rodrigues, C. M. 2010. Targeting the p53 pathway of apoptosis. Curr Pharm Des 16, 2493-2503.

Andrade, P. B., Barbosa, M., Matos, R. P., Lopes, G., Vinholes, J., Mouga, T. and Valentao, P. 2013. Valuable compounds in macroalgae extracts. Food Chem 138, 1819-1828.

Bennamara, A., Abourriche, A., Berrada, M., Charrouf, M., Chaib, N., Boudouma, M. and Garneau, F. X. 1999. Methoxybifurcarenone: an antifungal and antibacterial meroditerpenoid from the brown alga Cystoseira tamariscifolia. Phytochemistry 52, 37-40.

Bi, M. C., Rosen, R., Zha, R. Y., McCormick, S. A., Song, E. and Hu, D. N. 2013. Zeaxanthin Induces Apoptosis in Human Uveal Melanoma Cells through Bcl-2 Family Proteins and Intrinsic Apoptosis Pathway. Evid Based Complement Alternat Med 2013, 205082.

Boatright, K. M. and Salvesen, G. S. 2003. Mechanisms of caspase activation. Curr Opin Cell Biol 15, 725-731.

Bressenot, A., Marchal, S., Bezdetnaya, L., Garrier, J., Guillemin, F. and Plenat, F. 2009. Assessment of apoptosis by immunohistochemistry to active caspase-3, active caspase-7, or cleaved PARP in monolayer cells and spheroid and subcutaneous xenografts of human carcinoma. J Histochem Cytochem 57, 289-300.

Chau, G. Y., Lui, W. Y., Tsay, S. H., Chao, Y., King, K. L. and Wu, C. W. 2006. Postresectional adjuvant intraportal chemotherapy in patients with hepatocellular carcinoma: a case-control study. Ann Surg Oncol 13, 1329-1337.

Duriez, P. J. and Shah, G. M. 1997. Cleavage of poly(ADP-ribose) polymerase: a sensitive parameter to study cell death. Biochem Cell Biol 75, 337-349.

Fang, H. Y., Chokkalingam, U., Chiou, S. F., Hwang, T. L., Chen, S. L., Wang, W. L. and Sheu, J. H. 2015. Bioactive Chemical Constituents from the Brown Alga Homoeostrichus formosana. International Journal of Molecular Sciences 16, 736-746.

Ferenci, P., Fried, M., Labrecque, D., Bruix, J., Sherman, M., Omata, M., Heathcote, J., Piratsivuth, T., Kew, M., Otegbayo, J. A., Zheng, S. S., Sarin, S., Hamid, S. S., Modawi, S. B., Fleig, W., Fedail, S., Thomson, A., Khan, A., Malfertheiner, P., Lau, G., Carillo, F. J., Krabshuis, J., Le Mair, A. and World Gastroenterology, O. 2010. Hepatocellular carcinoma (HCC): a global perspective. J Clin Gastroenterol 44, 239-245.

Ferreres, F., Lopes, G., Gil-Izquierdo, A., Andrade, P. B., Sousa, C., Mouga, T. and Valentao, P. 2012. Phlorotannin extracts from fucales characterized by HPLC-DAD-ESI-MSn: approaches to hyaluronidase inhibitory capacity and antioxidant properties. Mar Drugs 10, 2766-2781.

Fink, S. L. and Cookson, B. T. 2005. Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. Infect Immun 73, 1907-1916.

Ghate, N. B., Hazra, B., Sarkar, R., Chaudhuri, D. and Mandal, N. 2014. Alteration of Bax/Bcl-2 ratio contributes to Terminalia belerica-induced apoptosis in human lung and breast carcinoma. In Vitro Cell Dev Biol Anim 50, 527-537.

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

Ip, B. C., Liu, C., Ausman, L. M., von Lintig, J. and Wang, X. D. 2014. Lycopene Attenuated Hepatic Tumorigenesis via Differential Mechanisms Depending on Carotenoid Cleavage Enzyme in Mice. Cancer Prevention Research 7, 1219-1227.

Kang, M. H. and Reynolds, C. P. 2009. Bcl-2 inhibitors: targeting mitochondrial apoptotic pathways in cancer therapy. Clin Cancer Res 15, 1126-1132.

Kimura, J. and Maki, N. 2002. New loliolide derivatives from the brown alga Undaria pinnatifida. J Nat Prod 65, 57-58.

Kirkin, V., Joos, S. and Zornig, M. 2004. The role of Bcl-2 family members in tumorigenesis. Biochim Biophys Acta 1644, 229-249.

Kroemer, G., Galluzzi, L. and Brenner, C. 2007. Mitochondrial membrane permeabilization in cell death. Physiol Rev 87, 99-163.

Liu, Y., Ding, Y., Huang, J., Wang, S., Ni, W., Guan, J., Li, Q., Zhang, Y., Ding, Y., Chen, B. and Chen, L. 2014. MiR-141 suppresses the migration and invasion of HCC cells by targeting Tiam1. PLoS One 9, e88393.

Lopes, G., Sousa, C., Silva, L. R., Pinto, E., Andrade, P. B., Bernardo, J., Mouga, T. and Valentao, P. 2012. Can phlorotannins purified extracts constitute a novel pharmacological alternative for microbial infections with associated inflammatory conditions? PLoS One 7, e31145.

Malumbres, M. 2012. Cell cycle-based therapies move forward. Cancer Cell 22, 419-420.

Peterson, Q. P., Hsu, D. C., Goode, D. R., Novotny, C. J., Totten, R. K. and Hergenrother, P. J. 2009. Procaspase-3 activation as an anti-cancer strategy: structure-activity relationship of procaspase-activating compound 1 (PAC-1) and its cellular co-localization with caspase-3. J Med Chem 52, 5721-5731.

Putt, K. S., Chen, G. W., Pearson, J. M., Sandhorst, J. S., Hoagland, M. S., Kwon, J. T., Hwang, S. K., Jin, H., Churchwell, M. I., Cho, M. H., Doerge, D. R., Helferich, W. G. and Hergenrother, P. J. 2006. Small-molecule activation of procaspase-3 to caspase-3 as a personalized anticancer strategy. Nat Chem Biol 2, 543-550.

Repeta, D. J. 1989. Carotenoid Diagenesis in Recent Marine-Sediments .2. Degradation of Fucoxanthin to Loliolide. Geochimica Et Cosmochimica Acta 53, 699-707.

Roos, W. P. and Kaina, B. 2006. DNA damage-induced cell death by apoptosis. Trends Mol Med 12, 440-450.

Soldani, C., Lazze, M. C., Bottone, M. G., Tognon, G., Biggiogera, M., Pellicciari, C. E. and Scovassi, A. I. 2001. Poly(ADP-ribose) polymerase cleavage during apoptosis: when and where? Exp Cell Res 269, 193-201.

Song, F., Fan, X., Xu, X., Zhao, J., Yang, Y. and Shi, J. 2004. Cadinane sesquiterpenes from the brown alga Dictyopteris divaricata. J Nat Prod 67, 1644-1649.

Spavieri, J., Allmendinger, A., Kaiser, M., Casey, R., Hingley-Wilson, S., Lalvani, A., Guiry, M. D., Blunden, G. and Tasdemir, D. 2010. Antimycobacterial, antiprotozoal and cytotoxic potential of twenty-one brown algae (Phaeophyceae) from British and Irish waters. Phytother Res 24, 1724-1729.

Taylor, H. F. and Burden, R. S. 1970. Identification of Plant Growth Inhibitors Produced by Photolysis of Violaxanthin. Phytochemistry 9, 2217-&.

Taylor, W. R. and Stark, G. R. 2001. Regulation of the G2/M transition by p53. Oncogene 20, 1803-1815.

Tibaduiza, E. C., Fleet, J. C., Russell, R. M. and Krinsky, N. I. 2002. Excentric cleavage products of beta-carotene inhibit estrogen receptor positive and negative breast tumor cell growth in vitro and inhibit activator protein-1-mediated transcriptional activation. J Nutr 132, 1368-1375.

Vizetto-Duarte, C., Pereira, H., Bruno de Sousa, C., Pilar Rauter, A., Albericio, F., Custodio, L., Barreira, L. and Varela, J. 2015. Fatty acid profile of different species of algae of the Cystoseira genus: a nutraceutical perspective. Nat Prod Res 29, 1264-1270.

Zubia, M., Fabre, M. S., Kerjean, V., Le Lann, K., Stiger-Pouvreau, V., Fauchon, M. and Deslandes, E. 2009. Antioxidant and antitumoural activities of some Phaeophyta from Brittany coasts. Food Chem 116, 693-701.

CHAPTER 6

OVERALL DISCUSSION

6.1 Overall discussion

In this thesis, we aimed to improve the knowledge of the biochemical composition of *Cystoseira* macroalgae in order to ascertain their suitability as a food, as part of a food product and/or as source of novel drug leads for the pharmaceutical industry.

The proximate biochemical composition and minerals profile of five macroalgal species belonging to the *Cystoseira* genus, namely *C. humilis*, *C. tamariscifolia*, *C. nodicaulis*, *C. compressa* and *C. baccata* were determined for the first time (Chapter 2). The nutritional value of the majority of marine macroalgae is primarily related to their ash content and with the carbohydrates levels, since they are rich in minerals and soluble and insoluble dietary fiber (Lahaye 1991, McDermid and Stuercke 2003, Ortiz et al. 2006). The biochemical composition of several brown macroalgae has been previously described by various authors and is summarized in Table 6.1. In accordance with these studies, the species investigated in this thesis have indeed presented high ash and carbohydrates contents. The protein and lipid levels were also in agreement with those reported in previous studies on brown algae (Holdt and Kraan 2011).

As shown in Table 6.1, there can be considerable variability in the proximate composition among algae of the same genus and even between individuals of the same species (e.g. *Dictyota ciliolata*) when studies carried out on samples from different geographical locations and different seasons are compared. The samples studied in Chapter 2 and 3 were collected in the middle/lower intertidal zones on the Algarve (Albufeira and Odeceixe, Portugal) coast in May 2010 during the low tide. The nutritional composition of marine algae can be influenced by nutrient concentration in seawater, water temperature and depth, amongst several other environmental factors such as the amount of photosynthetically active radiation. Therefore, harvest locations and dates are also important information that should be recorded by researchers. In addition, biochemical composition may also change according to the physiological state of the specimen as discussed in Chapter 2.

Macroalgae are particularly rich in minerals due to the fact that they absorb the elements from the surrounding seawater. Potassium, calcium and iron, for instance, tend to accumulate at much higher levels in macroalgae than in terrestrial plants (Hwang et al. 2013).

Table 6.1 – Proximate composition of a variety of brown algae (% DW).

Species	Moisture	Ash	Protein	Carbohydrates	Lipids
Colpomenia sinuosa	11.5	28.1	9.2	32.1	1.5
Dictyota ciliolata	–	33-47.2	4.1-10.7	15.2-20.3	7.1-7.8
Dictyota dichotoma	–	27.02	17.73	–	2.94
Feldmannia indica	–	45.1	7.4	17.7	3.6
Fucus spiralis	87.7	22.31	9.71	17.59	5.23
Hydroclathrus clathratus	–	49.4	4.2	18.3	2.9
Padina boryana	–	33.5-36.5	6.4-10.6	18.4-19.3	4.4-5.2
Padina gymnospora	–	36.6	9.86	1.86	–
Padina pavonica	–	33.08	11.83	–	1.79
Rosenvingea nhatrangensis	–	45.2-56.6	3.4-6.6	8.4-12.6	2.6-3.1
Sacchoriza polyschides	10.88	28.2	14.4	45.6	1.1
Sargassum decurrens	–	30.4	7.1	22.2	3.3
Sargassum filifolium	–	28.2	10.2	21.4	4.0
Sargassum filipendula	–	44.29	8.72	3.73	–
Sargassum ilicifolium	10.4	29.9	8.9	32.9	2.0
Sargassum muticum	9.64	22.9	16.9	49.3	1.45
Sargassum vulgare	14.66	14.20	15.76	67.8	0.45
Turbinaria conoides	–	34.4	5.9	19.7	2.3
Undaria pinnatifida	–	28.3	16.8	52.1	2.7
Cystoseira humilis	57.06	20.35	10.34	64.09	5.22
Cystoseira compressa	63.05	7.30	10.16	73.09	9.45
Cystoseira tamariscifolia	48.99	23.85	12.52	54.06	9.57
Cystoseira nodicaulis	58.95	13.45	9.20	73.04	4.31
Cystoseira baccata	51.43	19.10	12.46	42.48	10.92

Sources: (Robledo and Pelegrin 1997, Marinho-Soriano et al. 2006, Renaud and Luong-Van 2006, Rohani-Ghadikolaei et al. 2012, Tabarsa et al. 2012, Taboada et al. 2013, Paiva et al. 2014, Rodrigues et al. 2015, Vizetto-Duarte et al. 2015). –, information not available.

In Table 6.2, the mineral composition of a variety of brown algae, including the *Cystoseira* species evaluated in this thesis, is shown. It can be observed that K is often highly accumulated in brown macroalgae. In nutrition, particular importance is given to the Na/K ratio. Even under a Na-restricted diet, Na deficiency does not occur in humans (Strazzullo and Leclercq 2014). In contrast, excessive Na consumption has been related to increased risk of hypertension, cardiovascular diseases (CVD) and kidney problems among others (Galletti et al. 2014). Interestingly, adequate intakes of K have been

reported to protect against CVD (Geleijnse et al. 2007). Therefore, it is thought that the intake of food or food-products with favorable Na/K ratios, such as those of the *Cystoseira* macroalgae studied, could be included in healthy diets.

Table 6.2 – Mineral composition of a variety of brown algae.

Species	Na	K	Ca	Mg	Fe	Mn	Zn
	g kg^{-1}				mg kg^{-1}		
Alaria marginata	–	–	–	–	60	3.79	23.6
Dictyota acutiloba	–	72.6	10.3	13.6	438	0.012	16
Dictyota sandvicensis	–	55.7	18.1	9.1	608	0.021	13
Eisenia bicyclis	–	–	–	–	80	13.8	14
Fucus vesiculosus	–	–	–	–	520	5.77-27.9	14-24
Laminaria japonica	–	–	–	–	80	6.79	13
Laminaria saccharina	–	–	–	–	40	3.04	8.5
Laminaria spp.	9-60	13-106	5-30	5-20	40-800	–	–
Sargassum hemiphyllum	9.5	44.7	22.4	9.89	20.8	0.002	1.6
Sargassum echinocarpum	–	95	13.1	11.6	92	0.006	7
Undaria pinnatifida	16-70	55-63	11-30	10-30	80-400	–	–
Cystoseira humilis	7.56	29.78	23.70	12.90	171.88	152.18	41.16
Cystoseira compressa	8.94	60.02	13.08	18.53	149.74	14.27	9.43
Cystoseira tamariscifolia	16.36	16.87	25.82	6.56	508.06	398.46	105.99
Cystoseira nodicaulis	11.61	15.98	26.50	5.69	413.14	193.54	113.82
Cystoseira baccata	10.81	22.10	9.43	6.78	109.59	108.35	106.73

Sources: Mabeau and Florence 1993, McDermid and Stuercke 2003, Vizetto-Duarte et al. 2016.
–, information not available.

Caution must be applied, however, when considering the use of macroalgae as nutritional sources of minerals, due to the possible accumulation of toxic metals. Although toxicological research has showed that most metals in algal biomass are generally below the legislation limits allowed in several countries (Indergaard and Minsaas 1991), metal accumulation depends on several factors. Concentration of a given element in water can be a major factor, but others may be as important, such as interactions between elements, salinity, pH and also individual growth, modulating mineral concentration (Zbikowski et al. 2006). In addition, one has to take into account that the association of minerals with polysaccharides might limit the absorption of

minerals as well, modulating its bioavailability (Mabeau and Fleurence 1993). Having this in mind, *Cystoseira* species could be used, with prudence, in the formulation of mineral-rich formulations.

In Chapter 3, the FA profiles of *C. humilis*, *C. tamariscifolia*, *C. nodicaulis*, *C. compressa*, *C. baccata* and *C. barbata* were determined. Macroalgae have in general low lipid contents, containing 1–5% on a DW basis. However, Phaeophyceae have higher percentages than most green and red algae, and the *Cystoseira* species reported here also presented higher lipid levels (4.31–10.92%). Moreover, the nutritionally important C18 and C20 PUFA, including n-3 PUFA, are commonly present in brown algae in significant high amounts, ranging from 24 to 58% (Kumari et al. 2013), which is also a typical character of the class Phaeophyceae that differentiates them from the members of the Chlorophyta and Rhodophyta members (Pereira et al. 2012).

In this research, palmitic acid was the main FA detected for all species, which is in accordance with other Phaeophyceae (Pereira et al. 2012). As its name indicates, palmitic acid is a major component of palm oil, a widely used vegetable oil, found in food products such as margarine, cereals, sweets and baked goods. It is known that a high intake of palmitic acid may exert a negative effect on human health by raising the cholesterol blood levels (Clandinin et al. 2000) and by promoting the development of CVD (Mancini et al. 2015). However, it has been described that this effect can be counterbalanced by high levels of linoleic acid (Clandinin et al. 2000, French et al. 2002), which is present in relatively high amounts in the *Cystoseira* species included in this work. In fact, it was previously reported that oral administration of extracts from brown algae such as *Iyengaria stellata*, *Colpomenia sinuosa*, *Spatoglossum asperum* (Ara et al. 2002) and *Ecklonia stolonifera* (Yoon et al. 2008) had an anti-hyperlipidemic effect in terms of decreasing total cholesterol, low-density lipoprotein (LDL) cholesterol and triacylglycerols. Hyperlipidemia is considered to be a major risk factor for CVD, including atherosclerosis, myocardial infarction, heart attacks and cerebrovascular diseases. In addition, the *Cystoseira* algae studied here showed a lipid profile enriched in total MUFA and PUFA. Diets rich in MUFA and PUFA have been shown to decrease total cholesterol and LDL cholesterol (Hargrove et al. 2001). By potentially reducing hyperlipidemia, brown algae show once again their potential towards nutraceutical applications.

PUFA are known for having positive effects on human health and are vital elements in human nutrition. In fact, the mean ratio of PUFA/SFA recommended by the

British Department of Health is 0.45 or higher (HMSO 1994). Although many of the species analyzed in this work had high amounts of SFA, some species exhibited higher concentrations of PUFA, and PUFA/SFA ratios higher than 1 (*C. tamariscifolia*, *C. nodicaulis* and *C. compressa*). However, not all PUFA have beneficial effects on human health as excess n-6 PUFA are generally considered as pro-inflammatory or as promoters of other cell harmful effects. On the other hand, n-3 PUFA derivatives are thought to be less inflammatory or even anti-inflammatory (Calder 2006). Since the biosynthetic pathways of both n-3 and n-6 PUFA compete for the same enzymes, the health promoting effects are dependent on the n-6/n-3 ratio of PUFA obtained through diet. The World Health Organization (WHO) recommends a ∑n-6/∑n-3 ratio lower than 10 (Pereira et al. 2012). In this study, all *Cystoseira* algae except for one (*C. barbata*, 12.15) can be considered as a good source of dietary PUFA, since they showed ratios ranging between 0.29 and 6.73 (Vizetto-Duarte et al. 2015).

The type of FA consumed contributes to many processes in health and disease. Consumption of fatty acids can have a direct effect on stimulating or precluding atherosclerosis and coronary thrombosis due to their effect on blood cholesterol and LDL cholesterol concentrations as discussed above. Therefore, Ulbricht and Southgate (1991) proposed two indices that might better characterize the atherogenic and thrombogenic potential of the diet: the index of atherogenicity (IA) and index of thrombogenicity (IT). The IA indicates the relationship between the sum of the main SFA considered pro-atherogenic (favoring the adhesion of lipids to cells of the immune and circulatory systems), and of the main classes of unsaturated FA considered anti-atherogenic, inhibiting the plaque aggregation and diminishing the levels of esterified fatty acids, cholesterol and phospholipids (Ulbricht and Southgate 1991). The IT is an estimate of the effect of a given FA profile on the promotion of clot formation in blood vessels. This is defined as the relationship between the pro-thrombogenic (saturated C14:0, C16:0 and C18:0) and the anti-thrombogenic MUFA, n-6 and n-3 PUFA (Ulbricht and Southgate 1991). Stearic, myristic and palmitic FA (C12:0, C14:0 and C16:0, respectively) are known to be among the most atherogenic FA, whereas the longer chain stearic acid (C18:0) is believed to be thrombogenic, but neutral with respect to atherogenicity (Attia et al. 2015). In Chapter 3, it was also calculated the unsaturation index (UI), which accounts for the number of unsaturation of each FA (Kumari et al. 2013). As lipid unsaturation provides higher flexibility and fluidity to the bilayer of biological membranes, the high UI determined for *Cystoseira* algae suggests

that these macroalgae may also be beneficial for preventing a diverse array of diseases, such as type-2 diabetes (Weijers 2012) or Alzheimer's (Hosono et al. 2015), since membrane fluidity is known to affect the function of biomolecules (e.g. proteins) integrated or bound to the lipid bilayer.

The evaluation of the PUFA/SFA and ∑n-6/∑n-3 ratios as well as of the unsaturation, atherogenic and thrombogenic indices showed that *Cystoseira* species have beneficial nutritional value. Nonetheless, the profiles of *C. compressa*, *C. tamariscifolia* and *C. nodicaulis* were considered as the "healthiest" according to these parameters.

In summary, *Cystoseira* macroalgae could be regarded as highly nutritive food and/or incorporated in food supplements due to their high amounts of carbohydrates and minerals, especially of K, Ca and Fe, and a favorable Na/K ratio. Moreover, this study demonstrates that *Cystoseira* species can have beneficial effects if be incorporated in the formulation of low fat food and PUFA-rich nutraceuticals. The results described in this work, together with recent studies on the edibility of *Cystoseira* (Andrade et al. 2013), demonstrate that these algae may be alternative sources of valuable non-animal food and food products.

Another aspect explored in this thesis was the evaluation of antioxidant and anti-proliferative properties of extracts from different *Cystoseira* species, namely *C. humilis*, *C. tamariscifolia* and *C. usneoides* (Chapter 4). Aiming the evaluation of antioxidant activity, the total phenolic contents and the radical scavenging activity against DPPH and ABTS radicals were determined. DPPH and ABTS radical scavenging activity assays were selected as they are simple, rapid, sensitive and reproducible procedures (Lu et al. 2010, Zampini et al. 2010, Li et al. 2012). The tested algal extracts showed antioxidant activity in both bioassays. The hexane and diethyl ether extracts of *C. tamariscifolia* and the diethyl ether extract of *C. usneoides* had the highest antioxidant potential (Chapter 4). Brown algae, such as *Cystoseira* and *Fucus*, are known to contain molecules with antioxidant properties (Mhadhebi et al. 2011, Heffernan et al. 2015). In addition, Kosanić et al. (2015) analyzed acetone extracts of three *Cystoseira* species (*C. amentacea*, *C. barbata* and *C. compressa*) and found relatively strong scavenging activity in the acetone extract of *C. amentacea*. In another study, the lipid extracts of eight marine algae belonging to the genus *Cystoseira* (*C. amentacea* var. *amentacea, C. jabukae, C. crinita, C. amentacea* var. *stricta, C. elegans, C. algeriensis, C. elegans,* and *C. barbata*) were evaluated for their antioxidant activity in a micellar model system

by Ruberto et al. (2001). These authors found that all lipid extracts showed antioxidant activity and among them the *C. amentacea* var. *stricta* extract was the most active. The authors ascribed this activity to the presence in the extracts of tetraprenyltoluquinols namely cystoketal, strictaketal, amentaepoxide and amentol. In Chapter 4, demethoxy cystoketal was isolated from *C. tamariscifolia* and tested for antioxidant properties against DPPH and ABTS but the compound did not present significant antioxidant activity. Ruberto et al. (2001) reported that cystoketal seemed to be a compound responsible for the antioxidant activity. Although the two compounds are structurally related, it is known that molecules with slight differences in structure may have different physiological activities, including pharmacological effects, toxicology and metabolism (Chhabra et al. 2013). Moreover, Ruberto and collaborators (2001) used a micellar model that can afford different results than those determined by the DPPH or ABTS assays (Chandrasekara et al. 2016). In addition, the authors evaluated the antioxidant activity of the extract, but did not evaluate the antioxidant activity of the isolated compound. Therefore, other studies could be done to ascertain if cystoketal contributed for the observed antioxidant activity.

In our research work, the antioxidant activity was shown to correlate with the total phenolic content in the algal extracts. Phenols are important antioxidants because of their ability to scavenge free radicals such as singlet oxygen, superoxide and hydroxyl radicals. In accordance with our results, several studies have found a high correlation between antioxidant activities of algae and phenolic contents (Balboa et al. 2013, Farasat et al. 2014). On the other hand, studies such as that of Heo et al. (2005) reported that the antioxidant activity did not correlate with the total phenolic content in the algal extract. Extraction from algal biomass is usually not selective and the resulting extracts are complex mixtures of compounds. Different solvents, according to their polarity, may extract various compounds including pigments (carotenoids, chlorophylls *a* and *b*), alkaloids, phenolic compounds and essential oils. As many of these compounds have antioxidant activity, it is possible that synergistic effects may be at play (Balboa et al 2013).

In the past years, the importance of antioxidants in the protection of organisms against oxidative stress, and of food and food products against oxidation, has become evident in several areas including physiology, pharmacology, nutrition and food processing (Magalhaes et al. 2009). Compounds such as BHA, BHT, TBHQ and PG chemically synthesized are often used in food and pharmaceutical formulations as

antioxidants. However, the use of BHA and BHT has been restricted due to concerns regarding their toxic and carcinogenic effects (Lanigan and Yamarik 2002). Hence, there is a growing interest in new, safe and cheaper antioxidants for food applications, mostly of natural sources due to an increasing trend in consumer preferences towards natural antioxidants (Sanches-Silva et al. 2014). The high RSA obtained for *Cystoseira* extracts suggests that these macroalgae are potential sources of novel antioxidants.

In Chapter 4, *C. tamariscifolia* hexane extract (CTH) had the higher anti-proliferative activity in HepG2 cells among the other extracts, as demonstrated by its lower IC_{50}. Moreover, CTH was selective towards tumor cells when compared to non-tumoral ones.

Lower cell proliferation values in the MTT assay can be explained by either decreased cell proliferation and/or occurrence of cell death. Thus, proliferation of HepG2 cells treated with CTH was analyzed through the BrdU incorporation assay, whereas analysis of cell morphology and phosphatidylserine (PS) externalization were carried out to determine whether cell death occurred via apoptosis. Indeed, CTH inhibited proliferation and induced apoptosis of HepG2 cells (Chapter 5). Both anti-proliferative processes are thought to be one way to prevent carcinogenesis, as tumoral cells are considered to have limitless replicative potential and the ability to evade apoptosis (Hanahan and Weinberg 2011).

Furthermore, the application of *C. tamariscifolia* hexane extract was tested on 3D MCTS, because compounds with anti-tumoral potential must penetrate into tumor cell masses to reach cancer cells at adequate concentrations. Spheroid cultures are known to mimic more closely the properties of tumor tissue than monolayer cultures with regard to growth kinetics and metabolic rates (Herrmann et al. 2008). Tumor cell lines are in general more resistant to antineoplastic agents when the cells are grown as 3D MCTS rather than as monolayer cultures (Ivascu and Kubbies 2006). In addition, the susceptibility of multicellular spheroids to drugs has been reported to resemble the *in vivo* sensitivity (Hirschhaeuser et al. 2010). In our experiments, the tumoral spheroid completely lost its integrity after a 24h treatment, confirming the potential of CTH as an anti-tumoral agent in a 3D model.

Demethoxy cystoketal (**1**, trivial name) was isolated by bio-guided fractionation from CTH. Compound **1** was structurally elucidated by NMR and identified as an anti-proliferative molecule. Its structure was elucidated using ^{1}H and ^{13}C NMR data analysis and comparison with data previously described in the literature. All spectral data

showed to be identical to those reported by Valls et al. (1996). Additionally, the molecular mass of compound **1** was confirmed as $C_{27}H_{36}O_4$ by LRESIMS data analysis. Therefore, and as this compound corresponds to a known natural product isolated from other *Cystoseira* species (*C. amentacea*), chromane **1** was unequivocally identified as demethoxy cystoketal chromane, although NOESY was not recorded due to the low compound amount.

Terpenoids are particularly abundant in brown algae and are one of the most representative groups of secondary metabolites of these organisms. Many of these compounds possess various interesting biological properties that include cytotoxic, antioxidant, antifungal and antibacterial activities (Bennamara et al. 1999, Ayyad et al. 2003, Fisch et al. 2003).

In the 1980's and 1990's, Amico and collaborators performed a thorough screening of the chemistry of *Cystoseira* species (Amico 1995) and have reported the presence of cystoketal, a tetraprenylhydroquinol derivative, in *C. balearica* (Amico et al. 1984). In 1996, Valls et al. described two new cystoketal derivatives, demethoxy cystoketal chromane and cystoketal quinine, in the brown alga *C. amentacea* var. *stricta* collected off the French Riviera coast. However, in this thesis, we reported for the first time this compound in *C. tamariscifolia* and its anti-proliferative bioactivity.

In Chapter 5, we isolated isololiolide (trivial name) from *C. tamariscifolia* for the first time. The isolated compound displayed a $[M + Na]^+$ *quasi*-molecular ion peak at *m/z* 219.0993, indicating the molecular formula $C_{11}H_{16}O_3$, with four unsaturations. Again, using ^1H and ^{13}C NMR data analysis and comparison with data previously described in the literature (Kimura and Maki 2002), isololiolide was identified. Isololiolide is a loliolide derivative and both of them are carotenoid catabolites. Loliolide was first isolated from the plant *Fumaria officinalis* (Manske 1938) and its chemical synthesis has already been previously achieved (Rouessac et al. 1983). Recently loliolide and isololiolide have been found in other terrestrial plants such as *Vernonia cinerea* (Youn et al. 2014) and *Vitex leptobotrys* (Pan et al. 2014). These epimers were also identified in other brown algae such as *Undaria pinnatifida* (Kimura and Maki 2002), *Sargassum crassifolium* (Kuniyoshi 1985), *Dictyota dichotoma* (Ali et al. 2003), *Sargassum thunbergii* (Park et al. 2004), *Cladostephus spongiosus f. verticillatus* (El Hattab et al. 2008) and *Homoeostrichus formosana* (Fang et al. 2015). In fact, high concentrations of loliolide were found in the brown macroalgae *Taonia atomaria* and *Cutleria multifida* (Percot et al. 2009).

The origin of loliolide and isololiolide has been discussed by several authors. They suggested that loliolide is a degradation (Ghosal et al. 1976) or a photo-oxidation product of carotenoids such as fucoxanthin and zeaxanthin (Isoe 1972, Repeta 1989). Isololiolide, on the other hand, is thought to originate only from zeaxanthin (Fig. 6.1).

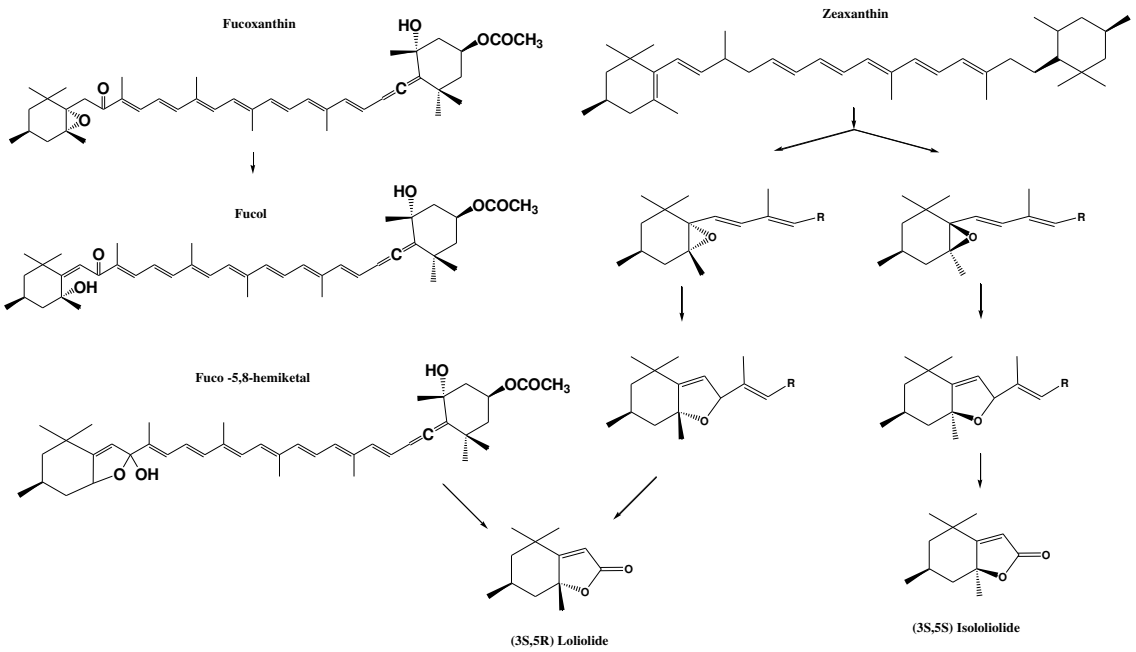

Fig.6.1 – Formation of loliolide and isololiolide from fucoxanthin and zeaxanthin.

From a biological point of view, loliolide is a phytotoxic compound and has various effects, such as inhibition of seed germination (Hiraga et al. 1997) and also of cyanobacterial growth (Xian et al. 2006). It was considered by the latter authors as an allelochemical, a compound produced by an organism that influences germination, growth, survival and/or reproduction of other organisms and that can be an important defense against herbivory. Loliolide has also demonstrated ant-repellent properties (Okunade and Wiemer 1985) and immunosuppressive activity (Duan et al. 2002).

In addition, metabolites of carotenoids such as fucoxanthinol had previously shown modulatory actions on viability, cell-cycle arrest and apoptosis. Interestingly, fucoxanthinol effects were even more pronounced than those of fucoxanthin (Martin 2015, Rwigemera et al. 2015).

In our study, isololiolide had anti-proliferative activity and was highly selective towards HepG2 cells, especially when comparing with non-tumoral human fibroblasts.

Furthermore, isololiolide disrupted the normal HepG2 cell cycle, inducing G2/M cell cycle arrest along with a decrease in the percentage of cells in S phase (Chapter 5).

Cells with an intact DNA damage response frequently arrest or die in response to DNA damage, thus reducing the likelihood of progression to malignancy. A transient or prolonged delay in cell cycle progression in either G1 or G2 phases allows the intervention of DNA repair mechanisms to achieve error-free DNA replication and correct chromosome segregation. This is essential for the maintenance of genomic integrity before entry into the S phase and mitosis (Sclafani and Holzen 2007).

Isololiolide significantly induced apoptosis in hepatocarcinoma HepG2 cells as demonstrated in Chapter 5. In hepatocytes, as well as in many other cell types, apoptosis mainly occurs through two major pathways: the extrinsic death receptor pathway and/or the intrinsic mitochondrial pathway (Guicciardi et al. 2013). In the death receptor pathway, and following the interaction with its cognate ligand, the receptors located at the cellular membrane recruit adaptor proteins such as initiator caspase-8, triggering the activation of caspases to orchestrate apoptosis. In the mitochondrial pathway, stimuli target mitochondria either directly or through the mediation of pro- or anti-apoptotic members of the Bcl-2 family. One of the most widely studied negative regulators of apoptosis is Bcl-2, thought to prevent the release of cytochrome *c* from the mitochondria. Indeed, it has been demonstrated that overexpression of Bcl-2 prevents the efflux of cytochrome *c* from mitochondria whilst protecting cells from apoptosis (Gogvadze et al. 2006). Isololiolide decreased anti-apoptotic Bcl-2 protein expression, suggesting that apoptotic stimulus through the intrinsic pathway may be occurring (Fig. 6.2).

Proaspase-3 (or caspase-3 zymogen) exists within the cytosol as an inactive dimer (Boatright and Salvesen 2003). Cleavage of procaspase-3 within their respective linker segments is required for caspase-3 activation. A decrease in procaspase-3 levels is due to its proteolysis, leading to caspase-3 activation. Our results showed that incubation of HepG2 cells with isololiolide resulted in a 2-fold decrease of procaspase-3 levels, strongly suggesting that procaspase-3 was processed to caspase-3 (Chapter 5). Active caspase-3 cleaves several substrates including PARP, and activates death effector molecules triggering the structural changes characteristic of apoptotic cells.

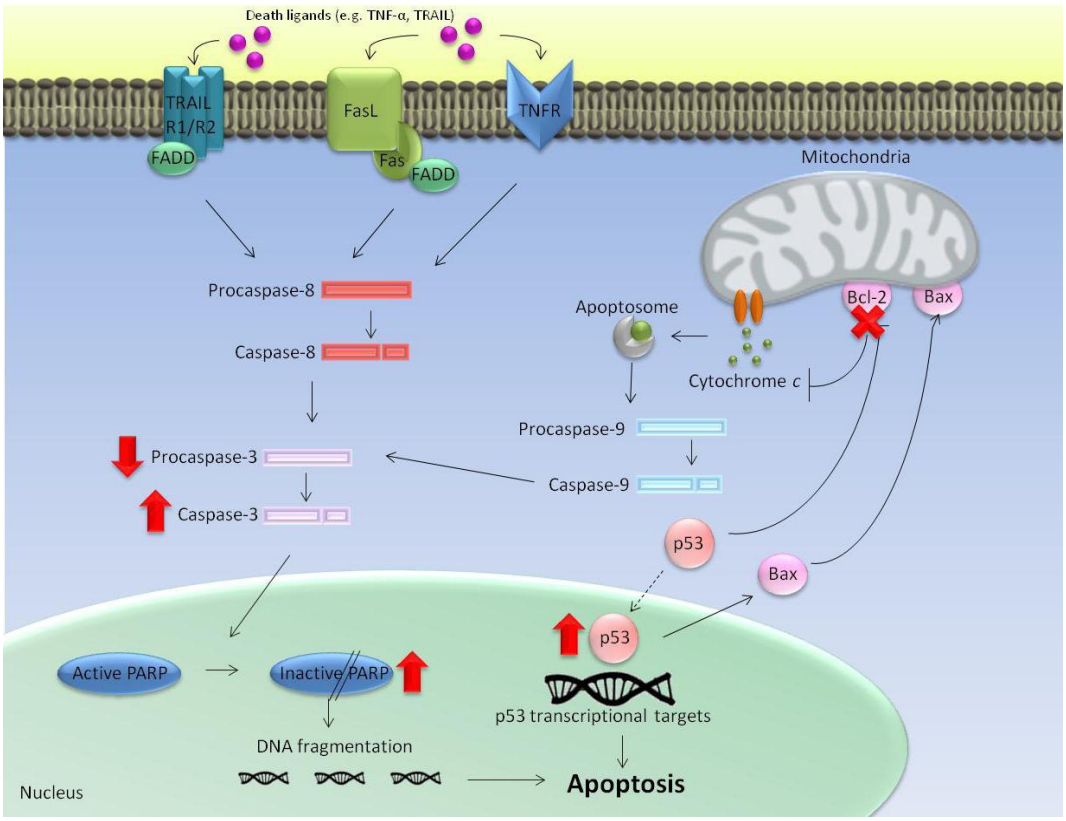

Fig. 6.2 – Suggestion of a model of how isololiolide affected the apoptotic pathways in HepG2 cells in the research performed in this thesis (red arrows and cross). Isololiolide treatment on HepG2 cells resulted in increased p53 expression, decreased procaspase-3 (strongly suggesting its activation to caspase-3), increased PARP cleavage and Bcl-2 downregulation.

In addition, exposure to isololiolide induced proteolysis of PARP in HepG2 cells. PARP is a substrate of caspase-3 and its cleavage has been considered to be indicative of functional caspase activation (Bressenot et al. 2009). PARP plays an active role in key biological processes, such as transcription and cell cycle regulation, response to DNA damage, apoptosis and maintenance of genome integrity. Targeting DNA repair with PARP inhibitors has shown a broad range of anti-tumor activities in patients with advanced malignancies (Nguyen et al. 2011).

Given the ability of p53 to induce both cell cycle arrest and apoptosis, we assessed its expression by western blot analysis after 24, 48 and 72h incubation periods with isololiolide. Interestingly, p53 protein expression increased after 24h of isololiolide treatment, a level that was maintained even after 72h. By arresting the cell cycle and allowing time for the repair of potentially lethal double-strand breaks, p53 maintains chromosomal integrity and improves the survival of damaged cells. In addition to enforcing cell cycle checkpoints, p53 also regulates a group of genes involved in DNA

recombination and repair (Gatz and Wiesmuller 2006). Indeed, p53 integrates multiple stress signals into a series of diverse anti-proliferative responses. Besides its function in cell cycle arrest, one of the most important p53 functions is its ability to activate apoptosis (Amaral et al. 2010). Given the profound proliferative advantage produced by loss of p53 function, it is not surprising that *p53* is the most commonly inactivated tumor suppressor gene in human cancer (Rivlin et al. 2011).

Overall, isololiolide treatment on HepG2 cells resulted in increased p53 expression, decreased procaspase-3 (strongly suggesting its activation to caspase-3) and increased PARP cleavage. These changes were further reinforced by the observed down-regulation of the anti-apoptotic/pro-survival Bcl-2 protein.

Based on this study, a timeline for the molecular events involved in isololiolide-induced apoptosis was constructed (Fig. 6.3).

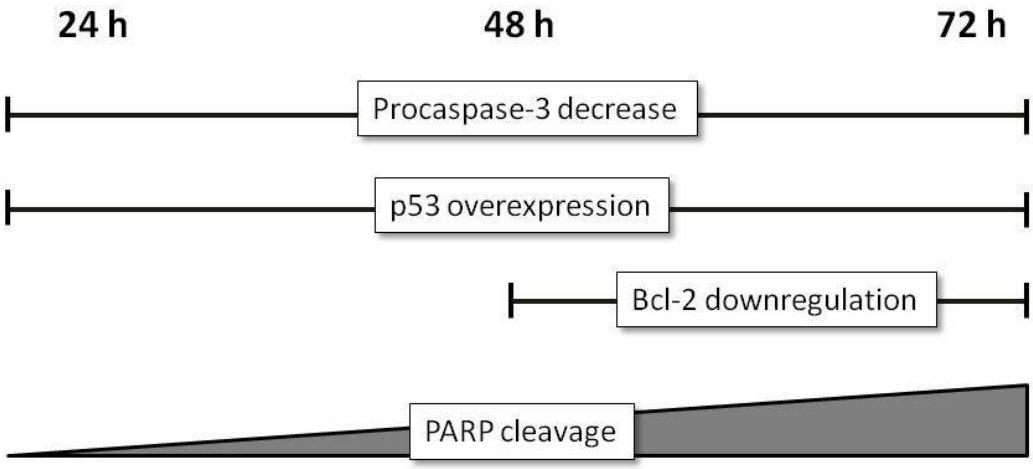

Fig. 6.3 – Temporal sequence of events during isololiolide-induced apoptosis in HepG2 cells. The sequence of events is shown linearly for simplicity.

Overexpression of p53, PARP cleavage and procaspase-3 decrease can be observed as early as 24h. Corresponding decrease in Bcl-2 protein expression is not observed until 48h. The delay between the Bcl-2 and the former protein expressions suggests that a separate regulatory mechanism might exist to control the timing of Bcl-2 downregulation. This may coincide with a cellular commitment to apoptosis that requires an integration of other signaling pathways. Moreover, increment of PARP cleavage (or inactivation) was expressed in a time-dependent fashion. These results suggest that there is a sequential activation of different apoptotic proteins at different times.

Taking all these results together, we can indeed conclude that *Cystoseira tamariscifolia* can be an important source of bioactive compounds, which could be used as pharmaceutical agents in the near future. However, the structure of natural bioactive compounds is often complex which hinders their identification, isolation and synthesis as well as a better understanding of the molecular mechanisms involved in their therapeutical potential. Moreover, insufficient yields can also become a limitation. Nonetheless, the wide spectrum of bioactivities found in marine algae underlines the important potential application of algal compounds in the pharmaceutical industry, which can complement and inspire the synthesis of novel drugs.

6.2 References

Ali, M. S., Pervez, M. K., Saleem, M. and Ahmed, F. 2003. Dichotenone-a and -b: two new enones from the marine brown alga *Dictyota dichotoma* (Hudson) Lamour. Nat Prod Res 17, 301-306.

Amaral, J. D., Xavier, J. M., Steer, C. J. and Rodrigues, C. M. 2010. The role of p53 in apoptosis. Discov Med 9, 145-152.

Amico, V. 1995. Marine brown algae of family Cystoseiraceae: Chemistry and chemotaxonomy. Phytochemistry 39, 1257-1279.

Amico, V., Cunsolo, F., Oriente, G., Piatelli, M. and Ruberto, G. 1984. Cystoketal, a new metabolite from the brown alga *Cystoseira balearica*. J Nat Prod 47, 947-952.

Andrade, P. B., Barbosa, M., Matos, R. P., Lopes, G., Vinholes, J., Mouga, T. and Valentao, P. 2013. Valuable compounds in macroalgae extracts. Food Chem 138, 1819-1828.

Ara, J., Sultana, V., Qasim, R. and Ahmad, V. U. 2002. Hypolipidaemic activity of seaweed from Karachi coast. Phytother Res 16, 479-483.

Attia, Y. A., Al-Harthi, M. A., Korish, M. A. and Shiboob, M. M. 2015. Fatty acid and cholesterol profiles and hypocholesterolemic, atherogenic, and thrombogenic indices of table eggs in the retail market. Lipids Health Dis 14, 136.

Ayyad, S. E., Abdel-Halim, O. B., Shier, W. T. and Hoye, T. R. 2003. Cytotoxic hydroazulene diterpenes from the brown alga *Cystoseira myrica*. Z Naturforsch C 58, 33-38.

Balboa, E. M., Conde, E., Moure, A., Falque, E. and Dominguez, H. 2013. *In vitro* antioxidant properties of crude extracts and compounds from brown algae. Food Chem 138, 1764-1785.

Bennamara, A., Abourriche, A., Berrada, M., Charrouf, M., Chaib, N., Boudouma, M. and Garneau, F. X. 1999. Methoxybifurcarenone: an antifungal and antibacterial meroditerpenoid from the brown alga *Cystoseira tamariscifolia*. Phytochemistry 52, 37-40.

Boatright, K. M. and Salvesen, G. S. 2003. Mechanisms of caspase activation. Curr Opin Cell Biol 15, 725-731.

Bressenot, A., Marchal, S., Bezdetnaya, L., Garrier, J., Guillemin, F. and Plenat, F. 2009. Assessment of apoptosis by immunohistochemistry to active caspase-3, active caspase-7, or cleaved PARP in monolayer cells and spheroid and subcutaneous xenografts of human carcinoma. J Histochem Cytochem 57, 289-300.

Calder, P. C. 2006. Polyunsaturated fatty acids and inflammation. Prostaglandins Leukot Essent Fatty Acids 75, 197-202.

Chandrasekara, A., Rasek, O.A., John, Chandrasekara, J.A. and Shahidi F. 2016. Solvent and extraction conditions control the assayable phenolic content and antioxidant activities of seeds of black beans, canola and millet. J Am Oil Chem Soc 93, 275.

Chhabra, N., Aseri, M. L. and Padmanabhan, D. 2013. A review of drug isomerism and its significance. Int J Appl Basic Med Res 3, 16-18.

Clandinin, M. T., Cook, S. L., Konard, S. D. and French, M. A. 2000. The effect of palmitic acid on lipoprotein cholesterol levels. Int J Food Sci Nutr 51 Suppl, S61-71.

Duan, H., Takaishi, Y., Momota, H., Ohmoto, Y. and Taki, T. 2002. Immunosuppressive constituents from *Saussurea medusa*. Phytochemistry 59, 85-90.

El Hattab, M., Culioli, G., Valls, R., Richou, M. and Piovetti, L. 2008. Apo-fucoxanthinoids and loliolide from the brown alga *Cladostephus spongiosus f. verticillatus* (Heterokonta, Sphacelariales). Biochem Syst Ecol 36, 447-451.

Fang, H-Y., Chokkalingam, U., Chiou, S-F., Hwang, T-S., Chen, S-L., Wang, W-L. and Sheu, J-H. 2015. Bioactive chemical constituents from the brown alga *Homoeostrichus formosana*. Int J Mol Sci 16, 736-746.

Farasat, M., Khavari-Nejad, R. A., Nabavi, S. M. and Namjooyan, F. 2014. Antioxidant Activity, Total Phenolics and Flavonoid Contents of some Edible Green Seaweeds from Northern Coasts of the Persian Gulf. Iran J Pharm Res 13, 163-170.

Fisch, K. M., Bohm, V., Wright, A. D. and Konig, G. M. 2003. Antioxidative meroterpenoids from the brown alga *Cystoseira crinita*. J Nat Prod 66, 968-975.

French, M. A., Sundram, K. and Clandinin, M. T. 2002. Cholesterolaemic effect of palmitic acid in relation to other dietary fatty acids. Asia Pac J Clin Nutr 11 Suppl 7, S401-407.

Galletti, F., Agabiti-Rosei, E., Bernini, G., Boero, R., Desideri, G., Fallo, F., Mallamaci, F., Morganti, A., Castellano, M., Nazzaro, P., Trimarco, B., Strazzullo, P. and Grp, M.-G. P. S. 2014. Excess dietary sodium and inadequate potassium intake by hypertensive

patients in Italy: results of the MINISAL-SIIA study program. J Hypertension 32, 48-56.

Gatz, S. A. and Wiesmuller, L. 2006. p53 in recombination and repair. Cell Death Differ 13, 1003-1016.

Geleijnse, J. M., Witteman, J. C., Stijnen, T., Kloos, M. W., Hofman, A. and Grobbee, D. E. 2007. Sodium and potassium intake and risk of cardiovascular events and all-cause mortality: the Rotterdam Study. Eur J Epidemiol 22, 763-770.

Ghosal, S., Singh, A.K. and Chaudhuri, R.K. 1976. Chemical constituents of Gentianaceae XX: Natural occurrence of (–) loliolide in *Canscora decussata*. J Pharm Sci 65, 1549-1551.

Gogvadze, V., Orrenius, S. and Zhivotovsky, B. 2006. Multiple pathways of cytochrome c release from mitochondria in apoptosis. Biochim Biophys Acta 1757, 639-647.

Guicciardi, M. E., Malhi, H., Mott, J. L. and Gores, G. J. 2013. Apoptosis and necrosis in the liver. Compr Physiol 3, 977-1010.

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

Hargrove, R. L., Etherton, T. D., Pearson, T. A., Harrison, E. H. and Kris-Etherton, P. M. 2001. Low fat and high monounsaturated fat diets decrease human low density lipoprotein oxidative susceptibility in vitro. J Nutr 131, 1758-1763.

Heffernan, N., Brunton, N. P., FitzGerald, R. J. and Smyth, T. J. 2015. Profiling of the molecular weight and structural isomer abundance of macroalgae-derived phlorotannins. Mar Drugs 13, 509-528.

Herrmann, R., Fayad, W., Schwarz, S., Berndtsson, M. and Linder, S. 2008. Screening for compounds that induce apoptosis of cancer cells grown as multicellular spheroids. J Biomol Screen 13, 1-8.

Heo, S-J., Cha, S-H., Lee, K-W., Cho, S.K. and Jeon Y-J. 2005. Antioxidant activities of Chlorophyta and Phaeophyta from Jeju Island. Algae 20, 251-260.

Hiraga, Y., Taino, K, Kurokawa, M., Takagi, R.and Ohkata, K. 1997. (−)-Loliolide and other germination inhibitory active constituents in *Equisetum arvense*. Nat Prod Lett 10, 181-186.

Hirschhaeuser, F., Menne, H., Dittfeld, C., West, J., Mueller-Klieser, W. and Kunz-Schughart, L. A. 2010. Multicellular tumor spheroids: an underestimated tool is catching up again. J Biotechnol 148, 3-15.

HMSO, UK. 1994. Nutritional aspects of cardiovascular disease (report on health and social subjects No. 46). London: HMSO.

Holdt, S. L. and Kraan, S. 2011. Bioactive compounds in seaweed: functional food applications and legislation. J Appl Phycol 23, 543-597.

Hosono, T., Mouri, A., Nishitsuji, K., Jung, C. G., Kontani, M., Tokuda, H., Kawashima, H., Shibata, H., Suzuki, T., Nabehsima, T. and Michikawa, M. 2015. Arachidonic or Docosahexaenoic Acid Diet Prevents Memory Impairment in Tg2576 Mice. J Alzheimers Dis 48, 149-162.

Hwang, E. S., Ki, K. N. and Chung, H. Y. 2013. Proximate composition, amino Acid, mineral, and heavy metal content of dried laver. Prev Nutr Food Sci 18, 139-144.

Indergaard, M. and Minsaas, J. 1991. Animal and human nutrition. *In*: Seaweed Resources in Europe: Uses and Potential, Guiry M.D. and Blunden G. (ed.). John Wiley & Sons, Chichester, pp. 21-64.

Isoe, S., Hyeon, S.B., Katsumura, S. and Sakan, T. 1972. Photo-oxygenation of carotenoids: II. The absolute configuration of loliolide and dihydroactinidiolide. Tetrahedron Lett 25, 2517-2520.

Ivascu, A. and Kubbies, M. 2006. Rapid generation of single-tumor spheroids for high-throughput cell function and toxicity analysis. J Biomol Screen 11, 922-932.

Kimura, J. and Maki, N. 2002. New loliolide derivatives from the brown alga *Undaria pinnatifida*. J Nat Prod 65, 57-58.

Kosanić, M., Ranković, B. and Stanojković, T. 2015. Biological potential of marine macroalgae of the genus *Cystoseira*. Acta Biol Hung 66, 374-384.

Kumari, P., Bijo, A. J., Mantri, V. A., Reddy, C. R. and Jha, B. 2013. Fatty acid profiling of tropical marine macroalgae: an analysis from chemotaxonomic and nutritional perspectives. Phytochemistry 86, 44-56.

Kuniyoshi, M. 1985. Germination inhibitors from the brown alga *Sargassum crassifolium* (Phaeophyta, Sargassaceae). Bot Mar 28, 501-503.

Lahaye, M. 1991. Marine-Algae as Sources of Fibers - Determination of Soluble and Insoluble Dietary Fiber Contents in Some Sea Vegetables. J Sci Food Agric 54, 587-594.

Lanigan, R. S. and Yamarik, T. A. 2002. Final report on the safety assessment of BHT(1). Int J Toxicol 21 Suppl 2, 19-94.

Li, W. J., Cheng, X. L., Liu, J., Lin, R. C., Wang, G. L., Du, S. S. and Liu, Z. L. 2012. Phenolic compounds and antioxidant activities of Liriope muscari. Molecules 17, 1797-1808.

Lu, J. M., Lin, P. H., Yao, Q. and Chen, C. 2010. Chemical and molecular mechanisms of antioxidants: experimental approaches and model systems. J Cell Mol Med 14, 840-860.

Mabeau, S. and Fleurence, J. 1993. Seaweed in food products: biochemical and nutritional aspects. Trends Food Sci Tech 4, 103-107.

Magalhaes, L. M., Santos, M., Segundo, M. A., Reis, S. and Lima, J. L. 2009. Flow injection based methods for fast screening of antioxidant capacity. Talanta 77, 1559-1566.

Mancini, A., Imperlini, E., Nigro, E., Montagnese, C., Daniele, A., Orru, S. and Buono, P. 2015. Biological and Nutritional Properties of Palm Oil and Palmitic Acid: Effects on Health. Molecules 20, 17339-17361.

Manske, R.H.F. 1938. The alkaloids of fumariaceous plants. Canadian J Res 16B, 438-444.

Marinho-Soriano, E., Fonseca, P. C., Carneiro, M. A. and Moreira, W. S. 2006. Seasonal variation in the chemical composition of two tropical seaweeds. Bioresour Technol 97, 2402-2406.

Martin, L. J. 2015. Fucoxanthin and Its Metabolite Fucoxanthinol in Cancer Prevention and Treatment. Mar Drugs 13, 4784-4798.

McDermid, K. J. and Stuercke, B. 2003. Nutritional composition of edible Hawaiian seaweeds. Journal of Applied Phycology 15, 513-524.

Mhadhebi, L., Laroche-Clary, A., Robert, J. and Bouraoui, A. 2011. Antioxidant, anti-inflammatory, and antiproliferative activities of organic fractions from the

Mediterranean brown seaweed *Cystoseira sedoides*. Can J Physiol Pharmacol 89, 911-921.

Nguyen, D., Zajac-Kaye, M., Rubinstein, L., Voeller, D., Tomaszewski, J. E., Kummar, S., Chen, A. P., Pommier, Y., Doroshow, J. H. and Yang, S. X. 2011. Poly(ADP-ribose) polymerase inhibition enhances p53-dependent and -independent DNA damage responses induced by DNA damaging agent. Cell Cycle 10, 4074-4082.

Okunade, A.L. and Wiemer, D.F. 1985. Loliolide, an ant-repellent compound from *Xanthoxyllum setulosum*. J Nat Prod 48, 472-473.

Ortiz, J., Romero, N., Robert, P., Araya, J., Lopez-Hernandez, J., Bozzo, C., Navarrete, E., Osorio, A. and Rios, A. 2006. Dietary fiber, amino acid, fatty acid and tocopherol contents of the edible seaweeds *Ulva lactuca* and *Durvillaea antarctica*. Food Chem 99, 98-104.

Paiva, L., Lima, E., Patarra, R. F., Neto, A. I. and Baptista, J. 2014. Edible Azorean macroalgae as source of rich nutrients with impact on human health. Food Chem 164, 128-135.

Pan, W., Liu, K., Guan, Y., Tan, G. T., Hung, N. V., Cuong, N. M., Soejarto, D. D., Pezzuto, J. M., Fong, H. H. and Zhang, H. 2014. Bioactive compounds from *Vitex leptobotrys*. J Nat Prod 77, 663-667.

Park, K.E., Kim, Y.A., Jung, H.A., Lee, H.J., Ahn, J-W., Lee, B-J. and Seo, Y-W. 2004. Three norisoprenoids from the brown alga *Sargassum thunbergii*. J Korean Chem Soc 48, 394-400.

Percot, A., Yalcin, A., Aysel, V., Erdugan, H., Dural, B. and Guven, K. C. 2009. Loliolide in marine algae. Nat Prod Res 23, 460-465.

Pereira, H., Barreira, L., Figueiredo, F., Custodio, L., Vizetto-Duarte, C., Polo, C., Resek, E., Engelen, A. and Varela, J. 2012. Polyunsaturated Fatty acids of marine macroalgae: potential for nutritional and pharmaceutical applications. Mar Drugs 10, 1920-1935.

Renaud, S. M. and Luong-Van, J. T. 2006. Seasonal variation in the chemical composition of tropical australian marine macroalgae. Journal of Applied Phycology 18, 381-387.

Repeta, D.J. 1989. Carotenoid diagenesis in recent marine sediments: II. Degradation of fucoxanthin to loliolide. Geochim Cosmochim Acta 53, 699-707.

Rivlin, N., Brosh, R., Oren, M. and Rotter, V. 2011. Mutations in the p53 Tumor Suppressor Gene: Important Milestones at the Various Steps of Tumorigenesis. Genes Cancer 2, 466-474.

Robledo, D. and Pelegrin, Y. F. 1997. Chemical and mineral composition of six potentially edible seaweed species of Yucatan. Botanica Marina 40, 301-306.

Rodrigues, D., Freitas, A. C., Pereira, L., Rocha-Santos, T. A., Vasconcelos, M. W., Roriz, M., Rodriguez-Alcala, L. M., Gomes, A. M. and Duarte, A. C. 2015. Chemical composition of red, brown and green macroalgae from Buarcos bay in Central West Coast of Portugal. Food Chem 183, 197-207.

Rohani-Ghadikolaei, K., Abdulalian, E. and Ng, W. K. 2012. Evaluation of the proximate, fatty acid and mineral composition of representative green, brown and red seaweeds from the Persian Gulf of Iran as potential food and feed resources. Journal of Food Science and Technology-Mysore 49, 774-780.

Rouessac, F., Zamarlik, H. and Gnonlonfoun, N. 1983. Synthese du (±) loliolide par polycyclisation de l'acide homogeranique. Tetrahedron Lett 24, 2247-2250.

Ruberto, G., Baratta, M.T., Biondi, D.M. and Amico, V. 2001. Antioxidant activity of extracts of the marine algal genus *Cystoseira* in a micellar model system. J Appl Phycol 13, 403-407.

Rwigemera, A., Mamelona, J. and Martin, L. J. 2015. Comparative effects between fucoxanthinol and its precursor fucoxanthin on viability and apoptosis of breast cancer cell lines MCF-7 and MDA-MB-231. Anticancer Res 35, 207-219.

Sanches-Silva, A., Costa, D., Albuquerque, T. G., Buonocore, G. G., Ramos, F., Castilho, M. C., Machado, A. V. and Costa, H. S. 2014. Trends in the use of natural antioxidants in active food packaging: a review. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 31, 374-395.

Sclafani, R. A. and Holzen, T. M. 2007. Cell cycle regulation of DNA replication. Annu Rev Genet 41, 237-280.

Strazzullo, P. and Leclercq, C. 2014. Sodium. Advances in Nutrition 5, 188-190.

Tabarsa, M., Rezaei, M., Ramezanpour, Z., Robert Waaland, J. and Rabiei, R. 2012. Fatty Acids, Amino Acids, Mineral Contents, and Proximate Composition of Some Brown Seaweeds. J Phycol 48, 285-292.

Taboada, C., Millan, R. and Miguez, I. 2013. Evaluation of marine algae Undaria pinnatifida and Porphyra purpurea as a food supplement: composition, nutritional value and effect of intake on intestinal, hepatic and renal enzyme activities in rats. J Sci Food Agric 93, 1863-1868.

Ulbricht, T. L. and Southgate, D. A. 1991. Coronary heart disease: seven dietary factors. Lancet 338, 985-992.

Valls, R., Mesguiche, V., Piovetti, L., Prost, M., Peiffer, G. 1996. Meroditerpenes from the brown alga *Cystoseira amentacea* var. *stricta* collected off the French mediterranean coast. Phytochem 41, 1367-1371.

Vizetto-Duarte, C., Pereira, H., Bruno de Sousa, C., Pilar Rauter, A., Albericio, F., Custodio, L., Barreira, L. and Varela, J. 2015. Fatty acid profile of different species of algae of the *Cystoseira* genus: a nutraceutical perspective. Nat Prod Res 29, 1264-1270.

Weijers, R. N. 2012. Lipid composition of cell membranes and its relevance in type 2 diabetes mellitus. Curr Diabetes Rev 8, 390-400.

Xian, Q., Chen, H., Liu, H., Zou, H. and Yin, D. 2006. Isolation and identification of antialgal compounds from the leaves of *Vallisneria spiralis* L. by activity-guided fractionation. Environ Sci Pollut Res Int 13, 233-237.

Yoon, N. Y., Kim, H. R., Chung, H. Y. and Choi, J. S. 2008. Anti-hyperlipidemic effect of an edible brown algae, *Ecklonia stolonifera*, and its constituents on poloxamer 407-induced hyperlipidemic and cholesterol-fed rats. Arch Pharm Res 31, 1564-1571.

Youn, U. J., Miklossy, G., Chai, X., Wongwiwatthananukit, S., Toyama, O., Songsak, T., Turkson, J. and Chang, L. C. 2014. Bioactive sesquiterpene lactones and other compounds isolated from *Vernonia cinerea*. Fitoterapia 93, 194-200.

Zampini, I. C., Ordonez, R. M. and Isla, M. I. 2010. Autographic assay for the rapid detection of antioxidant capacity of liquid and semi-solid pharmaceutical formulations using ABTS*+ immobilized by gel entrapment. AAPS PharmSciTech 11, 1159-1163.

Zbikowski, R., Szefer, P. and Latała, A. 2006. Distribution and relationships between selected chemical elements in green alga *Enteromorpha* sp. from the southern Baltic. Environ Pollut 143, 435-448.

CHAPTER 7

CONCLUSIONS

AND FUTURE PERSPECTIVES

7.1 Conclusions and future perspectives

Marine macroalgae possess a remarkable chemical and pharmacological potential for nutrition and drug discovery purposes. In this thesis, the main goal was to evaluate if *Cystoseira* species had the potential to be used in the nutra- and pharmaceutical industries. As a result, their biochemical composition and biomedical properties were determined and characterized.

In this context, biological questions were answered here for the first time and several concluding remarks can be drawn from the data obtained in this thesis, which are summarized in Fig. 7.1.

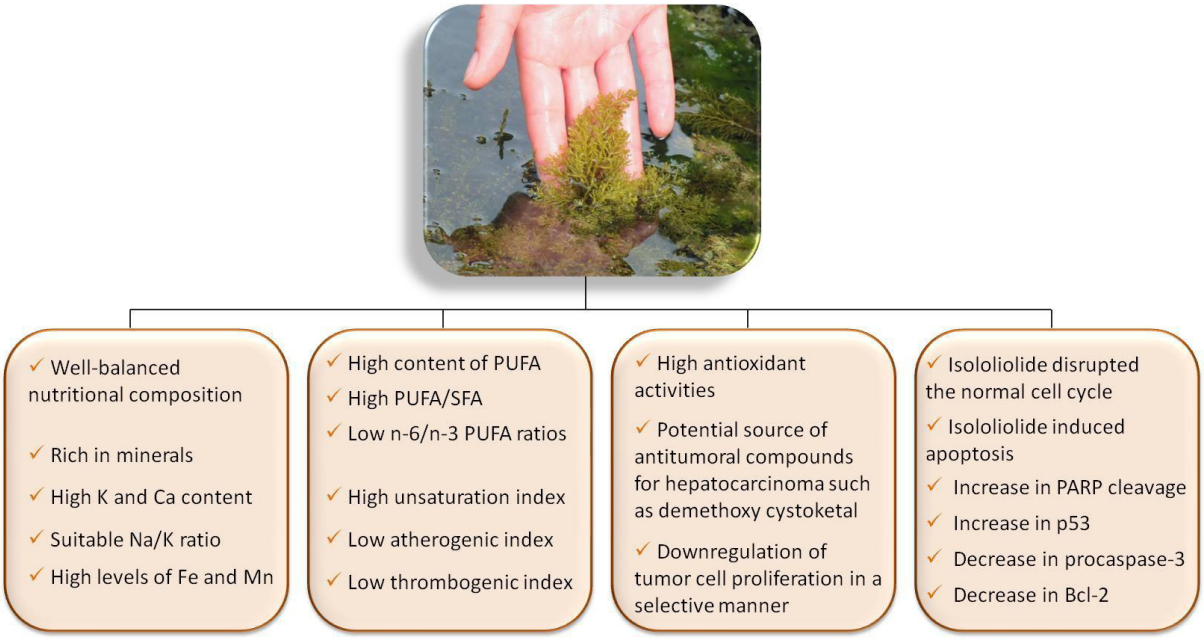

Fig. 7.1 – Summary of the main conclusions obtained with *Cystoseira* species in this thesis.

The nutritional proximate composition of five *Cystoseira* species was assessed and shows that these macroalgae have the characteristics of a healthy, nutritious food/food product. The fatty acid profile of six *Cystoseira* species also confirmed that they could be used in health-derived food products.

Cystoseira extracts exhibited antioxidant and anti-proliferative properties, especially the hexane extract of *C. tamariscifolia*. Two bioactive compounds with anti-proliferative activity and selectivity (demethoxy cystoketal and isololiolide) were found in *C. tamariscifolia* for the first time. Isololiolide showed to be a promising natural product that modulates molecular pathways involved in cell cycle and apoptosis, which

could be a valuable starting point in the quest for novel molecules for hepatocellular carcinoma treatment.

The outcomes of this thesis showed that some of the samples studied here have potential to become candidates for generating new nutra- and/or pharmaceuticals. Hopefully, the results here obtained will contribute to raise awareness of the importance of this renewable resource as a useful, full of potential genus of marine macroalgae.

As future research, a number of ideas can be considered. This work can, in the near future, be used to promote *Cystoseira* macroalgae as ingredients in gourmet salads or soups, among other preparations. *Cystoseira* lipids can be incorporated in the formulation of low fat food and PUFA-rich nutraceuticals. Future studies may also include the evaluation of controlled culture conditions in order to increase biomass and lipids (or other biomolecules) yield and commercial sustainability in, for instance, an integrated multi-trophic aquaculture. In addition, the extracts and compounds could be screened against a larger tumoral and non-tumoral panel cells and in models for other bioactivities such as anti-diabetic or neuroprotection. Moreover, for the compounds here isolated, it would be important to do absorption, distribution, metabolism and excretion (ADME) studies together with pharmacodynamics and pharmacokinetics.

As apoptosis is regulated by a complex network of signaling pathways, the effect of isololiolide on other key molecular players of the apoptotic cascade could also be assayed. Furthermore, other types of cell death such as autophagy could be evaluated. One interesting approach would be to assess co-treatment strategies with drugs currently used in the clinic, such as etoposide and doxorubicin among others, in order to try to improve efficacy. During the pre-clinical evaluation, *in vivo* studies, including the assessment of *in vivo* toxicity in terms of time and dose response, will have to be performed. Furthermore, there is also the possibility to optimize the existing compounds structures with structure-activity relationship (SAR) analyses.