CAROLINA AMARAL FRADE BRUNO DE SOUSA

MARINE ALGAE EXTRACTS AS SOURCE OF NATURAL ANTILEISHMANIAL COMPOUNDS

UNIVERSIDADE DO ALGARVE
FACULDADE DE CIÊNCIAS E TECNOLOGIA
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CAROLINA AMARAL FRADE BRUNO DE SOUSA

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Trabalho efectuado sob orientação de
Prof. Dr. João Varela
Prof. Dr. Lenea Campino
Prof. Dr. Fernando Alberício

UNIVERSIDADE DO ALGARVE
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“QUEM PLANTA TAMAREIRAS, NÃO COLHE TÂMARAS!”

Um jovem aproximou-se de um idoso
que plantava tamareiras no deserto e interpelou-o:
Por que é que o senhor perde tanto tempo a plantar o que não irá colher?
O senhor virou a cabeça e calmamente respondeu: “Se todos pensassem
como você, ninguém no mundo jamais colheria tâmaras. Se hoje sei o
sabor da tâmara é porque um dia alguém plantou uma tamareira.
O relevante não é quem beneficiará dos frutos, o importante
é o trabalho realizado. Não é perda de tempo, é sim
uma grande oportunidade de deixarmos um
legado para ser explorado.

Provérbio Árabe
adaptado de Vandi Dogado

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In the scope of this dissertation

Book chapters

1. Vizetto-Duarte, C.; Bruno de Sousa, C.; Rodrigues, M.; Custódio, L.; Barreira, L.; Varela, J. Marine Algae Bioactivities. *In* Marine Macro- and Microalgae - An Overview. F. Xavier Malcata, Isabel Sousa Pinto and A. Catarina Guedes (ed). CRC press, Taylor & Francis publishers, UK. *In press* ISBN 9781498705332 - https://www.crcpress.com/Marine-Macro-and-Microalgae-An-Overview/Malcata-Isabel-Guedes/p/book/ 9781498705332

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THESIS OVERVIEW

The present dissertation is organized in six chapters. An initial chapter with a general introduction (CHAPTER I), four chapters describing the original research work undertaken for this thesis, most published or submitted for publication in peer-reviewed scientific journals (CHAPTER II-V) and a final discussion of the main conclusions (CHAPTER VI).

CHAPTER I includes an introductory description of the covered issues, being divided in two subchapters addressing 1) the need of new antileishmanial agents and 2) the utility of the algae from the *Cystoseira* genus as source of bioactive compounds.

The chemical diversity of the genus *Cystoseira* as source of bioactive compounds has been elucidated by several authors. To better understand the chemical potentialities of this genus, CHAPTER II provides a comprehensive review of the compounds isolated from different *Cystoseira* species carried out under the aims of this thesis.

The potential of these algae as source of molecules useful against *Leishmania* parasites was explored under the scope of two additional publications. CHAPTERS III and IV describe the evaluation of the antileishmanial activity and cytotoxicity of several macroalgae species collected in coastal areas of Portugal and Spain, and the isolation and identification of compounds with antileishmanial properties in selected *Cystoseira* species, respectively.

In addition, and justified by the controversial taxonomic classification of the genus *Cystoseira* and by the importance of the accurate identification of the biomass used for phytochemical purposes, CHAPTER V describes the genetic assignment carried out for this genus. The results described in this chapter show the value of the molecular and chemical tools for the establishment of phylogenetic relationships between species and also for sample identification. The phylogeny of this genus is analysed and discussed.

Furthermore, in order to gain a general perspective on the utility of the *Cystoseira* genus as source of bioactive compounds against *Leishmania* parasites, the final CHAPTER VI includes an integrated discussion of the main conclusions retrieved from literature. Some perspectives for future research are also discussed.

ABSTRACT

This thesis aimed to identify *Cystoseira* macroalgae compounds displaying antileishmanial activity. Concerning the need to ensure the identification of the samples used for the drug screening, a second aim was determined to evaluate the usefulness of mitochondrial markers for identification of *Cystoseira*. A comprehensive review showed that marine algae, and in particular *Cystoseira*, are important sources of bioactive compounds, which can be used as antiparasitic agents (Chapter I and II). This work revealed that these algae contains compounds with antileishmanial activity. Forty five extracts from 15 species was submitted to bio-guided fractionation and its activity against *L. infantum* promastigotes and their cytotoxicity evaluated. Among the studied algae, *Cystoseira* extracts (*C. baccata*, *C. barbata*, *C. nodicaulis* and *C. tamariscifolia*) displayed the most interesting activities against this parasite (Chapter III). *C. baccata* hexane extract was further investigated, to the isolation of two active meroterpenoids: the (3*R*)- and (3*S*)-tetraprenyltoluquinone with an unknown structure, and the (3*R*)- and (3*S*)-tetraprenyltoluquinol previously isolated and active against *Leishmania* intracellular amastigote forms. Promastigote ultrastructural alterations, DNA fragmentation and mitochondrial potential variations suggest that the mechanism of action of these compounds interfere with the mitochondrial metabolism (Chapters IV). Moreover, the investigation of the chemical composition of the *Cystoseira* crude extracts showed that these algae contain fatty acids, triacylglycerols, carotenoids, steroids and meroterpenoids (Chapter III). This characterization complement published data, suggesting that these compounds might also be involved in the antileishmanial activity here unravelled (Chapter II). Concerning the identification of *Cystoseira*, samples from twenty-two *Cystoseira* species were analysed generating 135 new sequences of three mitochondrial regions (COI, 23S and mt-spacer). This work demonstrated that these three markers are suitable to distinguish these species. The results allowed for the correct identification of *Cystoseira* samples used for drug screening, encouraging the study of taxonomy and evolutionary elucidation of these brown algae using genetic tools (Chapter V).

Keywords: *Leishmania;* algae; *Cystoseira*; meroterpenoids; tetraprenyltoluquinol; tetraprenyltoluquinone mitochondrial markers, phylogeny

RESUMO

Este trabalho teve como objectivo a identificação de compostos de algas do género *Cystoseira* com actividade antileishmania. Perante a necessidade de assegurar a identificação das amostras utilizadas na pesquisa dos compostos activos, o segundo objectivo foi avaliar a utilidade de marcadores mitocondriais para a identificação de espécies de *Cystoseira*. A revisão alargada da bibliografia, mostrou que as algas marinhas, em particular as *Cystoseira*, são importantes fontes de produtos bioactivos com potencialidades antiparasitárias (Capítulos I e II). Os resultados revelaram que estas algas contêm compostos com actividade antileishmania. Quarenta e cinco extractos de 15 espécies de algas, submetidos a fraccionamento bioguiado, foram avaliados quanto à sua actividade contra promastigotas de *L. infantum* e a sua citotoxicidade. Os extractos das espécies de *Cystoseira* (*C. baccata*, *C. barbata*, *C. nodicaulis* and *C. tamariscifolia*) foram os que revelaram actividades antiparasitárias mais interessantes (Capítulo III). O extracto de hexano de *C. baccata* foi estudado, conduzindo ao isolamento de dois meroterpenóides activos: (3*R*)- and (3*S*)-tetrapreniltoluquinona com uma estrutura desconhecida, e (3*R*)- and (3*S*)-tetrapreniltoluquinol descrito anteriormente e activo contra formas amastigotas intracellulares de *Leishmania*. A observação de alterações ultraestruturais fragmentação do DNA e variações do potencial mitocondrial dos promastigotas sugerem que o mecanismo de acção destes compostos interfere com o metabolismo mitocondrial (Chapters IV). Para além destes resultados, a análise da composição dos extractos estudados, revelou que estas algas contém acidos gordos, triacilgliceróis, carotenoides, esteroides e meroterpenoides (Chapter III). Esta caracterização vem complementar a informação publicada, sugerindo que estes compostos podem também estar envolvidos na actividade antileishmania explorada neste trabalho (Capítulo II).

No que diz respeito à identificação das *Cystoseira*, foram analisadas amostras de vinte e duas espécies de *Cystoseira*, que geram 135 novas sequências nucleotídicas de três regiões mitocondriais (COI, 23S e mt-spacer). Este estudo demonstra que estes marcadores os são, de modo geral, eficientes a distinguir estas espécies. Os resultados permitiram a correcta identificação das amostras utilizadas na pesquisa dos compostos bioactivos, vindo encorajar o estudo da taxonomia e elucidação da historia evolutiva destas algas castanhas com recurso a ferramentas genéticas biologia molecular.

Palavras-chave: *Leishmania*; *Cystoseira*; algas; meroterpenoides; tetraprenyltoluquinol; tetrapreniltoluquinona; marcadores mitocondriais, filogenia

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LIST OF ABBREVIATIONS

$\Delta_{\psi m}$	mitochondrial membrane potential
^{13}C NMR	carbon-13 nuclear magnetic resonance spectroscopy
^{1}H NMR	proton nuclear magnetic resonance spectroscopy
23S	mitochondrial ribosomal DNA 23S subunit
ACE	angiotensin-converting enzyme
AChE	acetylcholinesterase
BALB/c	albino mouse laboratory-bred strain of the house mouse
BuChE	butyrylcholinesterase
CanL	canine leishmaniasis
CC_{50}	cytotoxic concentration that causes the death of 50% of the viable cells
$CDCl_3$	deuterated chloroform
CH_2Cl_2	dichloromethane
CHCL3	chloroform
CL	cutaneous leishmaniasis
COI	cytochrome c oxidase subunit I
COSY	correlation spectroscopy
DAD	diode-array detection
DALYs	disability-adjusted life years
DCL	disseminated cutaneous leishmaniasis
DEPT	distortionless enhancement by polarization transfer spectrometry
DHA	docosahexaenoic
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphates
DPPH	α,α-diphenyl-β-picrylhydrazyl
EPA	eicosapentaenoic acid
ESI	electrospray ionization
EtOAc	ethyl acetate
EtOH	ethanol
FBS	fetal bovine serum
FID	flame ionization detector
GC	gas chromatography
HAART	Highly Aactive antiRetroviral therapy
HIV	human immunodeficiency virus
HMBC	heteronuclear multiple-bond correlation spectroscopy
HPLC	High-performance liquid chromatography
HREIMS	high-resolution electron ionization mass spectrometry
HRESIMS	high-resolution electrospray ionization mass spectrometry
HRTLC	high resolution thin layer chromatography
HSQC	heteronuclear single-quantum correlation spectroscopy
IC_{50}	half-maximal inhibitory concentration

iNOS	inducible nitric oxide synthase
IR	infrared
ITS	internal transcribed spacer
JC-1	5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolylcarbocyanine iodide
LC/ESI-MS^n	liquid chromatography/electrospray ionization multistage mass spectrometry
LCL	localized cutaneous leishmaniasis
LPG	lipophosphoglycan layer
LPS	lipopolysaccharide
LREIMS	low resolution electron ionization mass spectrometry
LRESIMS	low-resolution electrospray ionization mass spectrometry
MCL	mucocutaneous leishmaniasis
MS	mass mpectrometry
Mt	mithocondrial
Mt-spacer	23S-$tRNA^{Val}$ intergenic spacer
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUFA	Monounsaturated Fatty Acids
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxygen
NOESY	nuclear overhauser effect spectroscopy
NPs	natural products
NTD	neglected tropical diseases
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PKDL	post kala-azar dermal leishmaniasis
PMM	peritoneal macrophages
PUFA	polyunsaturated fatty acid
RCF	relative centrifugal force
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	Rosewell Park Memorial Institute
SDS	sodium dodecyl sulphate
SEM	standard error of mean
SFA	saturated fatty acid
SiO2	silicon dioxide
TAG	triacylglycerol
THP-1	human monocytic leukemia cell line
TLC	thin-layer chromatography
TMS	tetramethylsilane
UV	ultraviolet
VL	visceral leishmaniasis
WHO	World Health Organization

INTRODUCTION

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1.1. THE NEED FOR NEW ANTILEISHMANIAL AGENTS

1.1.1. LEISHMANIASES

Leishmaniasis is a complex of zoonotic vector-borne diseases resulting from infection of human and animal vertebrate hosts by different species of euglenozoan kinetoplastid parasites of the genus *Leishmania* Ross, 1903, which are transmitted by several species of phlebotomine sand flies in the Old World, and in the New World (WHO, 2010; Ready, 2013).

Even though the signs of cutaneous leishmaniasis (CL) were already mentioned in ancient Assyrian texts dated from 2500 BC, the discovery of the etiological agent responsible for these ailments only occurred around the 18th century. Depictions of skin lesions and facial deformities that are typical of cutaneous and mucocutaneous leishmaniasis are represented in pre-Incan pottery from Ecuador and Peru. In this region, Inca texts from the 15th and 16th century and also reports from Spanish conquistadors described ulcers and skin lesions on agricultural workers in the Andes. This is usually refered to as “white leprosy” and “Andean sickness” (Marstellera et al., 2011; Mans et al., 2016).

With the advent of the microscopy, the parasite responsible for the cutaneous disease in the Old World was observed and described for the first time by D. Cunningham in 1885 and P. F. Borovsky in 1898. However, as they were worked in India and Russia their studies (reviewed by Cox, 2002) remained virtually unknown until 1903, when J. H. Wright revealed a similar discovery in a tropical ulcer observed in an Armenian child (Wright, 1903). Independently, and almost simultaneously, W. Leishman and C. Donovan discovered the causative agent of the visceral disease in spleens of patients with “Dum Dum fever” / ”Kala-azar” in 1900. This disease was originally confused with an acute form of malaria. In 1921, G. Vianna found that *Leishmania* from South America - the causative agent of cutaneous and disfiguring mucocutaneous forms of the disease, differed from those detected in India and Africa, suggesting they were different species. At this time, Etienne and Edouard Sergent demonstrated that *Phlebotomus* sand flies were responsible for the transmission of the *Leishmania* parasites in the Old World, and *Lutzomyia* sand flies were identified as *Leishmania* vectors in the New World in 1922 (reviewed by Cox, 2002 and Killick-Kendrick, 2010, 2013). Definitive proof that the bite of the phlebotomine sand fly was necessary for the transmission of the pathogen was achieved only two decades later (Adler et al., 1941).

With a large infection spectrum, ranging from the subclinical (not apparent) to localized (skin lesion), and disseminated (mucocutaneous and visceral), leishmaniases are a major

health problem worldwide (Alvar et al., 2012). Beyond its impact on human health, this parasite is also a serious veterinary problem in more than 70 countries due to canine leishmaniasis (Franco et al., 2011).

1.1.1.1. Epidemiology

A recent update of the World Health Organization (WHO) database on diseases caused by *Leishmania* identified 98 countries that reported endemic leishmaniases. It is estimated that about 2 million cases occur each year worldwide (Alvar et al., 2012). These data cover the different illnesses caused by these parasites, ranging from cutaneous forms (localized, mucocutaneous, and diffuse) to the potentially fatal visceral form. Being among the ten neglected tropical diseases (NTDs) which exert their largest burden on developing countries, these diseases are strongly associated with poverty (WHO 2010, 2015a). With mandatory report in only 34% of the endemic countries, it is estimated that 12 million people are infected by *Leishmania* parasites. However, the exact worldwide burden of the disease has not been determined (WHO, 2015a). Affecting particularly the world's poorest populations and causing chronic disability and poverty in low- and middle-income countries (Hotez and Pecoul, 2010), it was determined that these disorders have a worldwide impact of 2.35 million disability-adjusted life years (DALYs), 2.3% of which in the Americas (WHO, 2015b). The very low income of the affected population influences the prognosis of these diseases, since their treatment can cost more than 50% of the annual household earnings. This scenario has contributed to the increase of the level of poverty of families with at least one infected individual (Boelaert et al., 2009; Meheus et al., 2010).

In the past two decades, the expansion of the distribution area of this complex of diseases and an exponentially growing incidence of about 1.3 million/year new human cases has been observed. About 1 million of CL cases were mostly found in Afghanistan, Algeria, Brazil, Colombia, Iran, Pakistan, Peru, Saudi Arabia, Syria and Tunisia. The mucocutaneous form occurred mostly in Bolivia, Brazil and Peru, whereas 0.3 million cases of visceral leishmaniasis (VL) were mainly found in Bangladesh, Brazil, Ethiopia, India, Nepal, South Sudan and Sudan. Overall, 350 million people were at risk to become infected (Alvar et al., 2012; WHO, 2015a). Because of these numbers, CL has been ranked as an emerging and uncontrolled disease of category 1 (de Vries et al., 2015); simultaneously, and despite its lower prevalence, it is estimated that severe VL alone is responsible for 20,000-50,000 deaths per year (WHO, 2010; Alvar et al., 2012; WHO, 2015c).

The dispersion of leishmaniases depends on several different risk factors. Housing conditions, urbanization and other socioeconomic conditions influence the nutritional status and the overall health of the affected populations. In particular, health conditions involving immunosuppression can be an important risk factor. Other contributing factors are the increase in general traveling and migration to escape armed conflicts and the deleterious effects of climatic and environmental changes (Aagaard-Hansen et al., 2010; Argaw et al., 2013; Savoia, 2015). All these factors should be considered with more attention in terms of the One Health approach in order to reduce the prevalence and impact of these neglected vector-born diseases (Mansueto et al., 2014). Early diagnosis, treatment, vector control, disease surveillance, and education of the populations are key factors for prevention and control of infection (Savoia, 2015). It is also important to invest on the improvement of living conditions and control of reservoir populations.

In Europe, Asia, North Africa and South America, the infection caused by *L. infantum* constitutes an important zoonosis, with dogs being the main reservoir for human infection. Dogs are also the major host of *L. infantum* parasites and the disease is a serious public health and veterinary problem in the Mediterranean basin, being endemic in 22 countries (Albania, Algeria, Bosnia-Herzegovina, Croatia, Cyprus, Egypt, France, Greece, Israel, Italy, Libia, Malta, Monaco, Montenegro, Morocco, Portugal, Slovenia, Spain, Syria, Tunisia, Turkey; WHO, 2010). *L. infantum* is the causative agent of both CL and VL forms of human leishmaniasis in the Mediterranean basin (Campino et al., 2006). However, VL is the most frequent clinical form with about 900 human cases reported each year (Alvar et al., 2012). It has also been suggested that more than 2.5 million dogs are infected in Southwestern Europe (Moreno and Alvar, 2002).

The spread of leishmaniasis in Mediterranean region and up to Northern Europe is mentioned by different authors (Arce et al., 2013; Bart et al., 2013; Cortes et al., 2011; Gkolfinopoulou et al., 2013; Gramiccia et al., 2013; Harizanov et al., 2013; Lachaud et al., 2013; Šiško-Kraljević et al., 2013; Varani et al., 2013). The increase in the infection risk in this region is related with alterations in the epidemiological patterns due to several factors: climate change, which has resulted in increased exposure to the sand fly; migration from rural to urban and peri-urban areas; increased influx of migrants, travellers and dogs coming from endemic areas; and increased numbers of stray dogs (Campino et al., 2006; Dujardin et al., 2008; Ready, 2010; Miró et al., 2012; Mansueto et al., 2014; Savoia, 2015).

In the Mediterranean region, human infections ocurred mainly in children until the 1960-70s. However, the human immunodeficiency virus (HIV) brought new challenges, in particular during the 1980-90s, when *Leishmania*/HIV coinfection emerged, increasing the number of visceral cases, mostly in France, Italy, Portugal, and Spain (Monge-Maillo et al., 2014). At the end of 1996, the introduction in Europe of the Highly Active AntiRetroviral Therapy (HAART) led to a significant reduction in the number of cases of VL in immunocompromised patients of these countries - from 1440 cases (1990-1998) to 299 cases (2001-2006; Alvar et al., 2008). This study showed that Spain and Portugal were the countries with higher coinfection rates, with 130 and 98 coinfected patients, respectively, diagnosed in the latter period. Taking into account that human leishmaniasis are underreported in Portugal (Serrada, 2010), Campino and Maia (2010) reported 107 VL/HIV coinfected patients diagnosed between 2000-2009, showing that the incidence described by Alvar et al. (2008) remained stable in that period.

In Spain, between 1982 and 1995, a total of 1,574 accumulated human cases were reported (Gil-Prieto et al., 2011). In this period, leishmaniasis was declared as a disease notifiable to local health authorities. Between 1997 and 2011, 3,442 hospitalizations with leishmaniasis (82.6% VL and 3.4% CL) as first diagnosis were reported, 36.5% of which corresponding to HIV-positive patients (Herrador et al., 2015).

A WHO report (www.who.int/leishmaniasis/resources/SPAIN.pdf) states that the economic impact of leishmaniasis in the period of 1982-1995 represented more than 13 million euros. Various seroepidemiological canine leishmaniasis (CanL) surveys have been performed from North to South regions of Spain, reporting a prevalence between 1.1% and 35.6%, with the northern and eastern regions showing the highest values (Morillas et al., 1996; Amusátegui et al., 2004; Gálvez et al., 2010; Martín-Sánchez et al., 2009; Miró et al., 2012).

Between 2009-2012, the largest CL and VL outbreaks of leishmaniasis in Europe were reported in the Spainish Autonomous Region of Madrid, emphasizing the importance of the sylvatic cycle in the transmission of leishmaniasis in the peri-urban areas and reinforcing the importance of environmental measures to control this disease (Aguado et al., 2013; Arce et al., 2013; Gomez-Barroso et al., 2015).

In Portugal, VL is the most frequent clinical form, but a few CL cases have also been reported (Campino et al., 2006). With 20-30 visceral human cases reported yearly in immunocompetent patients (80% of them in children) is thus considered a hypoendemic

country (Campino and Maia, 2010), where three endemic foci have been identified (Alto Douro, Lisbon and Algarve Regions). Although sporadic cases occur all over the country, the higher number of human cases was reported in Lisbon Metropolitan area in 2001, especially in HIV patients, with an incidence of 0.2 cases per 100,000 inhabitants per year (Campino and Maia, 2010). In Portugal CanL has increased over the last two decades. Recent canine epidemiological surveys revealed a 6.31% global prevalence of anti-*Leishmania* antibodies in the general canine population (Cortes et al., 2012), value that reached nearly 20% in some localities (Cardoso et al. 2004; Sousa et al. 2011; Cortes et al., 2012). This is in agreement with a predictive study published on Western Europe that calculated a risk of CanL seroprevalence of 5-20 % in the Portuguese territory (Franco et al., 2011).

1.1.1.2. Clinical forms

Humans can be infected by more than 20 *Leishmania* species (WHO, 2010; Schönian et al., 2010). In the last 20 years, it has also been described the existence of natural hybrids resulting from the genetic recombination between different strains and species both in the New World (Belli et al., 1994; Dujardin, 1995; Delgado et al., 1997; Bañuls et al., 1999) and the Old World (Kelly et al., 1991; Hide and Bañuls, 2007; Ravel et al., 2006; Hamad et al., 2011).

Infectious species/strains are responsible for several disseminated and localized disease forms, ranging from the VL to the different skin disorders: cutaneous leishmaniases (CL; **Table 1.1.**). The occurrence of the clinical manifestations is clearly related with the virulence, tropism and pathogenicity of the *Leishmania* species involved (Bañuls et al., 2007). However, immune competence of the mammalian host strongly determines the outcome of the disease (WHO, 2010; Magill, 2015; Santos-Mateus et al., 2016).

The VL can be resultant from anthroponotic or zoonotic transmission. In the Old World, etiological agents of VL or *Kala-azar* are mainly species of the *L. donovani*-*L. infantum* complex. Being a systemic disease, chronic in the inhabitants of endemic areas where children are especially affected, this clinical form may be acute in travellers originated from *Leishmania*-free areas. VL can occur as an endemic (WHO, 2010), sporadic (Adhikari et al., 2010) or epidemic (Arce et al., 2013) disease, displaying different clinical manifestations and responses to therapy in each situation. Infections could be asymptomatic and may resolve spontaneously. However, because of the parasite tropism to the internal organs, some human hosts eventually develop clinical VL, in particular malnourished and immunosuppressed

individuals, such as HIV-positive patients. The parasite affects the liver, spleen, bone marrow and lymph nodes, typically inducing weight loss, anorexia, anemia, irregular fever, abdominal distension with spleno- and hepatomegaly and lymphadenopathy. Other symptoms, such as coughing, chronic diarrhea, darkening of the skin, chronic kidney disease, can also occur. The risk of thrombocytopenia and leukopenia increase the susceptibility to other infections. This disease is thus considered to be the most severe form of leishmaniasis. As a result of secondary infections and other complications, this disease is potentially fatal if not treated (Ezquerra, 2001; Assimina et al., 2008; CFSPH, 2009).

In East Africa and in the Indian subcontinent, where *L. donovani* is endemic, Post-kala-azar dermal leishmaniasis (PKDL) can eventually appear as sequelae of VL after a period of apparent healing (up to 50% in Sudan and 5-15% in Bangladesh; WHO, 2013a).

CL is the most common form of human leishmaniasis, varying between and within regions, according with the transmission cycle of the parasite, and genetic and immunological characteristics of the infected patients (Alvar et al., 2012). Involving only the skin, CL covers a number of clinical variants ranging from a single ulcer of spontaneous healing - i.e., localized cutaneous leishmaniasis (LCL) - to the presence of multiple mixed type (non-ulcerative) skin lesions with chronic evolution spread throughout the body – i.e., disseminated CL in anergic patients (DCL; WHO, 2010; Masmoudi et al., 2013). Depending on the infective species, papules and ulcers can occur, smooth nodules, plaques or hyperkeratotic flat wart-like lesions, sometimes together with regional lymphadenopathy. Skin lesions are usually painless; however, complications can occur if it becomes secondarily infected. Healing can be spontaneous or take more than a year, also depending on the *Leishmania* species and the immunological status of the patient, and some lesions can lead to social stigma (Ramdas et al., 2016) if they produce disfiguring scars (CFSPH, 2009). In the Old World, LCL also known as oriental sore, is caused by species of the subgenus *Leishmania* (*L.*) (Table 1.1; WHO, 2010; CDC, 2016).

Some zymodemes from the *L. donovani* complex reveal cutaneous tropism in the Mediterranean region (Campino and Abranches, 2002; Campino et al., 2005; Rhajaoui et al., 2012). Species from the *L. enriettii* complex apparently occur in Africa as well (Kwakye-Nuakoa et al., 2015). In the New World, CL causative agents belong to species of the subgenera *Leishmania* (*V.*) and *Leishmania* (*L.*) (**Table 1.1.**; CFSPH, 2009; Castro et al., 2016). Although occurring worldwide, DCL is rare and has higher incidence in the New World; in the Old World, it appears essentially associated with HIV-positive patients. Being difficult to treat, this form

Table 1.1. Vectors, pathology, geographic distribution, reservoirs and type of transmission cycle per *Leishmania* species (Adapted from CFSPH, 2009; Ready et al., 2013; Bates et al., 2015).

(Vectors) ***Leishmania*** **species**	**Geographic distribution**	**Reservoirs**	**Transmission / Clinical form**
(*Plebotomus* spp.)			
L.(L.) donovani	Bangladesh, Buthan, China, India; Ethiopia, Kenya, Nepal, Sudan, Uganda, Yemen	Man, Rodents, Canids	Anthroponotic / VL
L.(L.) infantum	Balkans, China, Mediterranean Europe, Middle East. North Africa, Southwest Asia	Canids	Zoonotic; Peridomestic / CL, VL
L.(L.) aethiopica	Ethiopia, Kenya, Uganda	Hyraxes	Zoonotic; Silvatic / CL
L.(L.) major	Arabic peninsula, Central Asia, Iran and neighbors, Kenya, Middle East, North Africa, Northwest India; Pakistan, Sub-Saharan Africa, Yemen, Sudan	Rodents	Zoonotic; Rural / CL
L.(L.) tropica	Afghanistan, India, Iran, Pakistan, Middle East, North Africa, Sub-Saharan Africa	Man, Canids, Hyraxes	Anthroponotic; Zoonotic / CL
(*Lutzomyia* spp.)			
L.(L.) infantum (Syn. L. chagasi)	Central and South America	Canids	Zoonotic; Peridomestic / VL, CL
L.(L.) amazonensis	South America	Rodents, Marsupials	Zoonotic; Silvatic / CL
L.(V.) braziliensis	South America	Rodents, Marsupials, Canids	Zoonotic; Peridomestic, Silvatic / CL
L.(V.) guyanensis	South America	Edentates, Rodents, Marsupials	Zoonotic; Silvatic / CL
L.(V.) lainsoni	Bolivia, Brazil, French Guiana, Peru, Suriname	Rodents	Zoonotic; Silvatic / CL
L.(V.) naiffi	Brazil; French Guiana; Panama	Edentates; Armadillo	Zoonotic; Silvatic / CL
L.(L.) mexicana	Central and South America; Southern United States	Rodents, Marsupials, Canids	Zoonotic; Silvatic / CL
L.(V.) panamensis	Belize, El Salvador, Northern Venezuela West of Andes	Marsupials, Rodents, Canids, Edentates	Zoonotic; Silvatic / CL
L.(V.) peruviana	Peru	Man, Rodents, Marsupials, Canids	Zoonotic; Peridomestic, Silvatic / CL
L.(V.) shawi	Brazil	Primates, Edentates	Zoonotic; Silvatic / CL
L. (V.) columbiensis	Columbia, Panama, Venezuela	Edentates	Zoonotic; Silvatic / CL
L. (V.) venezuelensis	Northern Venezuela	-	Zoonotic; Silvatic / CL

L. (L.) - Subgenus *Leishmania (Leishmania)*; *L. (V.)* - Subgenus *Leishmania (Viannia)*; VL - Visceral leishmaniasis; CL - Cutaneous leishmaniasis.

causes damage to internal tissues, changing the physical aspect of the patients and seriously influencing their psychological condition (Turetz et al., 2002; Purohit et al., 2012).

Mucocutaneous (MCL) or espundia, mostly caused by *L. braziliensis*, occurs mainly in Latin America, usually after a healed episode of CL. Starting with erythema and ulcerations at the nostrils with frequent bleeding, this infection induces a destructive inflammation of the upper respiratory tract. Affecting the mucosal membranes, it may perforate the nasal septum, causing severe disfigurement of the face, pharynx or larynx and even other mucosal tissues such as the genitalia (Goto and Lindoso, 2010; CFSPH, 2009; McGwire and Satoskar, 2014).

1.1.1.3. *Leishmania* parasite biology, reservoir host and vectors

Among the 35 species belonging to the genus *Leishmania*, ca. 20 of them are pathogenic to humans (Fraga et al., 2013). These parasites are classified in the order Kinetoplastidae, family Trypanosomatidae. They have been divided into two subgenera, *Leishmania* (*Leishmania*) and *Leishmania* (*Viannia*) (**Table 1.1.**), based on specificities related with their development in the sand fly midgut (Lainson et al. 1977 cited by Bates, 2007). This division has been confirmed by different authors using various isoenzymatic and molecular approaches (Bañuls et al., 1999; Campino et al., 2006; Fraga et al., 2010, 2013). Different isolates/strains are usually identified by their zymodemes or schizodemes profiles and classified by international codes (WHO, 2010). *Leishmania* nomenclature is being revised using highly discriminatory methodologies, such as multilocus sequencing and microsatellite typing (Schönian et al., 2010).

Spread all over the world, vectors of the pathogenic *Leishmania* species are insects belonging to the order Diptera, family Psychodidae and subfamily Phlebotominae (ECDPC, 2013). Within this subfamily, two genera and around 70 species have been implicated in the transmission of these pathogens and recognized as of medical importance, namely *Phlebotomus* and *Lutzomyia* in the Old and New World, respectively (Killick-Kendrick, 1999; Ready, 2013). In this system, these kinetoplastid parasites are maintained by multiple domestic and wild hosts from seven different mammal orders (Roque and Jansen, 2014; **Table 1.1.**). Within the mammal host reservoirs, two carnivore species are highlighted due to their close association with humans, namely dogs and cats. Although they can be infected with other *Leishmania* species, dogs are considered to be the most important domestic/ peridomestic reservoir hosts of *L. infantum* in Europe and South America, (Cortes et al., 2012; Dantas-Torres, 2012). The relevance of cats in the epidemiology of this parasite is currently being unraveled (Maia et al., 2011; Pennisi, 2013) and evaluated in several countries of the Mediterranean Basin and also in Brazil, where this zoonosis is endemic (Poli et al., 2002; Savani et al., 2004; Solano-Gallego et al., 2007; Maia et al., 2010, 2015).

Leishmania has a digenetic and heteroxenic life cycle involving two hosts (vertebrate and invertebrate) and two developmental stages (amastigote and promastigote). The sucess of the parasite transmission to a vertebrate host is dependent on the vector-parasite-host interaction, including the capacity of the vector to infect and adapt to the ecological niche of the vertebrate host (Ezquerra, 2001; Bates et al., 2015), and also the parasite species and dispersion strategies (ex: tropism). These factors influence the transmissibility competence (capacity to infect vectors) of a specific vertebrate species and consequently its role as a reservoir host (Roque and Jansen, 2014).

Biologically, the phlebotomine sand flies are silent, have crepuscular or nocturnal activity and females are predominantly exophagic (outdoor feeding) and exophilic (resting outdoors) (Killick-Kendrick, 1999), and undergo complete metamorphosis (egg, 4 larval stages and pupa; **Figure 1.1.**). The larvae are terrestrial, the ovoposition being held in sandy dark places with high relative humidity, constant temperature and rich in organic matter to ensure larvae feeding (Ezquerra, 2001). The form of the *Leishmania* that infects vertebrate hosts are the promastigotes, which live as extracellular parasites in the alimentary tract of the female insect vector. It has a fusiform shape with around 10-20 µm length and 1.5-3.0 µm width (**Figure 1.2A**). These forms are motile due to one flagellum that emerges from the front part of the cell body, allowing it to move in the insect gut and during the process of infection of the vertebrate host. Amastigotes, however, are obligate intracellular parasites of the mononuclear phagocytic system of mammalian hosts with tropism to macrophages. This form has a rounded shape (2.5-6.8 µm of length) and contains the same organelles as the promastigote form, although in a different arrangement. It has a vestigial, immobile flagellum (axoneme; **Figure 1.2.B**). As a kinetoplastid organism belonging to the phylum Euglenozoa, *Leishmania* cells have an ultrastructural organization of a eukaryote, which includes a cell membrane, nucleus, a single mitochondrion containing a network of mitochondrial DNA

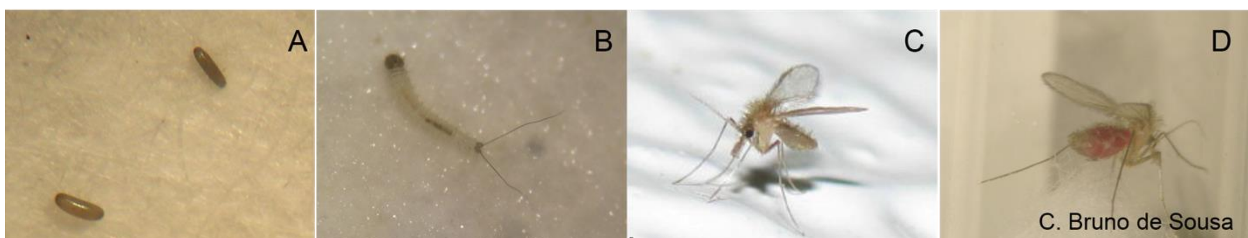

Figure 1.1. Sand fly biological stages. Eggs (A); First stage larvae (B); adult female at rest (C); Female with the abdomem engorged by the blood meal (D). Author’s photographs.

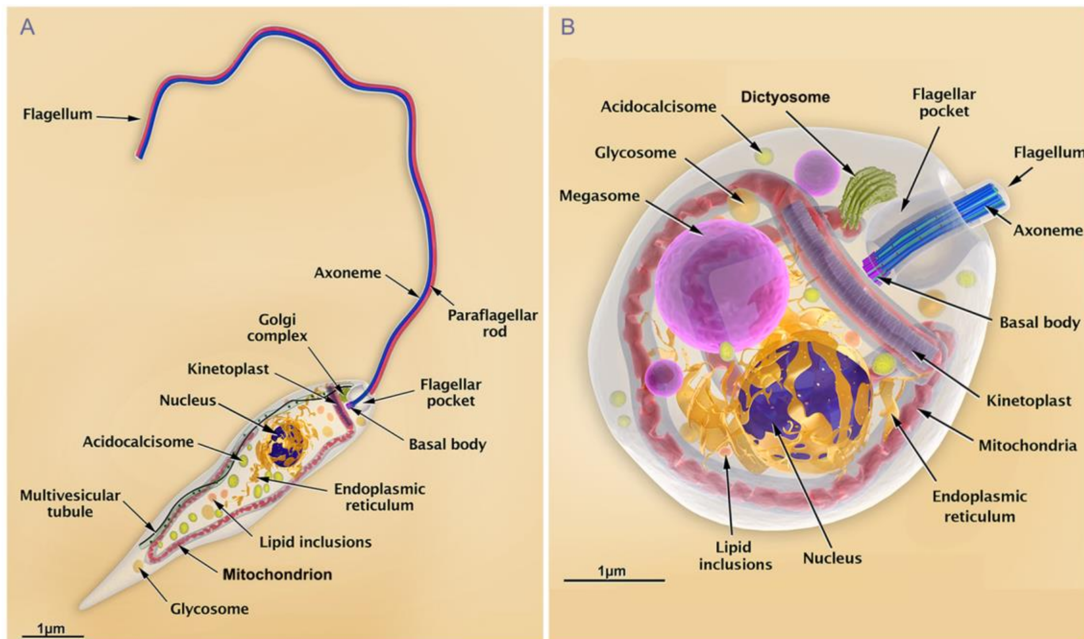

Figure 1.2. *Leishmania* biology - Structural organization of the promastigote (A) and amastigote (B) cells. Adapted from Teixeira et al. (2013).

(kinetoplast), cytoskeleton and endoplasmatic reticulum (Bates, 2007; Wheeler et al., 2011; Rodrigues et al., 2014a).

Description of this biological cycle has been deeply performed by many authors (revised by Dostálová and Volf, 2012) (**Figure 1.3.**). The infection of the sand fly by *Leishmania* starts when a female insect bites an infected reservoir, ingesting a pool of blood infected with amastigotes (Killick-Kendrick, 1999).

In the vector and to successfully achieve the transmission to the vertebrate, *Leishmania* has to overcome several obstacles to its development. Once in the midgut, blood meal and parasites are involved by the peritrophic matrix, starting its development, which is induced by the temperature drop and the pH increase (Bates and Rogers, 2004). Moreover, parasites have to face the action of midgut proteases and the oxidative stress caused by the heme digestion of the blood meal and sand fly immune reactions (Bates, 2007; Dostálová and Volf, 2012). The peritrophic matrix chemical composition (protein, glycoprotein, chitin) is specific to each sand fly species, influencing the selectivity for the *Leishmania* species that it can host (Walters et al., 1993).

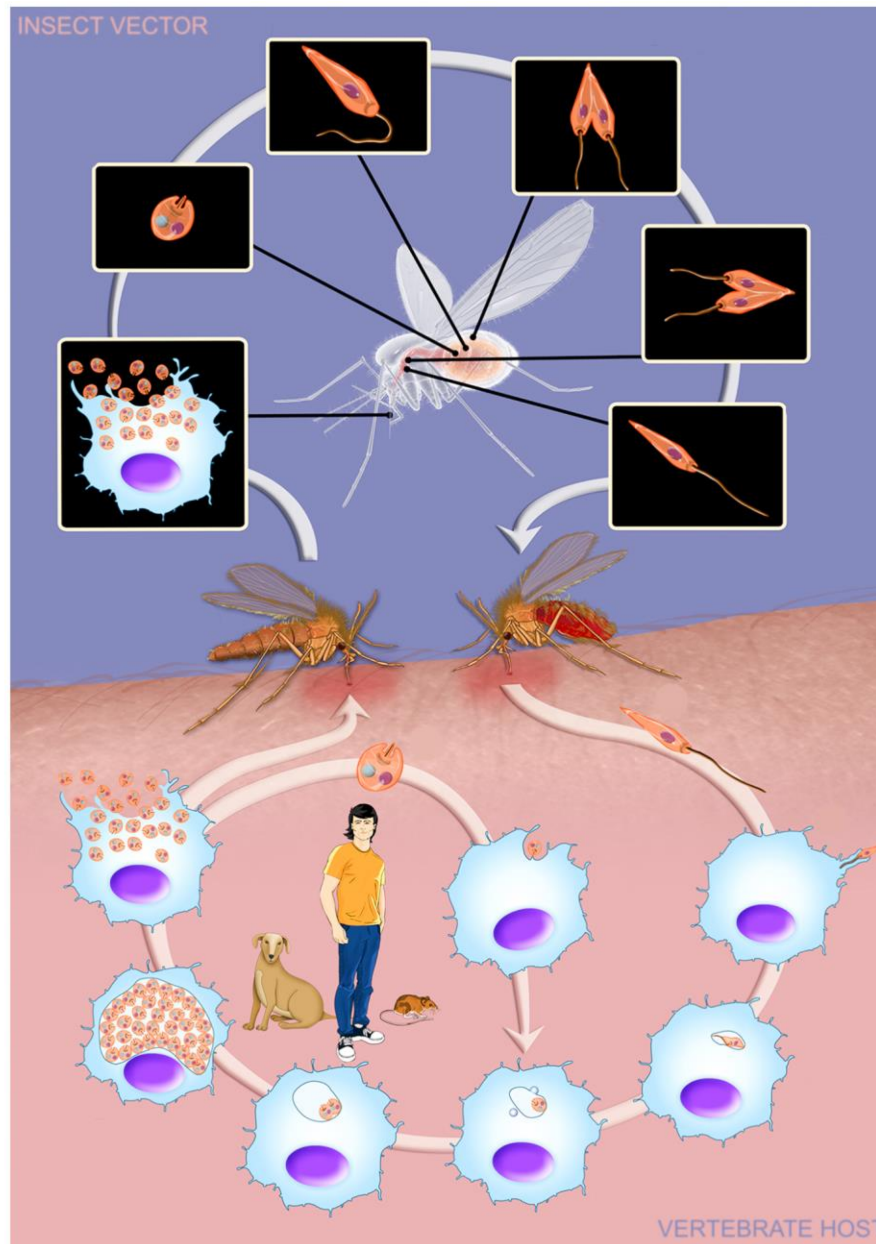

Figure 1.3. *Leishmania* life cycle. (1) The sand fly female bites an infected mammal and during the blood meal collects macrophages infected with amastigote forms of the parasite; (2) amastigotes transform into procyclic promastigotes that multiply in the midgut, becoming infective metacyclic promastigotes; (3) in another blood meal, the insect releases metacyclic promastigotes into other mammalian hosts; (4) metacyclic promastigotes infect macrophages, transforming into amastigotes that multiply in the parasitophorous vacuole; (5) the intense amastigote multiplication induces lysis of the macrophage; (6) amastigote being fagocitised by a macrophage (Adapted from Teixeira et al., 2013).

In addition to the morphological variations between the form infecting the vertebrate and the invertebrate hosts and to overcome all the barriers inside the hosts, *Leishmania* parasites undergo several behavioral and biochemical changes to sustain its life cycle. These changes differ between the various species and are key to its tropism and success as pathogens. During the intravectorial development, the parasite undergoes a sequence of

promastigote stages: procyclic, nectomonad, leptomonad, haptomonad and metacyclic. These stages differ in morphology, cell division capacity, motility, ability to bind to the tissues and infectivity (Bates, 2007; Dostálová and Volf, 2012). For example, the vertebrate host is infected by parasites in the metacyclic stage.

Once inoculated in the mammalian host, promastigotes present in the serum activate the host complement system, attracting macrophages to the inoculation site, being rapidly phagocytized. Once inside the cell, the promastigotes attach to the parasitophorous vacuole membrane and transform into amastigotes. Macrophages, neutrophils and B and T lymphocytes work together to lyse the parasites, inducing the release of cytokines and chemokines, complement-mediated lysis, production of nitric oxide and of other leishmanicidal factors (Silva et al., 1989; Sørensen et al., 1989; Guy and Belosevic, 1993; Brittingham et al., 1995; reviewed by de Almeida et al., 2003). Simultaneusly, parasites use complement factors to enable its recognition and promote fast internalization.

Inside the macrophage, lysosomes loaded with hydrolases merge with the phagosomes carrying parasites, generating acid phagolysosomes where reactive oxygen species (ROS) and nitric oxide (NO) are released together with lysosomal proteases in an acid environment (Bogdan et al., 1990) apparently adverse to the parasite. However, and despite this aggressive attack, amastigote intensively multiply by binary fission, inducing the burst of the phagocytic cell and releasing large amounts of new amastigotes, that are rapidly internalized by other macrophages (Kima, 2007).

Transformation into amastigotes is part of the parasite strategic mechanisms of adaptation to the extracellular environment and of resistance to the host immune system, ensuring its survival and succeful dispersion. To escape from the host immune response, *Leishmania* uses mechanisms involving surface membrane antigens, namelly the metalloproteinase of 63 kDa - (gp63) and the specific lipophosphoglycan layer (LPG; Brittingham et al., 1995; Sacks et al., 2000). Gp63 has suppressive activity of cytokines (e.g. IL-10) and regulates T-cell activation, allowing the oposonization of the infectious forms and its rapid phagocytosis (Guy and Belosevic, 1993), while LPG inactivates the hydrolytic enzymes, promoting the synthesis of ROS scavengers and inhibiting the NO generation through the down-regulation of NADPH oxidase and iNOS (inducible nitric oxide synthase) expression. As a result, parasite degradation is inhibited (Bogdan and Röllinghoff, 1998; Shio and Olivier, 2010; Gupta et al., 2013).

1.1.2. DRUGS AGAINST *LEISHMANIA* PARASITES

As already mentioned, in the absence of a vaccine, treatment and control of leishmaniasis relies on the use of chemotherapeutic agents. The advent of parasites resistance to the in-use molecules, together with a limited therapeutic index and significant toxicities, became a major concern. This has entailed new challenges to the use of existing drugs and prompted the search for more effective antiparasitic drugs able to withstand these resistances (Croft et al., 2006).

The use of natural products (NPs), currently recognized as tradicional medicine, has been reported since antiquity. Mesopotamian, Egyptian, Chinese and Indian registries reveal that plant-based products were used for the treatment of different diseases, including parasitic infections (Borchardt, 2002; Moo-Puc et al., 2008; Petrovska, 2012; Clausen and Demaitre, 2015). Over time, traditional knowledge has continued to play a crucial role in health care of many populations around the world (Sheng-Ji, 2001; Gurib-Fakim, 2006; Lifongo et al., 2014; Hosseinzadeh et al., 2015). Concerning its medical importance, the use of products of natural origin is recognized as a complementary strategy for the fight against NTDs, such as leishmaniasis, being included in the WHO traditional medicine strategy for the next decade 2014-2023 (WHO, 2001, 2003, 2013b).

Terrestrial plants have been used in traditional medicine as natural sources of antiprotozoal compounds (Wright and Phillipson, 1990), and contemporary science has acknowledged the importance of the NPs bioactivities, including a wide range of drugs are plant NPs or are derived from them. An example of the former is the antileishmanial amphotericin B, whereas the anti-malarials artesunate and mefloquine are based on natural scafolds (Watts et al., 2010).

Known since the ancient times, quinine and artemisinin, isolated from *Cinchona* spp. (reviewed in Achan et al., 2011) and *Artemisia annua* (reviewed in Faurant, 2011), respectively, are known as the most successful antiparasitic molecules from plant origin, remaining effective against one of the causative agents of malaria, the alveolate *Plasmodium falciparum* (Sullivan, 2013). Despite these successful cases, to the best of the author's knowledge, no plant source has traditionally been used for treatment of leishmaniasis. This could probably be related with the fact that, till the end of the 19th century, the symptoms associated with leishmaniasis were not matched with a given pathogen.

In recent years, growing interest in NP-based drug discovery has been observed. Many papers describe several studies assaying extracts and biochemicals against *Leishmania*

parasites (Newman and Cragg, 2012; Schmidt et al., 2012a,b; Cragg and Newman, 2013; Brito et al., 2013; Adebayo et al., 2013; Oryan, 2015).

As with land plants, the wide biodiversity of marine organisms and chemical diversity of their secondary metabolites have stimulated the search for novel natural molecules of marine origin for an array of biomedical purposes (Watts et al., 2010; Mayer et al., 2013; Blunt et al., 2016). The need for new products that could be used alone or in combination therapy with conventional drugs is explored below.

1.1.2.1. Conventional drugs

Available drugs of first and second choice include pentavalent antimonials, amphotericine B deoxycholate and its liposomal formulation, pentamidine, miltefosine and paramomycin (Croft and Olliaro, 2011; **Table 1.2.** and **Figure 1.4.**).

Table 1.2. Drugs in use for treatment of human leishmaniasis (Alvar et al., 2006; Croft et al., 2006; Gradoni et al., 2008; Croft and Olliaro, 2011; Singh et al., 2016).

Drug	**Properties**	**Administration and toxicity**	**Mode of action**
Pentavalent antimonials			
Sodium stibogluconate Meglumine antimoniate	Organo-metal complexes in polymeric forms.	Intravenous or intramuscular; GI, cutaneous (rash); myalgia, arthralgia, renal and cardiac toxicity.	Not completely understood. Act as prodrug, inhibits trypanothione reductase, and increase the ROS. Inhibits macromolecular biosynthesis in amastigotes.
Amphotericin B			
AmB deoxycholate	Polyene antibiotic, fermentation product of *Streptomyces nodus*.	Intravenous; Infusion-related (fever, chills, bone pain, rarely cardiac arrest), delayed hypokalaemia and impaired renal function.	Form complexes with sterols mainly ergosterols of parasite membrane leading to increase permeability inducing cell death.
Liposomal AmB	Unilamellar liposome.	Intravenous; Rare and minor (fever, rigor, backache).	Targeted delivery of drug to infected macrophages and kill the parasites as AmB
Miltefosine	Hexadecylphospho choline, alkyl phospholipid	Oral; GI (vomiting, diarrhoea, elevated liver enzymes), rash, nephrotoxicity, teratogenicity.	Modulate cell surface receptors and inositol metabolism of parasites, and cell death is mediated by apoptosis; Inhibits COI.
Pentamidine	Diamidine, as isethionate salt	Intramuscular; Diabetes, rare in VL: shock, myocarditis, death.	Inhibits mitochondrial topoisomerase II and the transcription process
Paromomycin	Aminoglycoside, fermentation product of *Streptomyces rimosus*.	Intramuscular for VL and topical for CL; Generally safe in VL, pain at injection site, cholear and renal toxicity.	Binds to 30S ribosomal subunit interfering with protein biosynthesis, inhibits RNA synthesis, decreases the membrane potential of parasite and inhibits respiration

GI - gastrointestinal; ROS - reactive oxygen species; COI - cytochrome C oxidase; AmB - amphotericine B; CL, cutaneous leishmaniasis; VL, visceral leishmaniasis; PKDL, post kala-azar dermal leishmaniasis.

Pentavalent antimonial family (Sb^v) includes sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantime®; **Table 1.2.** and **Figure 1.4**). Even though the response to these compounds varies from place to place, these drugs have been recommended by WHO as first-line treatment against CL and VL in most regions of the world, with the exception of Europe and the Indian Bihar State (Croft et al., 2006; Singh et al., 2012; Gradoni et al., 2008; WHO, 2016). The identification of parasite resistance to these drugs in the 1980's (Sundar, 2001; Chakravarty and Sundar, 2010) prevented their use in some hyper-endemic regions of India and Nepal, where around 70% of the patients appear refractory to the treatment (Singh et al., 2016). However, in the Mediterranean region, antimony resistance is not considered to be an issue (Gradoni et al., 2008). History of the parasite resistance to antimonials shows that problems are mainly associated with non-completion of the treatment and use of inadequate dosages. Still in 1980's, sodium stibogluconate was freely available in India, which has led to widespread misuse by medical practitioners, who often did not have proper qualification to prescribe it effectively. To minimize toxicity, initial small doses were recommended, leading to an increasing and irreversible tolerance of the parasite to the drug (Chakravarty and Sundar, 2010). Furthermore, as leishmaniasis has an exceptionally anthroponotic transmission cycle in the Indian subcontinent and in East Africa, faster selection and increment of the prevalence of the drug-resistant parasites in the infected population is promoted (Chakravarty and Sundar, 2010). Along with gastrointestinal, cutaneous, myalgia, arthralgia and renal symptoms, cardiac problems are the major side effects (Alvar et al., 2006). Not being completely understood, the processes involved in Sb^v resistance have been the subject of intensive research (reviewed in Ashutosh et al., 2007), being suggested that the mechanisms of action of these drugs implicate both parasite and the infected macrophage (Sundar and Chakravarty, 2015a; **Table 1.2.**).

To overcome the problems associated with resistance to antimonials, pentamidine (**Table 1.2.** and **Figure 1.4**) was used as a second-line drug to the treatment of refractory VL patients. However, its use has been drastically reduced due to declining efficacy (Croft et al., 2006). High cardiac and gastrointestinal toxicity, development of hypotension and diabetes mellitus and cure rates lower than that of amphotericine B have virtually restricted the use of this drug to combination therapies and secondary prophylaxis in HIV-VL co-infection (Das et al., 2001a, Rybniker et al., 2010).

After being firstly recommended for the treatment of patients refractory to antimonials in India (Thakur et al., 1993), amphotericin B deoxycholate (AmB; **Table 1.2.** and **Figure**

1.4), commercialized as Fungizone® and other names, depending on the country, is currently being used as first-line drug for VL treatment in endemic areas, as well as for CL and other complex forms of CL (WHO, 2016). AmB has high toxicity (high fever with rigor and chills, bone pain, thrombophlebitis, renal dysfunction, severe hypokalaemia, myocarditis and even cardiac arrest). As a result, the therapeutic programs need prolonged hospitalization for parentral administration and monitoring of the clinical parameters (Alvar et al., 2006; Sundar et al., 2008; Singh et al., 2016). Like fungi, the *Leishmania* cell membrane contains ergostane-based sterols. This property justifies the high selectivity of AmB towards this parasite (Chattopadhyay and Jafurulla, 2011). Being an antifungal agent, AmB acts on the ergosterol of the parasite cell membrane, changing its permeability and allowing the loss of intracellular components (Ramos et al., 1996). Although this seems to be the main mechanism of action of this drug, other mechanisms may also contribute to its activity (Chattopadhyay and Jafurulla, 2011).

AmB deoxycholate is a low-cost treatment; however, in the 1980s its toxicity boosted the development of less toxic and higher efficacy liposomal formulations: liposomal amphotericin B (AmBisome®), amphotericin B colloidal dispersion (Amphocil®) and

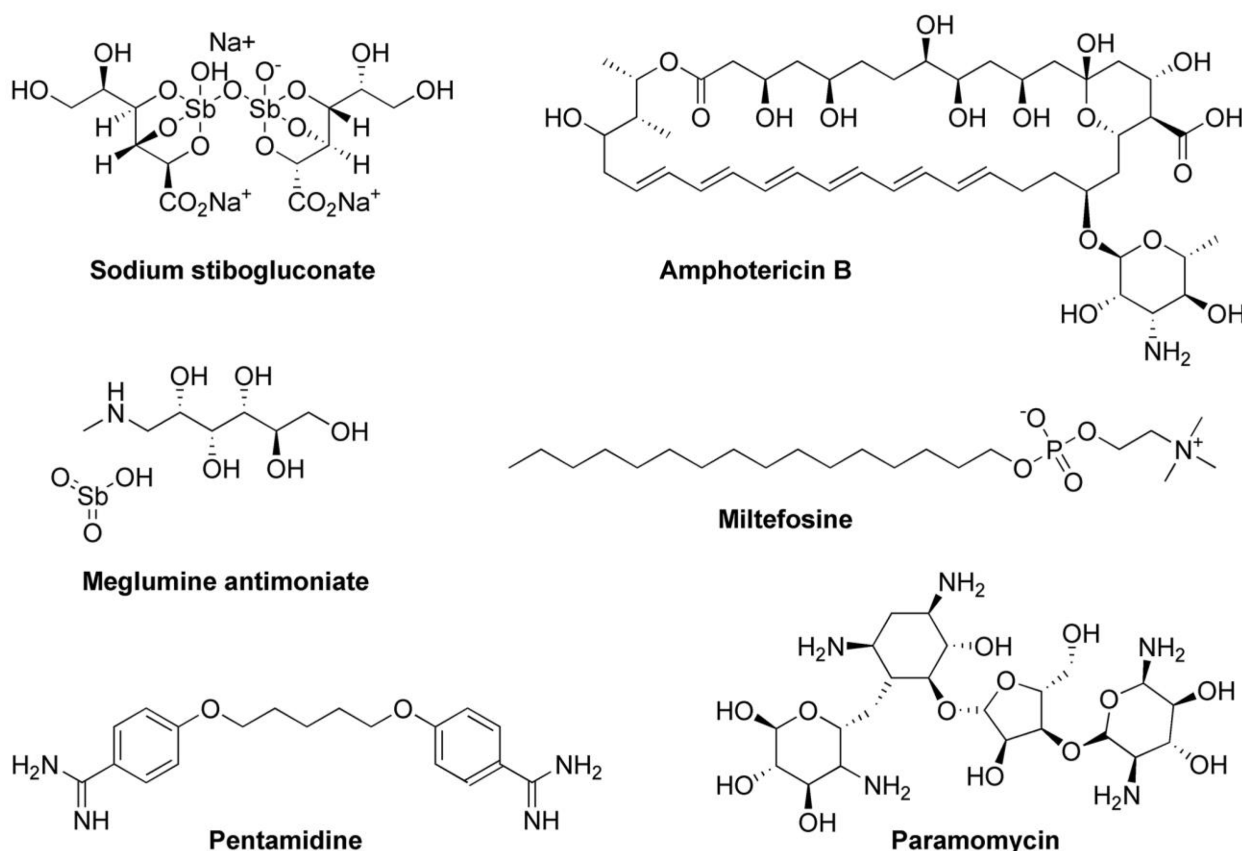

Figure 1.4. Chemical structures of the drugs with antileishmanial activity recommended for the treatment of leishmaniasis (https://pubchem.ncbi.nlm.nih.gov).

amphotericin B lipid complex (Albacete®; Sundar et al., 2004; **Table 1.2.** and **Figure 1.4**). AmBisome® is the standard treatment recommended by WHO for VL in several European countries and USA (Copeland and Aronson, 2015; WHO, 2016). Efforts are being made by WHO with the pharmaceutic companies to reduce the high costs of these drugs. However, this treatment is expensive, since it requires intravenous administration of several doses and follow-up of the patient to control the side effects. Stability of the product due temperature changes is also a problematic issue (Croft and Olliaro, 2011).

The alkyl phospholipid miltefosine (**Table 1.2.** and **Figure 1.4**), initially introduced as an antineoplasic agent, was later approved as the first oral drug to treat VL (Berman, 2005) and CL. Having a high efficacy, and low costs, this drug is commercialized for human use as Impavido® (Freitas-Junior et al., 2012). Despite its effectiveness, therapies require a long period of administration with some limited mild and temporary side effects (gastrointestinal dysfunctions, rash, nephrotoxicity) (Alvar et al., 2006) which contributes for the abandonment and non-compliance of the treatment by the patients (Rijal et al., 2013). Moreover, this compound has a long half-life in the body, which has also promoted the emergence of parasite resistance (Dorlo et al., 2012). Teratogenicity also compromises its use in women during reproductive age (Maes et al., 2013). Because of all these issues and to avoid the aforementioned risks, its use in therapeutic combinations is preferred (Murray et al., 2005). The strategies, effectiveness and costs of its use are being evaluated in various endemic regions (Dorlo et al., 2012; Singh et al., 2016). The mechanism of action of miltefosine involves the modulation of the cell surface receptors and inositol metabolism of parasites, and the inhibition of cytochrome *c* oxidase leading to parasite death through an apoptosis-like process due to the mitochondria membrane depolarization (Paris et al., 2004; Luque-Ortega and Rivas, 2007). Induction of changes in the lipid saturation, also interfere with ergosterol content, increasing the rigidity of the parasite plasma membrane (Saint-Pierre-Chazalet et al., 2009).

The antileishmanial activity of the aminoglycoside antibiotics (paramomycin or aminosidine, monomycin; **Table 1.2.** and **Figure 1.4**) was known since the 1960's. However, only in the 1990's its properties were highlighted after several clinical trials conducted in Kenya, Sudan and India, have shown its high efficacy and good tolerance against VL when used in mono- or in combination therapy with sodium stibogluconate (Singh et al., 2016). Being generally safe at therapeutic VL dosages, its parentral administration induces some side effects such as oto- and renal toxicity (Maes et al., 2013). Although it is known that aminoglycosides inhibit RNA synthesis, affect protein synthesis, decrease mitochondrial

membrane potential and inhibit cellular respiration of the parasite, further studies are required to fully elucidate their mechanisms of action (Chawla et al., 2011).

Several studies on VL suggest that multidrug therapies are safe and effective (Thakur et al., 1992; Das et al., 2001a; Sundar et al., 2008, 2011a,b). This strategy is recommended in order to increase the efficacy of the used drug, to delay the emergence of parasite resistance, to reduce the time of treatment and decrease costs (Singh et al., 2016).

Regarding CanL, and taking into account that none of the available drugs allow parasitological cure, several drugs are available (e.g. allopurinol, meglumine antimoniate, miltefosine, aminosidine, levamisole, domperidone). However, no standard treatment has been recommended for this disease. Allopurinol, alone or in combination with Sbv or miltefosine, is the main drug used for the control of CanL in Spain, Portugal and Italy (Solano-Gallego et al., 2011; Maia and Campino, 2013; Manna et al., 2015). Miltefosine showed to be therapeutically efficient and well tolerated in dogs infected with *L. infantum* (Woerly et al., 2009 cited by Andrade et al., 2011). However, long-term follow-up of dogs with leishmaniasis revealed that dogs treated with a combination therapy of meglumine antimoniate/allopurinol show higher stability of the laboratorial parameters than those treated with miltefosine/allopurinol. Therefore, it is possible that maintenance therapy with allopurinol might be crucial for stabilizing canine leishmaniasis (Manna et al., 2015). Allopurinol is a purine analog not used for human leishmaniasis, having low costs and good safety. It is recommended as first-line drug for the treatment of CanL (WHO, 2010). Its activity on *Leishmania* parasites is related with the inhibition of the enzyme hypoxanthine-guanine phosphoribosyl transferase of the purine pathway. *L. infantum* resistance against this drug was only described this year in dogs with CanL relapse (Yasur-Landau et al., 2016).

In the attempt at finding compounds able to overcome the toxicity, long-term and parentral therapies, efficacy and resistance issues concerning currently drugs used, the search for compounds with antileishmanial activity has been intense. Just from January 2010 to June 2013, 38 patents of compounds with antileishmanial activity, belonging to 37 different chemical classes, were registered, not including vaccines and peptides (reviewed by Rama et al., 2015). Event though a large body of research and patents have been published, most of these works do not elucidate mechanism of action of the proposed drugs, making difficult the comparative assessment of their effectiveness (reviewed in Jose et al., 2004, Monzote 2008, 2011 and Rama et al., 2015).

Despite the large number and chemical diversity of the registered patents and investigation work, it has been difficult to discover novel potential candidates for preclinical development, because molecules against *Leishmania* showing better results than those described above are seldom found. For example, sitamaquine, an oral 8-aminoquinoline, has been evaluated with high expectations for several years. Although it reached phase-II clinical trials (Sundar et al., 2011c), its development was abandoned in 2010 (Maes et al., 2013). In addition, the efficacy of antifungic azoles (ketoconazole, fluconazole, and itraconazole), shown to be active against Leishmania parasites trough the inhibition of sterol biosynthesis, is not high enough to support its use in monotherapy. However, these drugs remain potential candidates to be used in combination therapy (de Macedo-Silva et al., 2013, Maes et al., 2013).

Despite the significant advances made in the treatment of VL in the past decade, the impact of the scientific progress achieved in the field of leishmaniasis on clinical treatment was almost null (Singh et al., 2016). These justifies the importance and the need to reinforce the effort on the research and development of new antileishmanial therapeutics with appropriate screening procedures and use of recommended models in order to find a cheaper, less toxic, but more effective anti-*Leishmania* drug (Croft and Olliaro, 2011).

In view of the current therapeutic panorama, WHO has recently presented the scaffold priorities for leishmaniasis: improve rapid diagnostic tests, develop easy treatments for CL, vaccines and new therapeutics (WHO, 2015c), including the complementary use of products of natural origin (WHO, 2013b). In this context, the identification of novel or already known molecules, which allow the development of better therapeutic approaches for this disease, is a priority.

1.1.2.2. Marine natural products as scaffolds for new drugs

Natural products (NPs) are chemical compounds synthesized by an organism or a group of organisms that have the capacity to produce an effect on another biological target. They can also possess nutraceutical or toxicological properties that can be used in therapeutic procedures. NPs are usually secondary metabolites, i.e. molecules that are not essential to growth and are produced by the source organism to defend itself from environmental threats, including predators (Colegate and Molyneux, 2008). The same compounds can also be used to attract mates or to out-compete competitors for limited resources (Jaspars et al., 2016).

Exposed to several abiotic (such as pH, pressure, temperature, osmolarity) and biotic stresses, marine organisms produce a wide variety of specific secondary metabolites that enable them to respond to challenges posed by the marine environment. Covering around 70% of the Earth's surface, the marine ecosystem contains an immeasurable biodiversity (Mora et al., 2011), which has been recognized as a rich source of bioactive metabolites (Haefner, 2003; Cragg and Newman, 2013). Many of these chemicals have uncommon functional groups (such as isonitrile, dichloroimine, isocyanate, and halogenated functional groups) different from the ones commonly found in higher plants (Hu et al., 2012; Watts et al., 2010; Rocha-Martin et al., 2014).

The interest in the chemical wealth of marine organisms as a potential source of antiprotozoal agents has increased over the last decade (Fattorusso and Taglialatela-Scafati, 2009; Mayer et al., 2011; Tempone et al., 2011). Since the isolation of the first biologically active molecules in the late 1950's (Bergmann and Feeneyz, 1951), more than 28,000 compounds from marine organisms have been identified (Blunt et al., 2015). These compounds have been comprehensively compiled since 1984 in a series of periodic reviews organized by Faulkner (1977, 1984 untill 1990), Blunt et al. (2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2012, 2013, 2014, 2015, 2016), Mayer, 1998) and Mayer et al. (2000, 2002, 2004, 2005, 2007, 2009, 2011, 2013). Isolated from sponges (65.4%), fungi (15.0%), cyanobacteria (8.3%), algae (3.0%), actinomycetes (3.0%), corals (3.0%) and ascidians (2.3%; Watts et al., 2010), these compounds encompass a wide variety of chemical diversity such as alkaloids, peptides, polyketides, terpenes and other unidentified and uncharacterized structures that have demonstrated several pharmacological activities (antitumor, anti-inflammatory, anticoagulant, cardiovascular, nervous system, antibacterial, antifungal, antiviral, antiprotozoal). However, despite the promising results of many of the screening studies on marine species and from the thousands of compounds isolated from them, few compounds with antiprotozoal activity have been identified.

Several natural isolated structures have been used as models for the chemical synthesis of novel drugs. Currently, many synthetic compounds are based on natural scaffolds, demonstrating the importance of the discovery of compounds with different chemical skeletons, which can be used in the design of new and target-specific bioactive molecules. For this reason, the secondary metabolites produced by marine organisms have great value in drug discovery.

The evaluation and approval of drugs for medicinal use is simultaneously performed by the United States Food and Drug Administration (FDA; http://www.fda.gov/) and the European Medicines Agency (EMA; http://www.ema.europa.eu). At the moment, 22 marine-derived products are either approved or are at different phases of the clinical pipeline and a

larger number are under preclinical evaluation. Among these marine-derived products, only four have antiprotozoal purposes, two of which were isolated from macroalgae (**Figure 1.5.** and **Figure 1.6.**) and all of them are still at the beginning of the approval process (i.e. preclinical phase; **Table 1.3.**).

Table 1.3. Marine pharmacological pipeline: compounds, source marine organism and target disease. Adapted from Malve, 2016 and http://marinepharmacology.midwestern.edu.

Marine organism	Compound		Target disease
	Chemical class	**Name and clinical status**	
Algae			
	Glycolipid	Floridosides[1]	Inflammation
	Shikimate	Chrysophaentin A[1]	Bacterial infections
	Terpene	Bomophycolides[1]	Malaria
	Terpene	**4-acetoxydolastane[1]**	***Leishmania***
Bacteria			
	Alkaloid	Pulicatin A[1]	Nervous system
	β-lactone-γ lactam	Marizomib (salinosporamide A)[2]	Cancer
	Peptide	Arenamides A and B[1]	Inflammation
		Grassystatins A-C[1]	Immunity
		Soblidotin (TZT 1027)[4]	Cancer
		Tasidotin, synthadotin (ILX-651)[3]	Cancer
	Polyketide	*Pseudoalteromonas* sp. metabolites[1]	Bacterial infections
	Shikimate	Phenethylamine[1]	Bacterial infections
Bryozoa			
	Alkaloid	Peziza vesiculosa [1]β-carboline[1]	Fungal infections
	Polyketide	Bryostatin [2]	Cancer
Fish			
	Guanidinium alkaloid	Tetrodotoxin[4]	Chronic pain
	Omega-3 fatty acid	Omega-3-acid ethyl esters[5]	Hypertriglyceridemia
Fungi			
	Diketopiperazine	Plinabulin (NPI-2358)[3]	Cancer
Mollusks / Cyanobateria			
	Antibody drug conjugate (MMAE)	Brentuximab vedotin (SGN-35)[5]	Cancer, lymphoma
		Glembatumumab vedotin[3]	Breast cancer, melanoma
		Pinatuzumab vedotin (DCDT-2980S) and(DCDS-4501A)[2]	Non-Hodgkin lymphoma, chronic lymphocytic leukemia
		HuMax ® -TF-ADC[2]	Cancer for ovary, cervix, endometrium, prostate
Mollusks			
	Alkaloid	PM1004[2]	Cancer
	Depsipeptide	Elisidepsin[2]	Cancer
	Peptide	Ziconotide[5]	Pain
Soft corals			
	Diterpene glycoside	Pseudopterosins[2]	Wound healing
	Terpene	Capnellene[1]	Inflammation
		Gyrosanols[1]	Viral infections
Sponges			
	Alkaloid	Hymenidin[1]	Tuberculosis
	Macrolide	Eribulin mesylate (E7389)[5]	Breast cancer
	Nucleoside	Cytarabine, ara-C[5]	Cancer, leukemia
		Vidarabine, ara-A[5]	Anti-viral
	Peptide	Geodisterol sulfates[1]	Fungal infections
	PKS/NRPS	Calyculin A[1]	Nervous system
	Polyketide	Callyspongidiol[1]	Immunity
		Plakortin[1]	Malaria

Marine organism	Compound		Target disease
	Chemical class	Name and clinical status	
	Shikimate	Homogentisic acid[1]	Malaria
	Terpene	Dysideamine[1]	Nervous system
		Dysidine[1]	Diabetes
	Tripeptide	Hemiasterlin (E7974)[2]	Cancer
Tunicates			
	Alkaloid	Trabectedin (ET-743)[5]	Cancer
	Depsipeptide	Plitidepsin[4]	Cancer
Worms			
	Alkaloid	3-(2,4 dimethoxy) benzylidene-anabaseine (GTS-21)[3]	Cognition, Alzheimers disease, schizophrenia

MMAE - Monomethylauristatin E; PKS/NRPS - Polyketide synthases/Nonribosomal peptide synthases; [1] Pre-trial; [2] Phase I; [3] Phase II; [4] Phase III; [5] approved

The development of more marine natural products beyond those in the current pipeline promises important contributions to the medical and veterinary pharmacopeia (Mayer et al., 2010). This milestone involves the need for strengthening the NP research, optimizing the technology through the use of large scale, rapid, random, sensitive and reproducible screening methods (Malve, 2016).

An idea of the dimension of the research needed to obtain a compound that could successfully enter the biodiscovery pipeline is provided by the Pharma Sea project (http://www.pharma-sea.eu/), one of the current EU projects for the discovery of new bioactive marine compounds (Martins et al., 2014). This project aims to produce two compounds that could reach the preclinical evaluation. For that goal, complementary experts

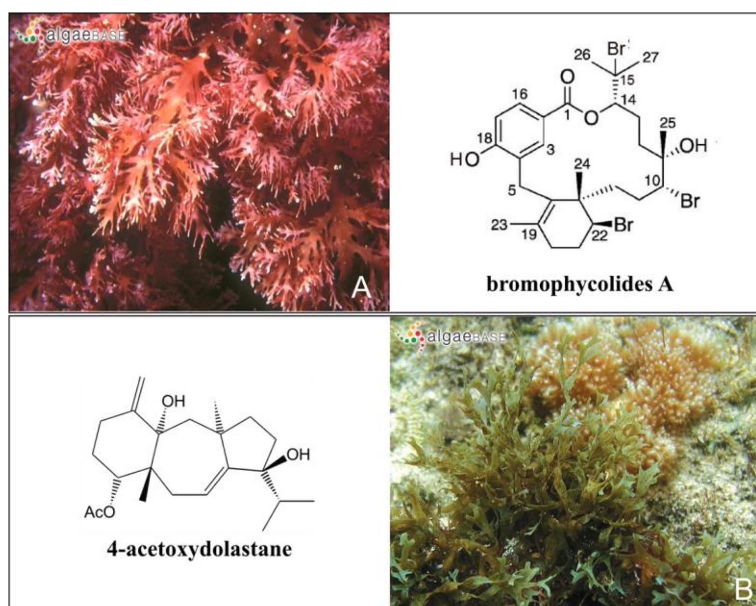

Figure 1.5. Chemical structures of the drugs isolated from algae with antitprotozoal activity and currently in the preclinical phase of the marine drugs pipeline: (A) bromophycolides isolated from the red alga *Callophycus serratus* and (B) the 4-acetoxydolastane isolated from the brown alga *Canistrocarpus cervicornis* (dos Santos et al., 2011; Stout et al., 2011; http://www.algaebase.org/).

from several scientific and economic areas (biology, genomics, natural product chemistry, bioactivity testing, industrial bioprocessing, legal aspects, market analysis and knowledge exchange) screened ca. 1,400 microbial marine strains and more than 15,000 extracts and fractions were already tested against a broad range of antimicrobial agents and central nervous system diseases (Jaspars et al., 2016). This example shows the difficulty in obtaining a bioactive compound wich can be inserted in the pipeline approval. However, from the 28,000 marine molecular entities discovered and deemed as clinically useful (Blunt et al., 2015), only seven are presently approved by the official entities (**Table 1.3.**), corresponding to 0.03% of the described natural products.

Concernig this thesis' aims and despite all the promising availability and novelity of the marine bioactive compounds described above, less extensive research has been carried out on the evaluation of the antiprotozoal potential of marine organisms, algae in particular. Only since the beginning of this century, there has been a focus on screening macroalgae for antiprotozoal activity, including that against *Leishmania* parasites (Tempone et al., 2011).

1.1.2.2.1 Macroalgae

Algae are cosmopolite photosynthetic organisms. Marine algae in particular have been used by Asian and Caribbean coastal comunities in the traditional medicine (Moo-Puc et al., 2008), and existing ancient Japanese and Chinese records have reported its use for antiparasitic treatments (Tseng and Chang, 1984). Moo-Puc et al., (2008) reviewed the folk use of 25 green (Chlorophyta), red (Rhodophyta) and brown algae (Heterokontophyta) species, identifying several medicinal properties (e.g. analgesic, anti-pyretic, anti-inflammatory, anti-neoplasic, urinary and respiratory diseases, cytotoxic and anti-proliferative, anti-mitotic and anti-microbial) including antihelmintic (*Jania capillacea*, *Gracilaria* spp. and *Hydropuntia cornea*) and antiprotozoan (*Dictyota caribaea*, *Laurencia microcladia*, *Sargassum fluitans*) activities.

Several studies demonstrated that macroalgae display biological properties, being promising sources of novel natural compounds for pharmaceutical purposes. In spite of the interest on this subject, very few published articles describe the identification of bioactive compounds from marine algae, in contrast with the literature on terrestrial plants (Schmidt et al., 2012a,b). Screened bioactivities include antioxidant (Ammar et al., 2015; Vizzeto-Duarte et al., 2016a), antiproliferative (Vizzeto-Duarte et al., 2016b), anti-inflammatory (Dimou et al., 2016), antibacterial (Al-Saifa et al., 2014), antiviral (Takebe et al., 2013), nutraceutic (Vizetto-Duarte et

al., 2015) and also antiparasitic properties against agents responsible for neglected protozoan diseases as leishmaniasis (reviewed by Torres et al., 2014).

Thus, screening efforts have explored extracts from several algae species belonging to the phyla Chlorophyta, Heterokontophyta and Rhodophyta for antileishmanial activity. A comprehensive review of these studies is presented in **ANNEX 1**.

These studies report the effects of crude extracts, obtained by different sequential extractions of 136 macroalgae species from European, Asian, Middle-East and South American countries, upon axenic promastigote (P) and amastigotes (AA) forms of different *Leishmania* species. Concerning the species from the Old World, *L. donovani* (Lakshmi et al., 2006; Orhan et al., 2006; Genovese et al., 2009; Allmendinger et al., 2010; Spavieri et al., 2010a,b; Süzgeç-Selçuk et al., 2010; Vonthron-Sénécheau et al., 2011) and *L. major* (Sabina et al., 2005; Fouladvand et al., 2011; Sabina and Aliya, 2011; Saher and Rahman, 2013) was the species used by most authors. In contrast, *L. infantum* was only reported once using extracts from one alga (Ainane et al., 2014). These data also show that the choice of the *Leishmania* species in screenings for novel active compounds is often related with the origin of the evaluated algae. For example, *L. braziliensis* (Bianco et al., 2013), *L. amazonensis* (Felício et al., 2010) and *L. mexicana* (Freile-Pelegrin et al., 2008) are usually species from the New Wold (**Table 1.1**) against which only extracts of algae collected on Brazil and Mexican coasts were evaluated. These co-localization trends between parasite and algal sources are also apparent for the dermotropic species *L. major*, usually found in central Asia, East Africa and Middle East, against which only Pakistani and Iranian algae were used in screening efforts for novel anti-parasitic compounds.

Another trend seen in these data (**ANNEX 1**) corresponds to the well-known phenomenon that marine organisms collected from different environments have different chemistries, which affects their biological activities (Spavieri et al. 2010a). For example, antileishmanial activity of three samples of *Ulva lactuca* and *Dictyota dichotoma* from different locations resulted in IC_{50} values between 5.9 and 12 µg/mL and 8.8 and 52.0 µg/mL, respectively, against *L. donovani* axenic amastigote forms, even though they were evaluated by means of the same screening method (Orhan et al. 2006, Spavieri et al. 2010a, Vonthron-Sénécheau et al. 2011). The observed discrepancies may stem from several factors, ranging from abiotic (e.g. salinity) and biotic (e.g. predation) components (Orhan et al. 2006; Spavieri et al. 2010a) to the use of different extraction methods and solvents, resulting in extracts of diverse chemical composition.

Regarding the activity of algal extracts against *Leishmania* promastigote forms, the lower IC_{50} values for Heterokontophyta, Rhodophyta and Chlorophyta were 10.9 µg/mL, 6.25 µg/mL, 34.0 µg/mL for *Turbinaria turbinata* (Freile-Pelegrin et al., 2008), *Osmundea pinnatifida* (Sabina et al., 2005; Sabina e Aliya, 2011), and *Caulerpa faridii* and *Codium flabellatum* (Sabina et al., 2005), respectively. The most potent activities against this parasite form were found in rhodophytes (**ANNEX 1**) with five species displaying IC_{50} < 25 µg/mL, namely *Laurencia microcladia* (Freile-Pelegrin et al. 2008), *Bostrychia tenella* (Felício et al., 2010), *Asparagopsis taxiformis* (Genovese et al., 2009), *Osmundea pinnatifida* and *Scinaia hatei* (Sabina et al., 2005; Sabina and Aliya, 2011). Rhodophyta are known to contain a wide range of secondary metabolites, such as halogenated mono- and diterpenes, sterols, alkaloids, polyphenols and sulphated sugars (Blunt et al., 2009), some of which showing antifungal and antibacterial activity. Genovese et al. (2009) suggested that the inhibitory properties of the red algae *Asparagopsis* could also be due to their contents in halogenated compounds.

From the phylum Heterokontophyta, only 6 species from 3 families have been screened. The most relevant results were observed with *Turbinaria turbinata*, *Dictyota caribaea* and *Lobophora variegata*, displaying antileishmanial activity with IC_{50} < 50 µg/mL (Freile-Pelegrin et al. 2008).

Chlorophyta was the phylum with less potent activities against *Leishmania* promastigotes. None of the 17 species studied had activities lower than 25 µg/mL. The lowest IC_{50} values were obtained with ethanolic extracts of *Caulerpa faridii* (IC_{50} = 34 µg/mL), *C. racemosa* (IC_{50} = 37.5 µg/mL) and *Codium flabellatum* (IC_{50} = 34 µg/mL; Sabina et al. 2005).

Regarding the clinically relevant stage of the parasite, most studies used axenic *L. donovani* amastigotes. The inhibitory effect of the tested marine macroalgae extracts ranged between 3.8-90.9 µg/mL, 9.5-85.6 µg/mL and 5.9.39.2 µg/mL for Heterokontophyta, Rhodophyta and Chlorophyta, respectively.

However, in contrast with the results observed with promastigote forms described above, higher activities were found in Heterokontophyta species, namely in *Bifurcaria bifurcata* (Spavieri et al., 2010a, Vonthron-Sénécheau et al., 2011), *Halidrys siliquosa* (Spavieri et al., 2010a), *Dictyota dichotoma* and *Dictyopteris polypodioides* (Vonthron-Sénécheau et al., 2011). All these species presented extracts with IC_{50} < 11 µg/mL. From the reviewed data, the Ulvaceae appears as one of the main sources, among chlorophytes, of antileishmanial compounds for the axenic amastigote form (Orhan et al. 2006, Spavieri et al. 2010b).

Within the 19 Rhodophyta families screened for antileishmanial on axenic amastigote forms, only 7 (Dumontiaceae, Rhodomelaceae, Ceramiaceae, Gelidiaceae, Plocamiaceae, Corallinaceae, Dasyaceae) showed activities with IC_{50} < 25 µg/mL (**ANNEX 1**).

Several metabolites such as terpenoids (Fisch et al., 2003; De-Paula et al. 2012), sesquiterpenes (Shimizu et al., 2015), phlorotannins (Steevensz et al., 2012) and steroids (Fleury et al., 1994) have frequently been reported in seaweeds. Hovewer, and despite all these efforts, the report of pure compounds active against *Leishmania* parasites isolated from marine algae has been scarce (**Table 1.4**).

Considering the data obtained with pure compounds isolated from marine seaweeds, two diterpenes, 4-acetoxydolastane ((4R,9S,14S)-4α-acetoxy-9β,14α-dihydroxydolast-1(15),7-diene) and the dolabelladienetriol diterpenes obtained from *Canistrocarpus cervicornis* (IC_{50} 12.3 µM; dos Santos et al., 2011) and *Dictyota pfaffii* (IC_{50} 43.9 µM; Soares et al., 2012), respectively, were shown to be active against intracellular *L. amazonensis* forms. Elatol, obtusol and triquitane were isolated from the red alga *Laurencia dendroidea* (dos Santos et al., 2010; Machado et al., 2011), being effective against the promastigote and intracellular amastigote forms of the same parasite species, though triquitane was less active. Sulphated polysaccharides, obtained from *Gayralia oxysperma*, *Gymnogongrus griffithsiae,*

Table 1.4. Compounds isolated in marine macroalgae active against *Leishmania amazonensis* parasites

Compound number, class and name		Algae species	Inhibitory concentration (µM)			References (a-f)
			P	**AA**	**IA**	
Diterpenes						
1	4-acetoxydolastane	*Canistrocarpus cervicornis*	6.1	36.8	12.3	(d)
2	dolabelladienetriol	*Dictyota pfaffii*	-	-	43.9	(e)
Sesquiterpenes						
3	elatol	*Laurencia dendroidea*	29.1	-	13.5	(c)
			4.0	-	0.45	(b)
4	obtusol		14.9		9.4	(c)
5	triquinane		195.5	-	217.4	(c)
Sulphated polysaccharides						
	galactana iota-nu-carragenana	*Eucheuma denticulatum*	-	-	10/98.0*	(a)
	galactana kappa-iota-nu-carragenana	*Gymnogongrus griffithsiae*	-	-	10/50.0*	(a)
	heteroraminana sulfatada	*Gayralia oxysperma*	-	-	10/55.0*	(a)
	n.n.	*Botryocladia occidentalis*	63.7#			(f)
	n.n.	*Caulerpa racemosa*	137.4#			(f)
	n.n.	*Solieria filiformis*	34.5#			(f)

P - promastigote; AA - axenic amastigote; IA - intracelullar amastigote; n.n. - no specified name; # Half inhibitory concentration (IC_{50}; µM); * Maximal concentration tested (µg/mL) / % cell inhibition. a – Marcolino, 2010; b - dos Santos et al., 2010; c - Machado et al.; 2011; d - dos Santos et al., 2011, e - Soares et al., 2012; f - Pires et al., 2013.

and *Eucheuma denticulatum* (Marcolino, 2010) and *Botryocladia occidentalis*, *Caulerpa racemosa*, *Solieria filiformis* (Pires et al., 2013) also revealed inhibitory activity against *L. amazonensis*. These macromolecules had already been recognized as secondary metabolites with important roles in algal physiology (Pires et al., 2013) and ecology, such as anti-herbivore activity and possible defence against infection by microorganisms (Marcolino, 2010; **Table 1.4**. **Figure 1.6**).

However, a note of caution must be mentioned regarding the choice of the life cycle stage of the parasite for screening efforts. For example, even though the aforementioned diterpenes and sesquiterpenes came from the same extract, they affected amastigotes and promastigotes differently. This result reiforces the importance of using the intracellular amastigote model as the *in vitro* model that better mimicks the response of the vertebrate host and the clinically relevant stage of the parasite for the evaluation of the efficacy of NPs against *Leishmania*.

Different bioactivity results were also reported using the same algal species, but tested in specific forms of the parasite. Dos Santos et al. (2011) observed a higher sensitivity of promastigotes (IC_{50} = 2.0 µg/mL) to the 4-acetoxydolastane isolated from *C. cervicornis* as compared with the intracellular form (IC_{50} = 4.0 µg/mL) and axenic amastigote forms (IC_{50} = 12.0 µg/mL) of *L. amazonensis* (**Table 1.4.**)

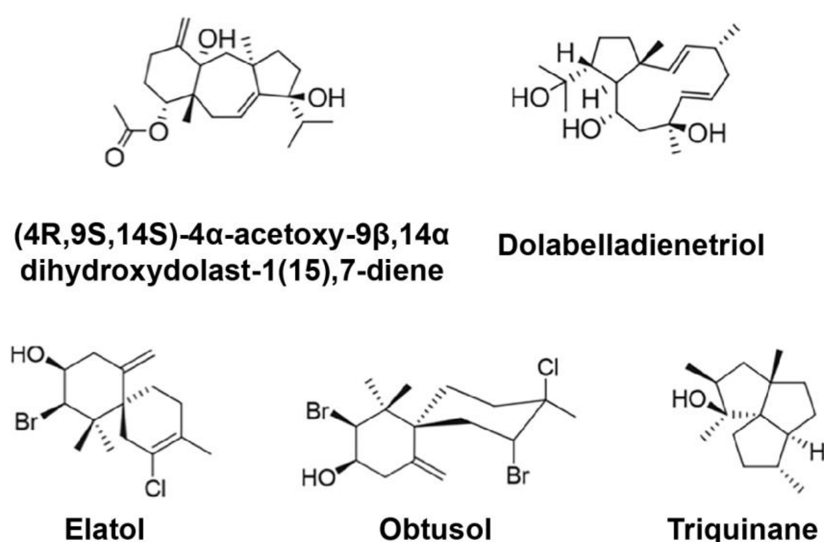

Figure 1.6. Chemical structures of diterpenes isolated from macroalgae with activity against *Leishmania amazonensis*.

1.2. *CYSTOSEIRA* ALGAE, A SOURCE OF BIOACTIVE COMPOUNDS

The genus *Cystoseira*, described by C. Agardh in 1820, is included in the family Sargassaceae (Order Fucales; Rousseau and de Riviers, 1999). Despite the existing taxonomic difficulties in the classification of this genus is currently accepted that it encompasses about 40 species, the majority occurring in the Mediterranean Sea and Atlantic-Mediterranean regions (García-Fernández and Bárbara, 2016; Guiry and Guiry, 2016). In the Iberian Peninsula, are recognized twenty-four specific and infraespecific taxa (García-Fernández and Bárbara, 2016).

Geographical distribution of algae species and genera usually reflects its evolutionary history (Garbary, 2001). Algae belonging to the genus *Cystoseira* originally emerged in the Thetis Sea, located between Eurasia and Africa, 80 million years ago during the late Cretaceous. As a consequence of continental drift, the Mediterranean was formed, being intermittently connected to the Atlantic Ocean. This enabled the colonization of the Mediterranean by the ancestors of *Cystoseira*. The isolation of Mediterranean from the Atlantic Ocean resulted in the hipersalinization of this sea and extinction of most marine organisms during the Cenozoic. At the beginning of Pliocene, when the Atlantic waters re-entered the Mediterranean through the Strait of Gibraltar, this sea was again colonized by *Cystoseira* algae. The colonization of this new but favorable ecosystem led to speciation, a process that is still ongoing (Piatelli, 1990 cited by Amico, 1995).

Being among the main species responsible for the habitat formation in the intertidal and lower sublittoral zones (Lotze et al., 2006; Thibaut et al., 2015; Bermejo et al., 2016), *Cystoseira* plays an essential role in the conservation of biodiversity and ecosystem functioning (Ballesteros, 1989; Giaccone et al., 1994). Marine *Cystoseira* forests provide habitat for other algae, invertebrates and fish (Bellan and Bellan-Santini, 1972; Bulleri et al., 2002; Cheminée et al., 2013; Bermejo et al., 2016), being a key element of the marine ecosystem (Ballesteros et al., 2007; Thibaut et al., 2014; Bermejo et al., 2013,2015).

Currently, many *Cystoseira* species are undergoing a strong demographic decline, which has been attributed to both local and global pressures (Thibaut et al., 2005; Mineur et al., 2015; Thibaut et al., 2015; Blanfuné et al., 2016). The water turbidity, eutrophication and pollution are among the changes that might explain the loss of biodiversity, including habitat-forming macrophyte species such as the *Cystoseira* spp. (Airoldi and Beck, 2007; Mangialajo et al., 2008; Sales et al., 2011). Considered as an important indicator of the marine environment ecological status on the Atlantic-Mediterranean regions (Ballesteros et al., 2007;

Díez et al., 2012), this decrease is a consequence of the sensitivity of these algae to the increasing anthropogenic activity (Thibaut et al., 2015; Bermejo et al., 2016).

In addition to its undeniable bio- and ecological importance, the algae from this genus have shown be carriers of a large number of chemical constituents from different classes (Amico, 1995; Valls et al., 1993a). This chemical diversity has been explored by several authors to unravel the complex phylogeny of this genus, but also for biochemical (Lodeiro et al., 2006) and biomedical discovery purposes (Calvo et al. 1986; Spavieri et al. 2010a; Pujol et al. 2012; Mhadhebi et al. 2014; de los Reyes et al. 2016; Bruno de Sousa et al., 2017).

1.2.1. SPECIES DIVERSITY AND DISTRIBUTION

Cystoseira belong to the phylum Heterokontophyta, whose members are often called "brown algae". They are known as brown due to the presence of carotenoids (e.g. fucoxanthine), which imparts a yellow-brownish color to plastids. Ultrastructurally, plastids contain 3-thylakoid lamellae and the chloroplast is surrounded by the endoplasmic reticulum, which is confluent with the nuclear envelope. Their mitochondria have tubular cristae; the cell walls contain alginic acid, fucoidine and cellulose; and the vacuoles storage β-glucan laminarin as food reserve (Gómez-Garreta, 2003; Kadam et al., 2014). Physodes, membrane-bound vesicles, contain phlorotannins; and reproductive cells present two heterokont lateral flagella (Gómez-Garreta, 2003).

Morphologically, *Cystoseira* have small dense clumps or tufts forming caespitose thalli linked to a single axis that is attached to the substratum by a conical disc or hapteron (**Figure 1.7**). The axis ends in a smooth or spinous apex. Radial (**Figure 1.7.-B3**) or distichous branches ramifications are (**Figure 1.7.-C3, E3**) abundant and can exhibit small spine-like or filiform appendages. Some characteristics are typical of specific species as, for example, the the presence of conical or ovoid tophules arranged along the axis or grouped in the apical zone in *C. nodicaulis* (**Figure 1.7.-D3**), the greenish-blue iridescence of the *C tamariscifolia* branches (**Figure 1.7.-E2**); and the aerocysts, either isolated or arranged in chains at the apices of the terminal branchlets of *C. baccata* (**Figure 1.7.-A2**) and *C. usneoides* (**Figure 1.7.-F2**).

Concerning the reproductive structures, the receptacles are variable in shape and develop at the upper parts of higher order branchlets, being ocasionally bifurcate or branched and with spine-like appendages; conceptacles, usually hermaphrodite, can be sazonally unisexual (Gómez-Garreta et al., 2001).

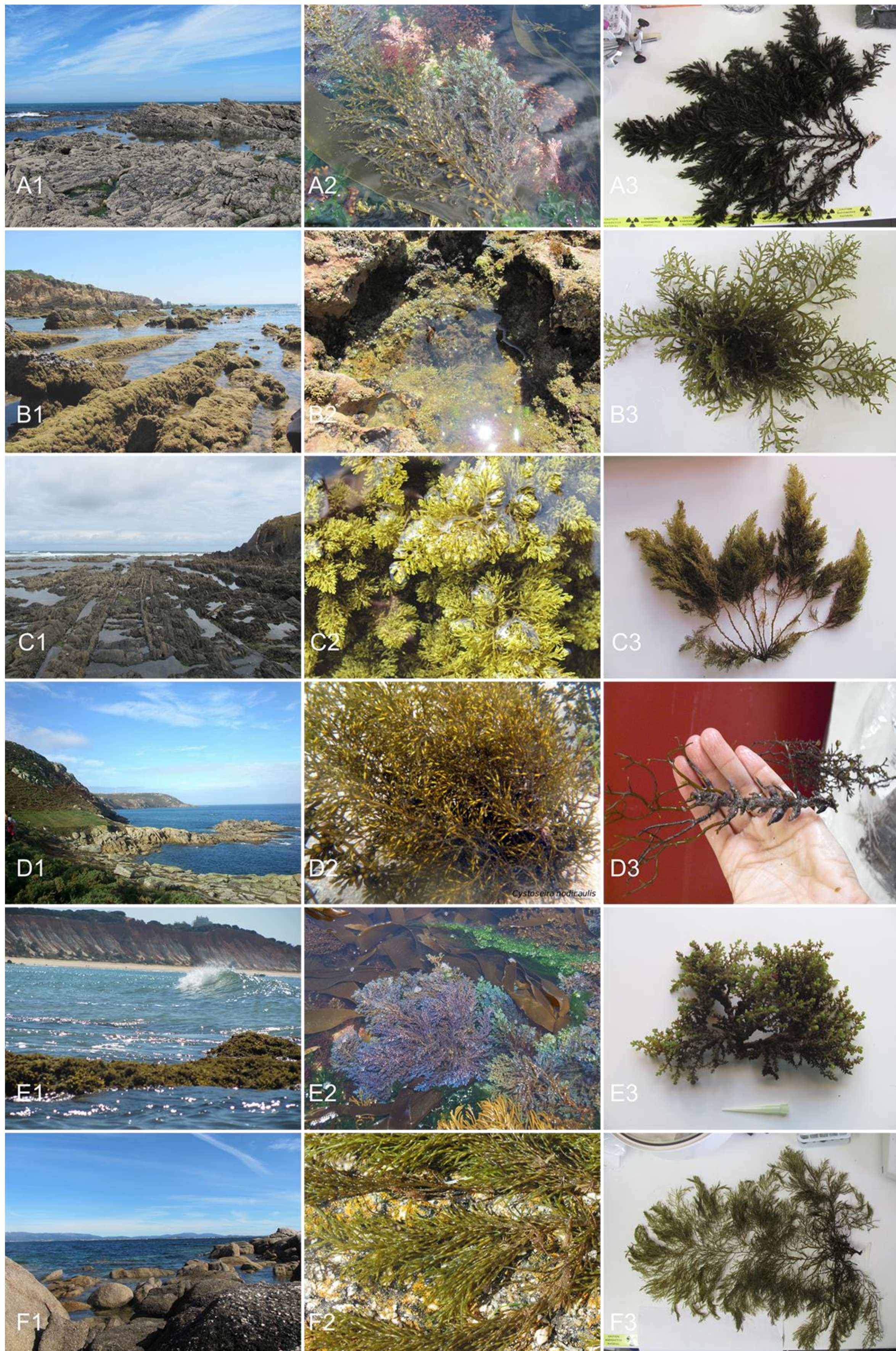

Figure 1.7. *Cystoseira* specimens (columns 2 and 3) and collection sites along the Iberian Atlantic coast (column 1). **A1-3**, *C. baccata* with its isolated aerocysts collected in lower intertidal of Areosa -

Viana do Castelo, Portugal (PT), together with *C. tamariscifolia* (A2); **B1-3**, *C. compressa* collected in the upper intertidal rocky pools of S. Rafael - Albufeira, PT; **C1-3**, *C. humilis* collected in the upper intertidal rocky pools of Almograve - Odemira, PT; **D1-3**, *C. nodicaulis* collected in the sheltered coast of Santa Mariña - Coruña, Spain, and its discriminating tophules in the main thalli (D3); **E1-3**, *C. tamariscifolia* collected in the lower intertidal of Olhos de Água – Albufeira (E1, E3) and Areosa – Viana do Castelo, PT where is possible to see the blue iridescence (E2); **F1-3**, *C. usneoides* with its abundant and chained aerocysts collected in O Grove - Pontevedra, Spain beach cast. In **A3**, **C3** and **E3** it is possible to see the caespitose thalli linked to a single axis that attach to the substratum. A,B,C3,E and F photographs were taken by the author, D2 was a courtesy of J. Cremades and D1 was obtained from http://www.caminodosfaros.com.

The easy acclimation and wide range of phenological and intra/inter species morphological variability of *Cystoseira* leds to successive alterations of the taxonomy and nomenclature of the genus since it was described by C. Agardh, in 1820. Aditionally, the ongoing active speciation, and hybridization of many of the species have often contributed to the occurrence of erroneous taxonomical assignments, and so the macroalgae currently classified as members of this genus still face challenges regarding their taxonomic assignment and classification (Draisma et al., 2010).

A biological characterization of Iberian *Cystoseira* species together with a detailed taxonomic description for the identification of specimens by means of keys was published by Gómez-Garreta et al. (2001). Recently, García-Fernández and Bárbara (2016) have summaryzed the discriminative morphological features for the identification of North Atlantic Iberian species.

The deep knwoledge and recognition of the morphological features of the species is of crucial importance for the acurate identification of the biomass used for phycochemical studies, justifying the need of synergistic collaborations of technicians from different areas.

The worldwide and Iberian distribution of the species of this genus was reviewed by Oliveras Plá and Gómez-Garreta (1989) and Gómez-Garreta et al. (2001), respectively. Recently, in a study of *Cystoseira* assemblages in Northern Atlantic Iberia, García-Fernández and Bárbara (2016) summarized the distribution of the species present within the Atlantic-Mediterranean region, indicating that 31 taxa from 24 species are present on the Iberian Peninsula coasts. Twenty species are only found in the Mediterranean Iberia, and two others are exclusive of the Atlantic Iberia, with 9 taxa being recognized as present in both Mediterranean Sea and Atlantic Ocean (**Table 1.5.**).

Table 1.5. Distribution of the Iberian *Cystoseira* species and infraespecific taxa (Adapted from García-Fernández and Bárbara, 2016)

Iberian Region	***Cystoseira* species and infraespecific taxa**
Mediterranean	*C. abies-marina*, *C. algeriensis*, *C. amentacea* var. *stricta*, *C. barbata*, *C. barbatula*, *C. brachycarpa*, *C. brachycarpa* var. *claudiae*, *C. compressa* f. *plana*, *C. crinita*, *C. elegans*, *C. foeniculacea* f. *latiramosa*, *C. foeniculacea* f. *tenuiramosa*, *C. funkii*, *C. mauritanica*, *C. mediterranea*, *C. pelagosae*, *C. sedoides*, *C. spinosa*, *C. spinosa* var. *tenuior*, *C. squarrosa*, *C. zosteroides*
Atlantic	*C. baccata, C. humilis var. myriophylloides*
Atlantic-Mediterranean	*C. barbata* f. *repens*, *C. compressa*, *C. foeniculacea*, *C. humilis*, *C. nodicaulis*, *C. sauvageauana*, *C. spinosa* var. *compressa*, *C. tamariscifolia*, *C. usneoides*

1.2.1.1. Taxonomy and specimen assignment

Taxonomic classification of *Cystoseira* is a controversial challenge (Gómez-Garreta et al., 1994; Ballesteros and Pinedo, 2004). The wide range of morphological plasticity, compounded by the active, ongoing speciation and hybridization of many species (Roberts, 1978 cited by Draisma, 2010) has led to erroneous taxonomical assignments of specimens collected at different locations and seasons (Jégou et al., 2010). This has become apparent from inconsistencies found between taxonomical classifications based on morphologic traits and current molecular data.

In order to overcome the problems in the correct identification of thalli caused by the morphological variability of these macroalgae, several studies tried to elucidate the genetic relationships of this genus and the related species using different genetic (Harvey and Goff, 2006; Susini et al., 2007; Draisma et al., 2010; Robvieux et al., 2012; Rožić et al., 2012) and chemical markers (Piatelli, 1990 cited by Amico, 1995; Valls et al., 1993a; Amico, 1995; Valls and Piovetti, 1995; Jégou et al., 2010). However, the classification of *Cystoseira* individuals and closely related macroalgae is yet to be fully resolved. First attempts to determine the taxonomy of these brown algae were mostly based on morphological traits. Chemotaxonomic approaches soon followed, showing the potential of using the chemical composition as a taxonomic classification tool (Valls et al. 1993a; Amico, 1995). Trends were observed, and a close agreement between chemistry and morphology has been suggested (reviewed by Amico, 1995). The study of characteristic diterpenoids in Atlantic and Mediterranean *Cystoseira* species allowed to define a chemotaxonomic classification based on the presence or not of diterpenes and meroditerpenes in its chemical composition (Valls et al., 1993b). In another approach, the analysis of the global chemical profile and the lipophilic composition of 5 Britanny *Cystoseira* species through LC/ESI-MS^n (liquid

chromatography/electrospray ionization multistage mass spectrometry) showed to be concordant with the phylogenetic relationships established by the nuclear ITS2 marker (Jégou et al., 2010). In spite of a partial concordance between the morphologic, chemical and genetic classifications, the taxonomy and phylogeny of this group of brown algae is yet to be fully explained (Draisma et al., 2010; Jégou et al., 2010).

Several studies have tried to elucidate the genetic relationships of this genus and related species using phylogenetic methods (Draisma et al., 2010; Rožić et al., 2012). Analysis of Fucales (Phaeophyceae) based on ribosomal DNA (rDNA) sequences led to the merging of the Cystoseiraceae with the Sargassaceae (Rousseau and de Reviers, 1999). In 2010, the polyphyly of the genus *Cystoseira* was demonstrated. Using mitochondrial genetic markers, the authors proposed the division of the genus into 6 different groups, along with other entities belonging to Sargassaceae (Draisma et al., 2010). Several members of the genus were reclassified as belonging to the genera *Sirophysalis*, *Polycladia* and *Stephanocystis* based on the conjugation of these data with the morphologic and embryonic development characteristics. In this study, the mitochondrial ribosomal DNA 23S subunit (mt23S) genetic marker proved to be useful to delineate genera (Draisma et al., 2010). Conversely, the mitochondrial intergenic spacer (mt-spacer) between the 23S-tRNA^{Val} intergenic spacer mt23S gene and the tRNA^{Val} gene, encompassing the tRNA^{Lys} gene, was either not used for phylogenetic inference due to its extreme intergeneric variability (Draisma et al., 2010) or proved not to be suitable to resolve closely related *Cystoseira* (Rožić et al., 2012). As a result, the group of species that retained the original classification was found to be polyphyletic, clustering into at least three different genera.

The mt23S genetic marker proved to be useful to define genera (Draisma et al., 2010). A set of 10 additional mitochondrial, plastid and nuclear markers has also been used to investigate the evolutionary history of brown algae at the ordinal level (Silberfeld et al., 2010). However, to date, full infrageneric resolution of the genus and related Sargassaceae macroalgae has yet to be achieved and the taxonomy of the *Cystoseira* species is still to be completed. More comprehensive genetic studies are needed to fully clarify the phylogeny of *Cystoseira*.

The results here described show the value of molecular and chemical tools in a taxonomical context, for the establishment of species phylogenetic relationships and also for sample identification (Jégou et al., 2010). The phylogeny of this genus is analysed and discussed in CHAPTER V.

1.2.2. CHEMICAL COMPOSITION AND POTENTIALITIES FOR DRUG DISCOVERY

Chemistry of the *Cystoseira* species has been studied since 1976 (Fattorusso et al., 1976). Three important reviews on the chemistry of secondary metabolites isolated from algae of the former Cystoseiraceae family, to which the genus *Cystoseira* once belonged, were published by Piatelli (1990) cited by Amico, 1995), and Valls and Piovetti (1995). These works described the chemistry and chemotaxonomy of these species, mainly based on the diterpenoids and meroditerpenes diversity. Recent studies show that globally the chemical constitution of these brown algae include diterpenoids (Ayyad et al., 2003), meroditerpenoids (de los Reyes et al., 2013), tetraprenyltoluquinol derivatives (Fisch et al., 2003), fatty acids, sterols (Andrade et al., 2013) and terpenes (Kamenarska et al., 2002) (CHAPTER II).

The bio- and chemical diversity of this genus suggest that *Cystoseira* macroalgae have great potential for the discovery of novel compounds with biomedical relevance (Valls et al., 1993a). Various *Cystoseira* species have been investigated for their pharmaceutical potential and, among those studies, extracts from these algae were shown to possess antibacterial (Bennamara et al., 1999; Süzgeç-Selçuk et al., 2010; Spavieri et al., 2010a), antifungal (Bennamara et al., 1999), antiprotozoal (Spavieri et al., 2010a; Süzgeç-Selçuk et al., 2010), antioxidant (Fisch et al., 2003; Mhadhebi et al., 2014), cytotoxic (Ayyad et al., 2003; Spavieri et al., 2010b), antiviral (Urones et al. 1992a), anti-proliferative (Urones et al. 1992b; Mhadhebi et al. 2014) and anti-inflammatory (Mhadhebi et al., 2014; de los Reyes et al., 2013) activities. In addition, several of the compounds (e.g. phlorotannins and diterpenes) identified in different species of *Cystoseira*, but deemed as not specific to these species, exhibited diferent biomedical properties, such as antiviral and antitumoral (Gupta and Abu-Ghannam, 2011; Lopes et al., 2013; Stiger-Pouvreau et al., 2014). *Cystoseira indica* sulphated polysaccharides have also shown antiviral activity (Pujol et al., 2012). In addition, *Cystoseira* fatty acids, sterol and hydrocarbons displayed specific activities against enzymes (α - glucosidase, acetylcholinesterase and butyrylcholinesterase) and free radicals (α,α-diphenyl-β-picrylhydrazyl - DPPH, nitric oxide, superoxide and hydroxyl; Andrade et al., 2013).

Nevertheless, only a limited number of papers evaluating the antileishmanial activity of novel *Cystoseira* species have been published. To date, only *C. baccata*, *C. barbata*, *C. tamariscifolia* and *C. crinita* extracts have been tested against *Leishmania* parasites, showing to be active against *L. donovani* axenic amastigotes (Spavieri et al., 2010a; Süzgeç-Selçuk et al., 2010; **ANNEX 1**). *C. tamariscifolia* was also evaluated against the VL agent, *L. infantum*, however, without interesting results (Ainane et al., 2014). Despite the lack of knowledge on the

antileishmanial properties of the genus *Cystoseira*, the published results suggest that this genus is a potential source of compounds with activity against *Leishmania* parasites. This study was conducted under the aims of this tesis and is described in the CHAPTERS III and IV.

1.2.3. BIOMASS IDENTIFICATION FOR PHYCOCHEMICAL STUDIES

Discovery of NPs requires an unequivocal identification of the investigated biological material and specific sampling strategies in order to efficiently survey the chemical diversity of the target organisms (Amico, 1995; Bucar et al., 2013; Leal et al., 2016). As important as the precise identification of the sampling location, the characterization of the chemical structure and the bioactivity of the identified molecules, the taxonomical identification of the biological entities used is crucial to guarantee the reproducibility of the performed research (Leal et al., 2016).

The accurate taxonomic identification of the biomass is relevant for the effectiveness of the discovery of NPs, because compounds of interest can be specific of a given species. This aspect also ensures that the studied organism is not under protection programs, and therefore its abundance allows its exploitation (Bruno de Sousa et al., *in prep.*; Leal et al., 2016). Moreover, it allows the researchers to positively identify the biological material if additional sampling is necessary for large-scale isolation of the active molecules (Cordell et al., 1993). Thus, accuracy of the identification of biomass used for isolation and identification of NPs is a crucial issue for the reproducibility and reliability of the obtained results, as well as for the implementation of conservation measures for the target species. Classification of algal species is not an easy task, being in some cases difficult to define trustworthy attributes to accurately describe a given group of species, as for example those belonging to the genus *Cystoseira* (Gómez-Garreta et al., 2001).

Biological classifications or taxonomy are dynamic systems that attempt to classify organisms according to their similarities and lack of it. Since the beginning, different principles have been adopted concerning the concept of species (Mayr and Bock, 2002; Taylor, 2009). Initially, these systems assumed that individuals morphologically similar and/or capable of interbreeding were nearest relatives. However, there is the perception that this assumption is not always correct and that there are individuals with similar morphological traits that can be observed in distantly related taxonomic groups, whilst the contrary may also be applied, since organisms with different morphologies can be more closely related than other individuals with a more similar morphology (Coleman, 2009). Towards the need to

clarify these specificities, other criteria were required to develop a different taxonomic classification that could clarify the diverse phylogenetic relations. Several biochemical approaches were developed, such as studying the taxonomy of organisms through their chemical profiles (Valls et al., 1993a; Jégou et al., 2010). However, the use of molecular genetics proposed for the first time by Zuckerkandl and Pauling (1965) became the most used procedures for taxonomic purposes. Since then, the development of molecular and bioinformatics technologies, which use DNA sequence analysis as data, has exponentially increased the resolution of the phylogenetic inference, bringing the ability to process, analyse and integrate large amounts of molecular information, very useful to elucidate the phylogenetic relationships between individuals and populations (Delsuc et al., 2005).

1.2.3.1. Phylogenetic markers

DNA sequences contain the information suitable for taxonomy and phylogenetic studies using appropriate bioinformatic tools. DNA sequencing and PCR related techniques are applied to target specific regions and comparison of homologous sequence data of the studied individuals making possible to access information about changes in nucleotide composition. Numerous parts of the genome can be compared between individuals, and some genetic sequences can be used as phylogenetic reference markers (Delsuc et al., 2005).

Several molecular markers have been studied by different authors to unravel brown algae phylogenetic relationships and the evolutionary history of brown algae in the last decade. Specifically, within the Sargassaceae, the family to which the genus *Cystoseira* belongs, nuclear (Rousseau et al., 2001; Harvey and Goff, 2006; Jégou et al., 2010; Silberfeld et al., 2010) plastid (Cho et al., 2006; Silberfeld et al., 2010, 2014) and mitochondrial (Silberfeld et al. 2010, 2014; Draisma et al., 2010; Rožić et al., 2012) markers have been used.

In our study, we used molecular markers that reflect the changes in three specific regions of the mitochondrial DNA, namely the mt23S, mt-spacer and cytochrome oxidase gene subunit I (COI). The mt23S and the mt-spacer are two of the most used mitochondrial markers (Draisma et al., 2010; Coyer et al., 2006; Rožić et al., 2012). These were combined with plastidial sequences coding for the photosystem II thylakoid protein D1 (*psb*A; Draisma et al., 2010; Rožić et al., 2012), enabling the authors to show the polyphyly of the *Cystoseira* genus, among other Sargassaceae genera. Phylogenetic inference using the photosystem I coding *psaA* gene confirmed the integration of Cystoseiraceae family within the Sargassaceae

(Cho et al., 2006). COI is a well-known molecular tool used for the identification, i.e. DNA barcoding, of different metazoan species (Arif and Khan, 2009; Hebert et al., 2003a; Aly, 2014). The mutation rate of this gene is high enough to distinguish species that are closely related and, for that reason, it has been successfully used in the barcoding of animals (Hebert et al., 2003a; Rubinoff, 2006) as well as red (Saunders, 2005; Le Gall and Saunders, 2010; Sherwood et al., 2010a) and brown algae, including Phaeophyceae (Mattio and Payri, 2010; McDevit and Saunders, 2009; Saunders and McDevit, 2013). Despite its regular use, the utility of this marker for the intraspecific identification of *Cystoseira* species has not been evaluated to date.

1.3. AIMS OF THIS STUDY

Because of the high cost, toxicity, declining efficacy and parasite resistance of the available drugs, the need for novel drugs for treatment of leishmaniasis, which affects millions of people and dogs worldwide, is urgent. Marine biodiversity is nowadays recognized as a source of novel products, to be used alone or in combination therapies with available drugs, which is a promising alternative to anti-*Leishmania* therapy and control.

The *Cystoseira* macroalgae, occurring mainly in the Atlantic-Mediterranean coasts, have been screened for several biomedical activities; however, few reports describe the evaluation of their antileishmanial potential. Despite the several studies on its chemical diversity, no information was available on the compounds responsible for the inhibitory effects of their extracts on the *Leishmania* parasite. Thus, a study of the antileishmanial potential of this algal genus was considered to be highly relevant. Biological sources used to obtain compounds with biomedical properties require the proper identification of that source. This question is especially important in species with a high degree of morphological plasticity such as the *Cystoseira*. In this genus, identification of thalli down to the species level is not always consensual and molecular-based techniques for the positive identification of the specimens used for drug screening are urgently needed.

Therefore, the present thesis has two main objectives:

- Identify compounds of the *Cystoseira* macroalgae displaying antileishmanial activity with potential for further development studies in the context of antileishmanial therapy and;

- Evaluate the usefulness of mitochondrial markers, as molecular aids in the identification of macroalgae belonging to the *Cystoseira* genus in order to ensure the correct assignment of a sample for drug screening purposes.

CYSTOSEIRA ALGAE (FUCACEAE): UPDATE ON THEIR CHEMICAL ENTITIES AND BIOLOGICAL ACTIVITIES

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CYSTOSEIRA ALGAE (FUCACEAE): UPDATE ON THEIR CHEMICAL ENTITIES AND BIOLOGICAL ACTIVITIES

Carolina Bruno de Sousa[1], Katkam N. Gangadhar[1,2], Jorge Macridachis[1], Madalena Pavão[1], Thiago R. Morais[3,4], Lenea Campino[5], João Henrique G. Lago[3,4,*], João Varela[1,*]

[1] Centro de Ciências do Mar, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

[2] Institute of Chemical and Biological Technology, New University of Lisbon, 2780-157 Oeiras, Lisbon, Portugal

[3] Centro de Ciências Naturais e Humanas, Universidade Federal do ABC, São Paulo 09210-180, Brazil;

[4] Instituto de Ciências Ambientais, Químicas e Farmacêuticas, Universidade Federal de São Paulo, São Paulo 09972-270, Brazil.

[5] Global Health and Tropical Medicine Centre, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, 1349-008 Lisboa, Portugal

* Corresponding authors:

Centre of Marine Sciences, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal. Tel.: +351-289-800-051; Fax: +351-289-800-051. *E-mail address*: jvarela@ualg.pt (J. Varela).

Departamento de Ciências Exatas e da Terra, Instituto de Ciências Ambientais, Químicas e Farmacêuticas, Universidade Federal de São Paulo, 09972-270, Diadema, SP, Brazil. Tel.: +55-(11)-3091-6513; *E-mail address*: joao.lago@unifesp.br (J.H.G. Lago).

2.1. ABSTRACT

Cystoseira is a genus of marine brown algae belonging to the Sargassaceae family and composed by about 40 species, which is distributed along the Eastern Atlantic and Mediterranean coasts. The biological potential of *Cystoseira* genus has been investigated and antifouling, anti-bacterial, antifungal, antiviral, cytotoxic, antioxidant, antitumoral, and antiprotozoal activities have been reported. Chemically, the genus *Cystoseira* contains a wide variety of secondary metabolites, such as terpenoids, steroids, phlorotannins and phenolic compounds. Additionally, other chemical components for instance carbohydrates, triacylglycerols and fatty acids, pigments as well as vitamins have been isolated and identified in this genus. Some of the isolated compounds were associated with the reported pharmacological properties. Important reviews on the chemistry of secondary metabolites from *Cystoseira* are published, however, given the current importance of the natural products of marine origin it was considered pertinent to update these revisions in the light of current knowledge. In this review, we provide a comprehensive overview of the compounds isolated and identified after 1995 from the different species of *Cystoseira*, compiling more than 200 compounds isolated, together with their therapeutic potentialities.

Keywords: Macroalgae; *Cystoseira*; Natural products; Chemistry; Biological activity.

2.2. INTRODUCTION

Marine resources represent approximately 25% of the Earth's biodiversity (Mora et al., 2011). The adaptation to the extremely pH, pressure, temperature and osmolarity challenging conditions induced the production of several secondary metabolites by the living organisms (Colegate and Molyneux, 2008). Among the marine organisms, macroalgae are nowadays receiving increasing attention due to their economic and ecological role as well as because of their important role in the actual panorama of drug discovery, resultant of the wide diversity of biological and specific molecules produced by these organisms (Bourgougnon and Stiger-Pouvreau, 2011; Leal et al., 2016).

Cystoseira C. Agardh, 1820 is a polyphyletic genus of marine macroalgae of the Sargassaceae family. Distributed along the Atlantic-Mediterranean coasts, this genus currently encompasses around 40 species (García-Fernández and Bárbara, 2016; Guiry and Guiry, 2016; **Figure 1**). Being essential for the biogenic structure of the marine forests these species ensure food and shelter to numerous species of marine organisms that co-habiting the rocky reefs and have economic value for man (Cheminée et al., 2013; Bermejo et al., 2016). Members of this genus are known to produce various secondary metabolites from different categories such as terpenoids, fatty acids, triacylglycerols, steroids, phlorotannins, phenolic compounds and polysaccharides (Amico, 1995; Valls and Piovetti, 1995).

The bio- and chemical diversity of the genus, suggest that *Cystoseira* macroalgae have great potential for the discovery of novel compounds with biomedical relevance (Amico, 1985a; Valls et al., 1993a). Extracts of different *Cystoseira* species have been evaluated for their several activities, such as antimycobacterial and antiprotozoal (Spavieri et al., 2010a; Bruno de Sousa et al., *in prep.*), antiviral (Pujol et al., 2012) and antifungal (Calvo et al., 1986) properties. Additionally, cytotoxic (Spavieri et al., 2010a), antioxidant (Vizetto-Duarte et al., 2016a) and antitumoral (Vizetto-Duarte et al., 2016a,b) potentials were also reported.

Four important reviews on the chemistry of secondary metabolites isolated from Cystoseiraceae algae, the family where the genus *Cystoseira* was once considered to belong, were published by Piatelli (1990) cited by Amico (1995), Valls and Piovetti (1995) and Gouveia et al. (2013a). Since these publications, relevant taxonomic changes have been proposed to this family that changed its position to the current family Sargassaceae (Rousseau and de Reviers, 1999; Cho et al., 2006). Other studies also led to the reclassification of some *Cystoseira* species from the Pacific Ocean in other genera (Draisma et al., 2010).

The current importance of natural products from marine origin, and in particular of the nutritional value and pharmacological applications of *Cystoseira* sp., led us to considered pertinent to update the available reviews regarding the global chemical constitution and biological aspects of these algae. Moreover, a compound of interest often has crossed bioativities, therefore, the knowledge of other recognized bioativities provides for a better understanding of the pharmacological potential of a certain species. In this scope, this review provides a comprehensive overview of the compounds isolated after 1995 from the different species from the current genus *Cystoseira*.

2.3. CHEMICAL CONSTITUENTS AND BIOLOGICAL ACTIVITIES OF *CYSTOSEIRA* SP. ALGAE

Chemical and biological aspects of 214 compounds isolated from sixteen studied *Cystoseira* species are shown in **Table 2.1.**, in which and are compiled the compounds isolated from each species and some of the respective recognized biological activities. The metabolites found in major number were lipids followed by terpenoids (including meroterpenoids), steroids, carbohydrates, phlorotannins, phenolic compounds, pigments and vitamins. Other several compounds were identified and the respective chemical and biological aspects are described below.

Table 2.1. Chemical and biological studies of genus *Cystoseira*. (Numbering of compounds will be used throughout the chapter).

No	Compound class and name	Species (Origin)	Reference	Biological activity
Terpenoids				
1	Cystoseirol monoacetate	*C. myrica*[b]	32	Cytotoxic
2	Dictyol F monoacetate	*C. myrica*[b]	32	Cytotoxic
3	Dictyone	*C. myrica*[b]	32	Cytotoxic
4	Dictyone acetate	*C. myrica*[b]	32	Cytotoxic
5	Isodictytriol monoacetate	*C. myrica*[b]	32	Cytotoxic
6	Pachydictyol	*C. myrica*[b]	32	Cytotoxic
7	3,7-dimethyl-1,6-octadiene-3-ol- 2-aminobenzoate	*C. crinita*[l]	245	-
8	Hexahydrofarnesylacetone	*C. crinita*[l]	245	-
9	Dihydro-3-hydroxy-3-hydroxymethyl-2(3H)-furanone	*C. crinita*[l]	245	-
10	Dihydroactinidiolide	*C. crinita*[a]	245,336	-
11	Isololiolide	*C. tamariscifolia*[i]	503	Antiproliferative503,anti-germinative[232], anti-cyanobacterial[525]
Meroterpenoids				
12	14-epi-amentol triacetate	*Cystoseira* sp.[j]	357	-
13	Amentol	*Cystoseira* sp.[j]	357	-
14	Amentol chromane diacetate	*Cystoseira* sp.[j]	357	-
15	Cystoseirone diacetate	*Cystoseira* sp.[j]	357	-
16	Preamentol triacetate	*Cystoseira* sp.[j]	357	-
17	Demethoxy cystoketal chromane	*C. tamariscifolia*[i] *C. amentacea var. stricta*[c]	502 495	Antiproliferative

No	Compound class and name	Species (Origin)	Reference	Biological activity
18	Cystoazorol A	*C. abies-marina*[i]	191	Antioxidant, cytotoxic
19	Cystoazorol B	*C. abies-marina*[i]	191	Antioxidant
20	Cystoazorone A	*C. abies-marina*[i]	191	Cytotoxic
21	Cystoazorone B	*C. abies-marina*[i]	191	Cytotoxic
22	2-[(2′E,6′E,10′E,14′Z)-5′-hydroxy-15′-hydroxymethyl-3′,7′,11′-trimethylhexadeca-2′,6′,10′,14′-tetraenyl]-6-methyl hydroquinone	*C. crinita*[f]	165	Antioxidant, cytotoxic
23	2-[(2′E,6′E,10′E)-5′-oxo-13′-hydroxy-3′,7′,11′,15′-tetra-methylhexadeca-2′,6′,10′,14′-tetraenyl]-6-methyl hydroquinones	*C. crinita*[f]	165	Antioxidant
24	2-[(2′E,6′E,10′E)-5′-oxo-3′,7′,11′,15′-tetramethyl hexadeca-2′,6′,10′,14′-tetraenyl]-6-methyl hydroquinone	*C. crinita*[f]	165	Antioxidant
25	2-[(2′E,6′Z,10′E,14′Z)-5′-Oxo-15′-hydroxymethyl-3′,7′,11′-trimethylhexadeca-2′,6′,10′,14′-tetraenyl]-6-methyl hydroquinone	*C. crinita*[f]	165	Antioxidant, cytotoxic
26	2-[(2′E,6′Z,10′E)-5′-oxo-13′-hydroxy-3′,7′,11′,15′-tetra-methylhexadeca-2′,6′,10′,14′-tetraenyl]-6-methyl hydroquinones	*C. crinita*[f]	165	Antioxidant
27	2-[(2′E,6′Z,10′E)-5′-oxo-3′,7′,11′,15′-tetramethylhexadeca-2′,6′,10′,14′-tetraenyl]-6-methyl hydroquinone	*C. crinita*[f]	165	Antioxidant
28	2-[(2′E,6′E)-5′-oxo-3′,7′,11′-trimethyldodeca-2′,6′10′-trienyl]-6-methylhydroquinone	*C. crinita*[f]	165	Antioxidant
29	2-[(2′E,6′Z)-5′-oxo-3′,7′,11′-trimethyldodeca-2′,6′10′-trienyl]-6-methylhydroquinone	*C. crinita*[f]	165	-
30	5-oxo-cystofuranoquinone	*C. crinita*[f]	165	Antioxidant
31	5-oxo-isocystofuranoquinone	*C. crinita*[f]	165	Antioxidant
32	2,12-diepineobalearone	*C. amentacea var. stricta*[k]	331	-
33	4'-methoxy-(2E)-bifurcarenone	*C. amentacea var. stricta*[c]	331	Cytotoxic
34	Methoxybifurcarenone	*C. amentacea var. stricta*[c] *C. tamariscifolia*[h]	331 52, 331	Antifungal[52], anti-bacterial[52]
35	Merediterpenoid 1	*C. baccata*[h]	341	-
36	Merediterpenoid 2	*C. baccata*[h]	341	-
37	Merediterpenoid 3	*C. baccata*[h]	341	-
38	Merediterpenoid 4	*C. baccata*[h]	341	Antifouling
39	Merediterpenoid 5	*C. baccata*[h]	341	Antifouling
40	Merediterpenoid 6	*C. baccata*[h]	341	-
41	Merediterpenoid 7	*C. baccata*[h]	341	Antifouling
42	Cystodione A	*C. usneoides*[h]	133	Anti-inflammatory, antioxidant
43	Cystodione B	*C. usneoides*[h]	133	Anti-inflammatory, antioxidant
44	Cystodione C	*C. usneoides*[h]	133	Antioxidant
45	Cystodione D	*C. usneoides*[h]	133	Antioxidant, anti-inflammatory
46	Cystodione E	*C. usneoides*[h]	133	Antioxidant
47	Cystodione F	*C. usneoides*[h]	133	Antioxidant
48	Cystodione G	*C. usneoides*[j]	132	Antioxidant
49	Cystodione H	*C. usneoides*[j]	132	Antioxidant
50	Cystodione I	*C. usneoides*[j]	132	Antioxidant
51	Cystodione J	*C. usneoides*[j]	132	Antioxidant
52	Cystodione L	*C. usneoides*[j]	132	Antioxidant

No	Compound class and name	Species (Origin)	Reference	Biological activity
53	Cystodione M	*C. usneoides*[j]	132	Antioxidant
54	Cystone A	*C. usneoides*[j]	132	Antioxidant
55	Cystone B	*C. usneoides*[j]	132	Antioxidant
56	Cystone C	*C. usneoides*[j]	132	Antioxidant
57	Cystone D	*C. usneoides*[j]	132	Antioxidant
58	Cystone E	*C. usneoides*[j]	132	Antioxidant
59	Cystone F	*C. usneoides*[j]	132	Antioxidant
60	Cystomexicone A	*C. abies-marina*[j]	165	Antioxidant
		C. usneoides[h]	133	
61	Cystomexicone B	*C. abies-marina* [j]	165	Antioxidant
		C. usneoides[h]	133	
62	Usneoidone E	*C. usneoides*[h,j]	133,132	Anti-inflammatory, antioxidant
63	Amentadione-1′-methyl ether	*C. usneoides*[h,j]	133,132	Antioxidant
64	Usneoidone Z	*C. usneoides*[h]	133	Anti-inflammatory, antioxidant
65	6-*cis*-amentadione-1′-methyl ether	*C. usneoides*[h]	133	Antioxidant
66	(3R)-tetraprenyltoluquinone	*C. baccata*[i]	85	Antileishmania
67	(3S)-tetraprenyltoluquinone	*C. baccata*[i]	85	Antileishmania
68	(3R)-tetraprenyltoluquinol	*C. baccata*[i]	85	Antileishmania
69	(3S)-tetraprenyltoluquinol	*C. baccata*[i]	85	Antileishmania
Steroids				
70	Ergost-5-en-3β-ol	*C. adriatica*[g]	248	-
71	Stigmast-5-en-3β-ol	*C. adriatica*[g]	248	-
72	22-dehydrocholesterol	*C. adriatica*[g]	248	-
73	Androst-5-en-3-ol	*C. crinita*[l]	245	-
74	Chol-5-en-3β-ol	*C. crinita*[l]	245	-
75	23,24-dinor-chol-5-en-3β-ol	*C. crinita*[lz]	245	-
76	23,24-dinor-chol-5,20-dien-3β-ol	*C. crinita*[l]	245	-
77	24-isopropylcholesta-5,22-dien-3β-ol	*C. crinita*[l]	245	-
78	24-ethylcholesterol	*C. crinita*[l]	245	-
79	Isofucosterol	*C. crinita*[l]	245	-
80	Stigmasterol	*C. crinita*[a]	336	Anti-cancer[259], antiviral[372,]
		C. adriatica[g]	248	anti-atherosclerosis[284],
				anti-osteoarthritic[173]
81	Saringasterol	*C. adriatica*[g]	248	-
		C. barbata[a]	336	
		C. crinita[a]		
82	Fucosterol	*C. adriatica*[g]	248	Antioxidant[24,281], cholinesterase
		C. barbata[a]	336	inhibitor[24,529], anti-diabetic[24,240,282], anti-
		C. compressa [d,k]	335	cancer[237], anti-obesity[242], anti-
		C. crinita[a,l]	24,245	inflammatory[239], anti-atopic[228], anti-
		C. nodicaulis[i]	24	photohaging[227,257], anti-osteoporotic[39],
		C. tamariscifolia [i]		hepatoprotective[216], ACE inhibitor[205],
		C. usneoides[i]		antifungal[268], cholesterol reducer[216],
				antileishmanial[39]
83	Chalinasterol	*C. adriatica*[g]	248	-
84	Brassicasterol	*C. crinita*[l]	245	-
85	24-nor-chol-5-en-3β-ol	*C. crinita*[l]	245	-
86	24-nor-chol-5,22-dien-3β-ol	*C. crinita*[l]	245	-
87	Pregn-5-en-3-ol	*C. crinita*[l]	245	-
88	Saoussazine	*C. compressa*[k]	335	-
89	3-keto-22-epi-28-nor-cathasterone	*C. myrica*[b]	207	Cytotoxic
90	Cholest-4-ene-3,6-di-one	*C. myrica*[b]	207	Cytotoxic
Phlorotannins and Phenolic Compounds				
91	7-phloroethol	*C. humilis*[c]	456	Antioxidant, HAase inhibitor
		C. tamariscifolia [i]	164	
92	Fucophloroethol	*C. baccata*[c]	456	Antioxidant, HAase inhibitor
		C. usneoides[i]	164	
93	Fucodiphloroethol	*C. tamariscifolia* [i]	164	Antioxidant, HAase inhibitor
94	Fucotriphloroethol	*C. usneoides*[i]	164	Antioxidant, HAase inhibitor

No	Compound class and name	Species (Origin)	Reference	Biological activity
95	Phloroglucinol	*C. baccata*[c]	456	Antioxidant[24,108,246] anti-diabetic[24348],
		C. compressa[j]	108	cholinesterase inhibitor[24]
		C. foeniculacea[j,c]	108,804	
		C. humilis[c]	56	
		C. nodicaulis[i]	33,456	
		C. tamariscifolia[i,c]	24	
		C. usneoides[i]	24,456	
			24	
96	Benzoic acid	*C. abies-marina*[i]	191	Antioxidant[191], anti-inflammatory[191],
		C. crinita[l]	245	antifungal[226]
97	Phenol-2,4-bis-(1,1-dimethylethyl)	*C. barbata*[l]	364	Anti-quorum sensing[365], anti-biofilm[365]
98	Ketone	*C. abies-marina*[j]	162	-
Carbohydrates				
99	Mannitol	*C. nodicaulis*[i]	24	Antioxidant, anti-diabetic,
		C. tamariscifolia[i]		cholinesterase inhibitor
		C. usneoides[i]		
100	Fucoidans	*C. compressa*[k]	22	Anti-inflammatory, oedema inhibition,
		C. crinita[k]	22	gastroprotective, antioxidant,
		C. sedoides[k]	22	anti-allergic[280], anti-cancer[280]
101	Uronic acid	*C. compressa*[k]	22	Anti-herpetic[303]
		C. crinita[k]	22	
		C. sedoides[k]	22	
		C. indica[e]	303	
102	Xylose	*C. indica*[e]	303	Anti-herpetic
103	Mannose	*C. indica*[e]	303	Anti-herpetic
104	Fucose	*C. indica*[e]	303	Anti-herpetic
105	Galactose	*C. indica*[e]	303	Anti-herpetic
106	Glucose	*C. indica*[e]	303	Anti-herpetic
Triacylglycerols[*]				
107	C14:0/C16:1/C16:1	*C. brachycarpa*[f]	382	-
108	C16:1/C16:1/C16:1	*C. brachycarpa*[f]	382	-
109	C18:1/C18:1/C18:1	*C. brachycarpa*[f]	382	-
110	C16:0/C18:1/C18:10	*C. brachycarpa*[f]	382	-
111	C18:1/C18:2/C16:0	*C. brachycarpa*[f]	382	-
112	C16:0/C16:0/C20:4	*C. brachycarpa*[f]	382	-
113	C16:0/C16:0/C20:5, ω-3	*C. brachycarpa*[f]	382	-
114	C16:0/C18:4, ω-3/C20:5, ω-3	*C. brachycarpa*[f]	382	-
115	C14:0/C16:1/C16:0	*C. brachycarpa*[f]	382	-
116	C16:0/C16:1/C16:1	*C. brachycarpa*[f]	382	-
117	C16:0/C16:0/C18:1	*C. brachycarpa*[f]	382	-
118	C14:0/C14:0/C16:0	*C. brachycarpa*[f]	382	-
119	C14:0/C16:0/C16:0	*C. brachycarpa*[f]	382	-
120	C16:0/C16:0/C16:0	*C. brachycarpa*[f]	382	-
121	C16:0/C16:0/C18:0	*C. brachycarpa*[f]	382	-
122	C16:0/C18:0/C18:0	*C. brachycarpa*[f]	382	-
123	C16:0/C18:1/C18:0	*C. brachycarpa*[f]	382	-
124	C14:0/C16:0/C18:1	*C. brachycarpa*[f]	382	-
125	C14:0/C18:1/C18:1	*C. brachycarpa*[f]	382	-
Saturated Fatty Acid (SFA)				
126	Caproic acid (C6:0)	*C. barbata*[a]	366	-
127	Pelargonic acid (C9:0)	*C. crinita*[l]	245	-
128	Capric acid (C10:0)	*C. crinita*[l]	245	-
		C. barbata[a]	366	
129	Lauric acid (C12:0)	*C. barbata*[a]	366	-
		C. humilis[i]	504	
130	Myristic acid (C14:0)	*C. baccata*[i]	504	-
		C. barbata[a,i]	366,	
		C. brachycarpa[f]	504,382	
		C. compressa[d,i]	247,504	

No	Compound class and name	Species (Origin)	Reference	Biological activity
		C. crinita[a,l]	233,245	
		C. humilis[i]	504	
		C. nodicaulis[i]	504	
		C. tamariscifolia[i]	504	
131	Pentadecyclic acid (C15:0)	*C. baccata*[i]	504	-
		C. barbata[i]	504	
		C. compressa[i]	504	
		C. crinita[l]	245	
		C. humilis[i]	504	
		C. nodicaulis[i]	504	
		C. tamariscifolia[i]	504	
132	Palmitic acid (C16:0)	*C. baccata*[i]	504	Antifungal[241], antiviral[279], anti-bacterial[528]
		C. barbata[a,i]	366,	
		C. brachycarpa[f]	504,	
		C. compressa[d,i]	382	
		C. crinita[a,l]	247,504	
		C. humilis[i]	233,245	
		C. nodicaulis[i]	504	
		C. tamariscifolia[i]	24,504	
		C. usneoides[i]	24,504	
			24	
133	Margaric acid (C17:0)	*C. baccata*[i]	504	-
		C. barbata[a,i]	366,	
		C. brachycarpa[f]	504,382	
		C. humilis[i]	504	
		C. nodicaulis[i]	504	
		C. tamariscifolia[i]	504	
134	Stearic acid (C18:0)	*C. baccata*[i]	504	Anti-diabetic, cholinesterase inhibitor
		C. barbata[a,i]	366,504	
		C. brachycarpa[f]	382	
		C. compressa[d,i]	247,504	
		C. crinita[a,l]	233,245	
		C. humilis[i]	504	
		C. nodicaulis[i]	504	
		C. crinita[i]	24,504	
		C. usneoides[i]	24	
135	Arachidic acid (C20:0)	*C. baccata*[i]	504	-
		C. barbata[a]	366,504	
		C. compressa[i]	504	
		C. crinita[a]	233	
		C. nodicaulis[i]	504	
		C. tamariscifolia[i]	504	
136	Heneicosylic acid (C21:0)	*C. barbata*[a]	366	-
137	Behenic acid (C22:0)	*C. baccata*[i]	504	-
		C. barbata[a,i]	366,504	
		C. compressa[i]	504	
		C. nodicaulis[i]	504	
		C. tamariscifolia[i]	504	
138	Tricosylic acid (C23:0)	*C. barbata*[a]	366	-
139	Lignoceric acid (C24:0)	*C. baccata*[i]	504	-
		C. barbata[a,i]	366,504	
		C. compressa[i]	504	
		C. tamariscifolia[i]	504	
Monounsaturated Fatty Acids (MUFA)				
140	Myristoleic acid (C14:1)	*C. barbata*[a]	366	-
141	Palmitoleic acid (C16:1)	*C. baccata*[i]	504	-
		C. barbata[a,i]	366,504	
		C. brachycarpa[f]	382	
		C. compressa[d,i]	247,504	

No	Compound class and name	Species (Origin)	Reference	Biological activity
		C. crinita[a,l]	233,245	
		C. humilis[i]	504	
		C. nodicaulis[i]	504	
		C. tamariscifolia[i]	504	
142	Oleic acid (C18:1)	*C. baccata*[i]	504	Anti-inflammatory[188], cancer preventive[188,] anti-androgenic[188], anti-diabetic[340], cholinesterase inhibitor[391], antifungal[501], anti-bacterial[105], antioxidant[510]
		C. barbata[a,i]	366,504	
		C. brachycarpa[f]	382	
		C. compressa[d,i]	247,504	
		C. crinita[a,l]	245,24	
		C. humilis[i]	24	
		C. nodicaulis[i]	504	
		C. tamariscifolia[i]	504	
			504	
143	*cis*-10-heptadecenoic acid (C17:1)	*C. barbata*[a]	366	-
144	Eicosenoic acid (C20:1)	*C. crinita*[l]	245	-
		C. humilis[i]	504	
		C. nodicaulis[i]	504	
		C. crinita[i]	504	
145	Erucic acid (C22:1)	*C. brachycarpa*[f]	382	-
Polyunsaturated Fatty Acids (PUFA)				
146	Hexadecatrienoic acid (C16:3)	*C. crinita*[l]	245	-
		C. tamariscifolia[i]	504	
147	Hexadeca-4,7,10,13-tetraenoic acid (C16:4)	*C. crinita*[1]	245	-
148	Linoleic acid (C18:2)	*C. baccata*[i]	504	Anti-cancer[440]
		C. barbata[a,i]	366,504	
		C. brachycarpa[f]	382	
		C. compressa[d,i]	247,504	
		C. crinita[a,l]	233,245	
		C. humilis[i]	504	
		C. nodicaulis[i]	504	
		C. tamariscifolia[i]	504	
149	α-Linolenic acid (C18:3, n-3)	*C. barbata*[a]	366	Anti-inflammatory[223], anti-bacterial[223]
		C. crinita[l]	233,245	
150	γ-Linolenic acid (C18:3, n-6)	*C. crinita*[l]	233	Anti-inflammatory[249,250], endocrine precursor[398], skin protective[250], anti-rheumatoid arthritis[530], anti-multiple sclerosis[327], schizophrenia preventive[493], premenstrual syndrome preventive[398], infant diet supplement[504]
		C. compressa[i]	504	
		C. tamariscifolia[i]	504	
151	Stearidonic acid (C18:4)	*C. brachycarpa*[f]	382	-
		C. compressa[d]	247	
		C. crinita[l]	245	
152	Eicosadienoic acid (C20:2)	*C. baccata*[i]	504	-
		C. barbata[a,i]	371,504	
		C. compressa[i]	504	
		C. nodicaulis[i]	504	
		C. tamariscifolia[i]	504	
153	Eicosatrienoic acid (C20:3)	*C. baccata*[i]	504	-
		C. barbata[i]	504	
		C. compressa[i]	504	
		C. humilis[i]	504	
		C. nodicaulis[i]	504	
		C. tamariscifolia[i]	504	
154	Eicosatetraenoic acid (C20:4)	*C. brachycarpa*[f]	382	-
155	Arachidonic acid (C20:4)	*C. baccata*[i]	504	Cholinesterase inhibitor[391], endocrine precursor[381], lipoprotein precursor[508], hemorheologic agent[508], diet supplement[508], cardioprotective[508]
		C. barbata[i]	504	
		C. brachycarpa[f]	382	
		C. compressa[i]	504	

No	Compound class and name	Species (Origin)	Reference	Biological activity
		C. crinita[a]	233	
		C. humilis[i]	504	
		C. nodicaulis[i]	504	
		C. tamariscifolia[i]	504	
156	Eicosapentaenoic acid (C20:5)	*C. baccata*[i]	508	Cholinesterase inhibitor[391], anti-
		C. barbata[a,i]	366,	inflammatory[431], anti-cancer[276],
		C. compressa[i]	508,504	immunomodulatory[431], endocrine
		C. humilis[i]	504	precursor[381], cardioprotective[276], anti-
		C. nodicaulis[i]	504	atherosclerosis[79], hyperlipidemia
		C. crinita[a]	233	preventive[270], schizophrenia preventive[152],
		C. brachycarpa[f]	382	diet supplement[508], anti-bacterial[223],
		C. tamariscifolia[i]	504	antileishmanial[500]
157	Docosapentaenoic acid (C22:5)	*C. crinita*[l]	245	-
158	Docosahexaenoic acid (C22:6)	*C. barbata*[a]	366	Cardioprotective[276], anti-cancer[276], diet supplement[508], food additive[508], anti-bacterial[223]
Pigments and Vitamins				
159	β-carotene	*C. barbata*[a]	366	Anti-inflammatory[33], antioxidant[354], antiproliferative[231]
160	Astaxanthin	*C. barbata*[a]	366	Antioxidant[356,33], antitumoral[476], anti-inflammatory[33,215], cataract protective[203], cardioprotective[155], Immunomodulatory[287], anti-bacterial[507,269], hepatoprotective[107]
161	Fucoxanthin	*C. brachycarpa*[f]	382	Anti-inflammatory[256], antioxidant[411], anti-cancer[218,231,256,264,265,351], anti-obesity[313,339], anti-diabetic[339], anti-angiogenic[458], antimalarial[1], hepatoprotective[522], ocular-protective[439], skin-protective[490,437]
162	Chlorophyll *a*	*C. brachycarpa*[f]	382	Antioxidant[221]
163	α-tocopherol	*C. barbata*[a]	366	Antioxidant[258,366], cellular signaling[81,530], gene expression regulator[81,530], antigenotoxic[397]
164	Retinol	*C. barbata*[a]	366	Anti-aging[263]
165	Ergocalciferol	*C. barbata*[a]	366	Citotoxic[106], endocrine regulator[485], Ca/P homeostatic[413]
Others				
166	3-bromo-2-chloro-1-propanol	*C. barbata*[a]	336	-
167	1-bromo-2-chloroethane	*C. barbata*[a]	336	-
168	1,1,2-trichloroethane	*C. barbata*[a]	336	-
169	1,1,2,2,-tetrachloroethane	*C. barbata*[a]	336	-
170	Hexachlorobutadiene	*C. barbata*[a]	336	-
171	Dimethylformamide	*C. barbata*[a]	336	-
172	Heptane	*C. barbata*[l]	364	-
173	Octane	*C. barbata*[l]	364	-
174	Undecane	*C. barbata*[l]	364	-
175	Tridecane	*C. barbata*[l]	364	-
176	Tetradecane	*C. barbata*[l]	364	-
177	Pentadecane	*C. barbata*[l]	364	-
178	Hexadecane	*C. crinita*[l]	245	Anti-inflammatory[371], , thermogenic regulator[225]
179	Heptadecane	*C. crinita*[l]	245	
180	Octadecane	*C. crinita*[l]	245	-
181	Nonadecane	*C. crinita*[l]	245	-
182	Eicosane	*C. barbata*[l]	364	-
183	Heneicosane	*C. barbata*[l]	364	-
184	Docosane	*C. barbata*[l]	364	-
185	Tricosane	*C. barbata*[l]	364	-

No	Compound class and name	Species (Origin)	Reference	Biological activity
186	Tetracosane	*C. barbata*[l]	364	-
187	Pentacosane	*C. barbata*[l]	364	-
188	Hexacosane	*C. barbata*[l]	364	-
189	Heptacosane	*C. barbata*[l]	364	-
190	Octacosane	*C. barbata*[l]	364	-
191	Nonacosane	*C. barbata*[l]	364	-
192	Triacontane	*C. barbata*[l]	364	-
193	Tritriacontane	*C. barbata*[l]	364	-
194	Tetratriacontane	*C. barbata*[l]	364	-
195	Pentatriacontane	*C. barbata*[l]	364	-
196	1-octene	*C. barbata*[l]	364	-
197	1-heptadecanamin	*C. barbata*[l]	364	Anti-bacterial[459]
198	2,4-dimethyl-1-heptene	*C. barbata*[l]	364	-
199	Chloroacetic acid	*C. crinita*[l]	245	-
200	Chloroacetic acid, ethyl ester	*C. crinita*[l]	245	-
201	1-chloro-2-ethoxy-2-methoxyethane	*C. crinita*[l]	245	-
202	1-chloro-2,2-diethoxyethane	*C. crinita*[l]	245	-
203	1,1-dichloro-2,2-diethoxyethane	*C. crinita*[l]	245	-
204	Dimethyl disulfide	*C. crinita*[l]	245	-
205	Thioacetic acid-O-ethyl ester	*C. crinita*[l]	245	-
206	1,3-butanediol	*C. crinita*[l]	245	-
207	2,3-butanediol	*C. crinita*[l]	245	-
208	Glycerol	*C. crinita*[l]	245	-
209	2-hydroxypropanoic acid	*C. crinita*[l]	245	-
210	4-hydroxypentanoic acid	*C. crinita*[l]	245	-
211	2,3-dihydroxy palmitic acid, propyl ester	*C. crinita*[l]	245	-
212	2-ethylhexylphtalate	*C. compressa*[k]	335	-
213	Cinnamic acid	*C. crinita*[l]	245	-
214	Proline	*C. nodicaulis*[i]	24	Anti-diabetic, cholinesterase inhibitor
		C. tamariscifolia[i]	24	
		C. usneoides[i]	24	

*indicates number of carbons in side chain and presence of unsaturation to each original fatty acid from triacylglycerol. [a] Bulgaria; [b] Egypt; [c] France; [d] Greece; [e] India; [f] Italy; [g] Montenegro; [h] Morocco; [i] Portugal; [j] Spain; [k] Tunisia; [l] Turkey; HAase - hyaluronidase; and ACE - angiotensin-converting enzyme

2.3.1. TERPENES

The terpenoids cystoseirol monoacetate (**1**), dictyol F monoacetate (**2**), dictyone (**3**), dictyone acetate (**4**), isodictytriol monoacetate (**5**), and pachydictyol (**6**) were identified in *Cystoseira myrica* from Egypt. These compounds were tested against three different mouse cell lines (fibroblast NIH3T3 and the cancer cell lines SSVNIH3T3 and KA3IT), and displayed moderate cytotoxic activity against the KA3IT (IC_{50} 5 µg/ml) and reduced cytotoxicity towards the normal cells NIH3T3 (Ayyad et al., 2003). Structures of compounds **1-11** are presented in **Figure 2.1.**.

Other terpenoids, such as 3,7-dimethyl-1,6-octadiene-3-ol-2-aminobenzoate (**7**), hexahydrofarnesylacetone (**8**), dihydro-3-hydroxy-3-hydroxymethyl-2(3H)-furanone (**9**) and dihydroactinidiolide (**10**) were identified in *Cystoseira crinita* from Bulgaria (Milkova et al., 1997), and Turkey (Kamenaska et al., 2002). However, no biological activity was reported to compounds **7-10.** Furthermore, Isololiolide (**11**)**,** isolated from *C. tamariscifolia*, proved to be

cytotoxic against gastric cancer cells and selectively cytotoxic on human hepatocellular

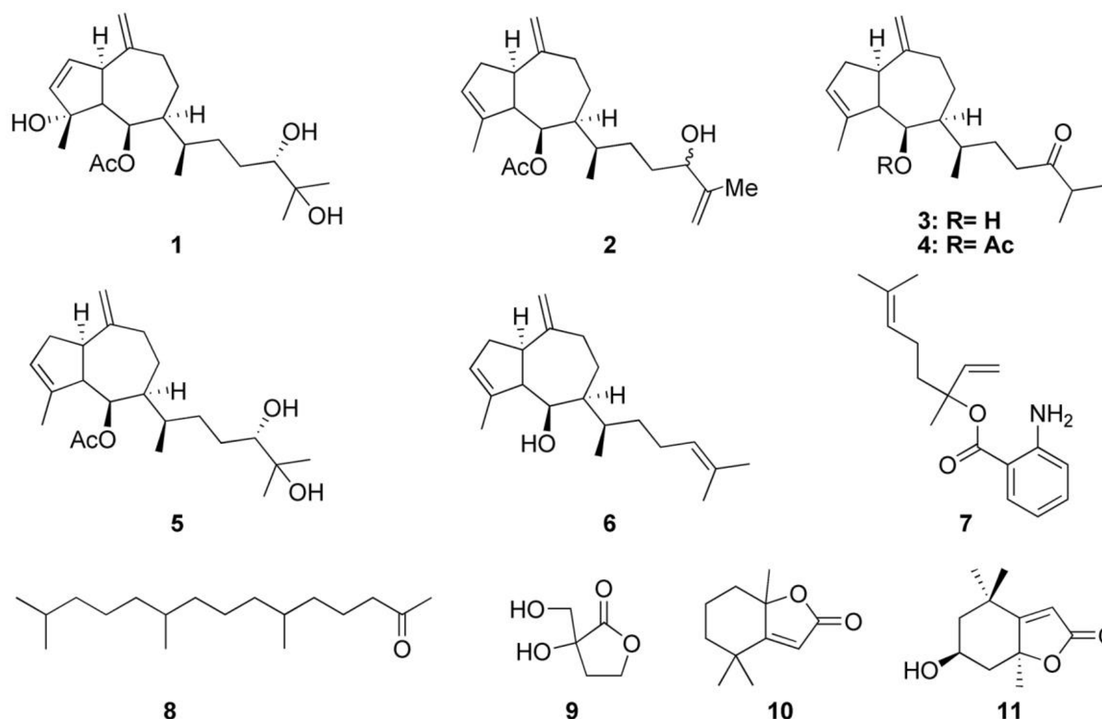

Figure 2.1. Structures of terpenoids **1-11** isolated from *Cystoseira* algae

carcinoma cells comparing with non-tumoral human fibroblasts (Vizetto-Duarte et al., 2016b).

2.3.2. MEROTERPENOIDS

The merediterpenoids 14-epi-amentol triacetate (**12**), amentol (**13**), amentol chromane diacetate (**14**), cystoseirone diacetate (**15**) and preamentol triacetate (**16**) were isolated from a *Cystoseira* sp. specimen nearby the Spanish Canary Islands (Navarro et al., 2004). Demethoxy cystoketal chromane (**17**), was found in *C. tamariscifolia* and *C. amentacea* var. *stricta* from Portugal and France respectively (Valls et al., 1996; Vizetto-Duarte et al., 2016a), revealing antiproliferative activity against hepatocellular carcinoma cells (Vizetto-Duarte et al., 2016a). Cystoazorol A (**18**), cystoazorol B (**19**), together with the meronorsesquiterpenoids cystoazorone A (20), cystoazorone B (21) were found for the first time in *C. abies-marina* algae collected in the São Miguel Island, Azores - Portugal (Gouveia et al., 2013b). Compounds **18**, **20** and **21** exhibited inhibitory activity against HeLa cells and **18** and **19** moderate antioxidant DPPH radical scavenging activity (Gouveia et al., 2013b). Structures of compounds **12-21** are presented in **Figure 2.2.**.

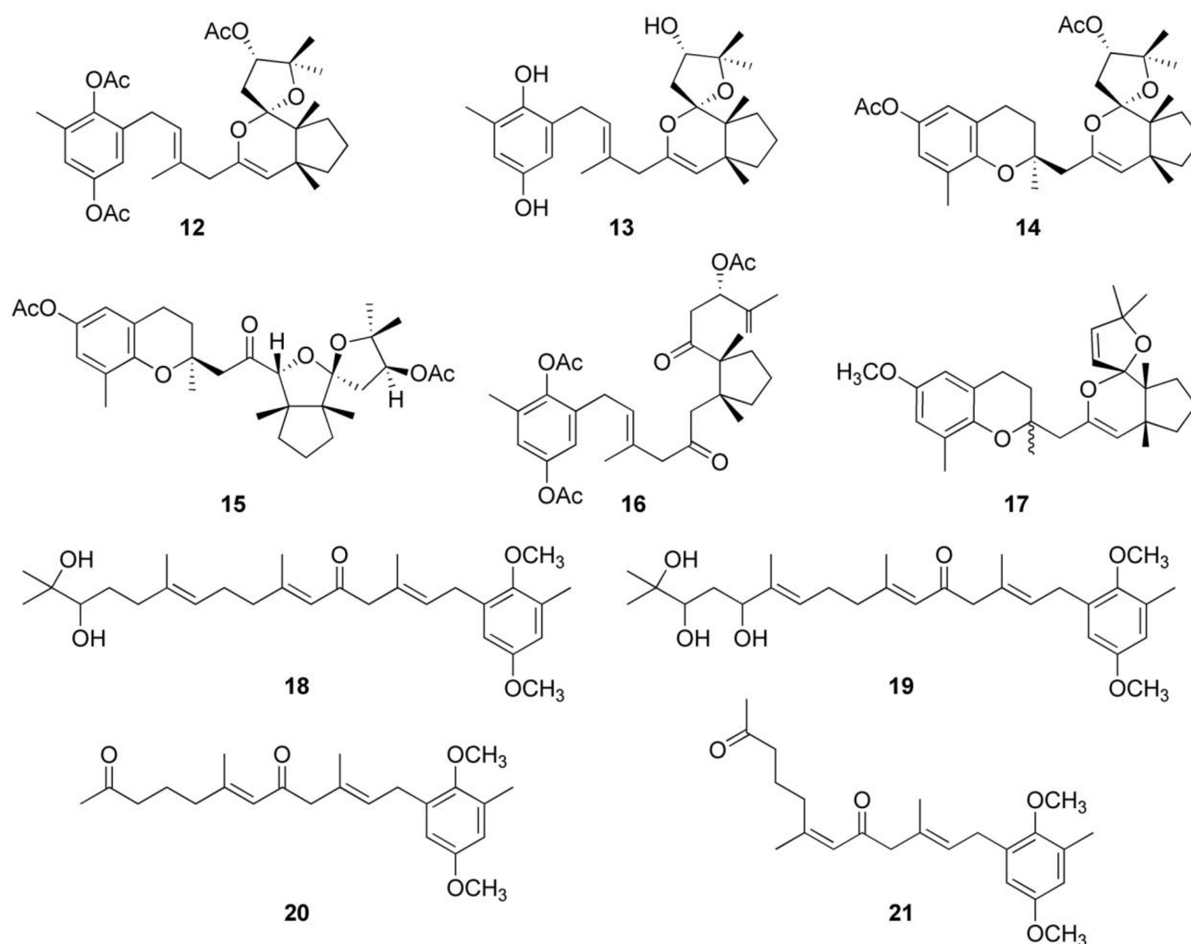

Figure 2.2. Structures of meroterpenoids **12-21** isolated from *Cystoseira* algae

Tetraprenyltoluquinol derivatives **22-27**, triprenyltoluquinol derivatives **28-29**, and tetraprenyltoluquinone derivatives **30-31** were isolated for the first time in the brown alga *C. crinita* (Fisch et al., 2003). Antioxidant properties of these compounds were evaluated by different methods revealing that hydroquinones have powerful antioxidant activity comparable to that of α –tocopherol. Structures of compounds **22-31** are showed in **Figure 2.3.**.

Related compounds 2,12-diepineobalearone (**32**), 4'-methoxy-(2E)-bifurcarenone (**33**) and the methoxybifurcarenone **34**, were isolated from *C. amentacea* var *stricta* specimens collected on France and Tunisia. Compound **34** was also isolated in *C. tamariscifolia* (Bennamara et al., 1999) and inhibited the development of the fertilized eggs of the common sea-urchin *Paracentrotus lioidus* (Mesguiche et al., 1997). Furthermore, this compound displayed antifungal activity against three tomato pathogenic fungi (*Botrytis cinerea*, *Fusarium oxysporum* sp. *mycopersici* and *Verticillium alboatrum*) and anti-bacterial activity against *Agrobacterium tumefaciens* and *Escherichia coli* (Bennamara et al., 1999).

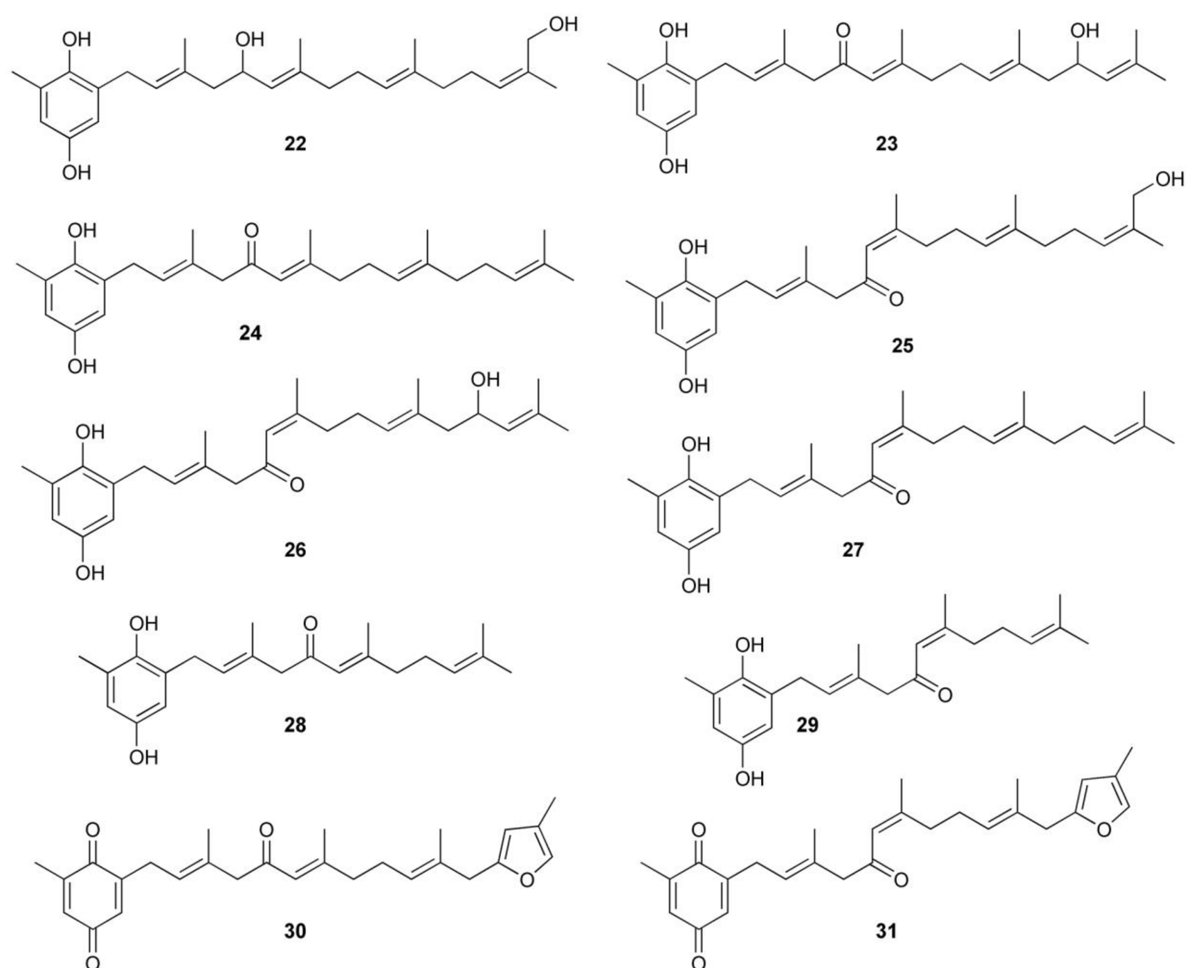

Figure 2.3. Structures of meroterpenoids **22-31** isolated from *Cystoseira* algae

Seven meroditerpenoids (**35-38**) and their derivatives (**39-41**) were found in the brown alga *C. baccata* harvested in Moroccan Atlantic coast. Compounds **38**, **39** and **41** were not toxic against larvae of sea urchins and oysters, and to possess interesting antifouling activities (inhibition of microalgae growth, macroalgal settlement, and mussel phenoloxidase activity; Mokrini et al., 2008). Eighteen new meroterpenoids, cystodiones A-M (**42-53**) and cystones A-F (**54-59**) were isolated for the first time in the *C. usneoides* collected in the Moroccan and Spanish coasts. All these compounds and other already known meroterpoids (**60-65**) reveal radical-scavenging activity, although **42**, **43**, **48**, **49**, **63** and **65** showed strong radical-scavenging activity. Moreover, inhibition of the production of the proinflammatory cytokine TNF-α in LPS-stimulated THP-1 human macrophages and anti-inflammatory activity were also observed in compounds **48**, **56**, **62** and **64** (de los Reyes et al., 2013, 2016).

32 33 34

35 36 37

38 39 40 41

42 43

44 45

46 47

48 49

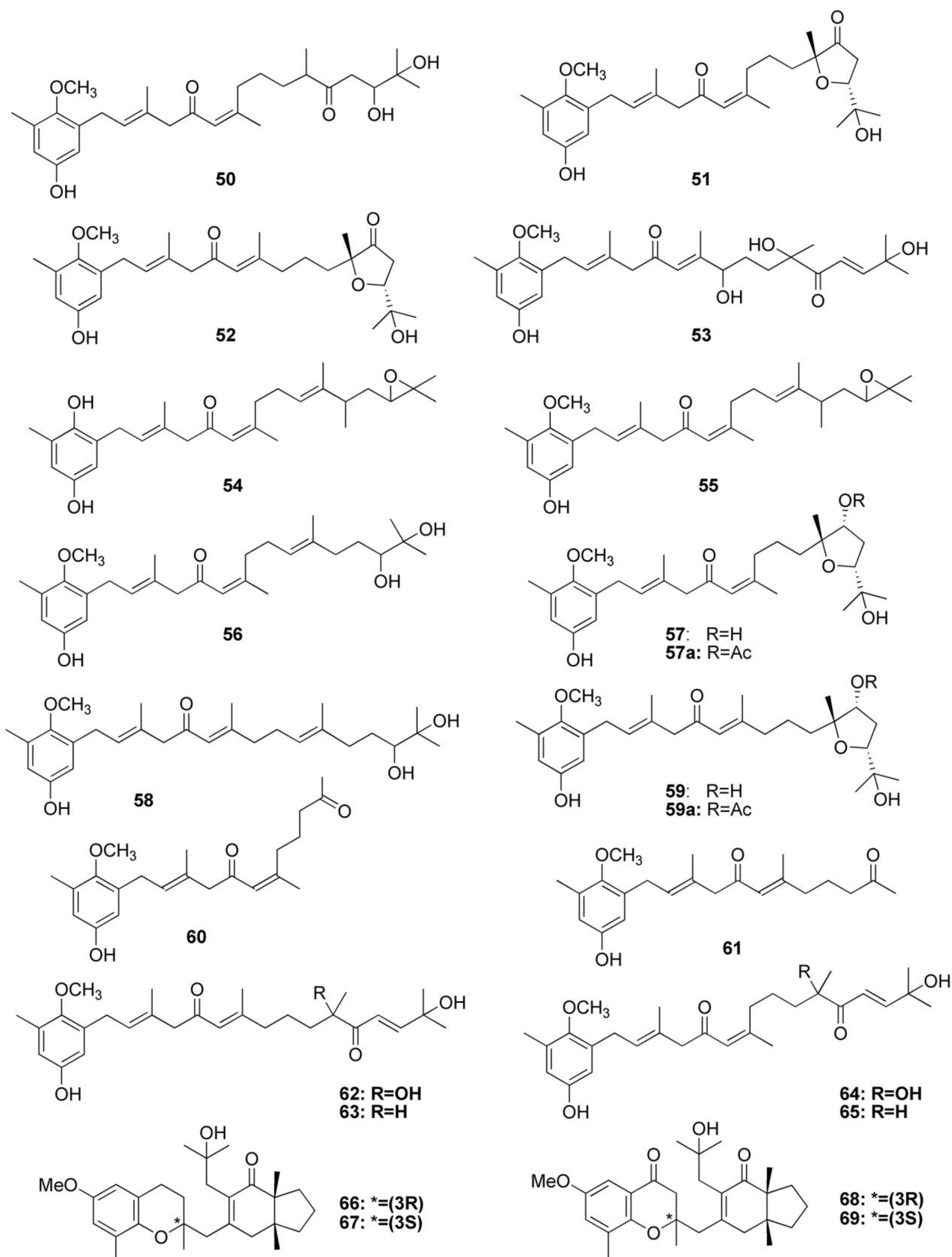

Figure 2.4. Structures of meroterpenoids **32**-**69** isolated from Cystoseira.

The novel (3R)- and (3S)- tetraprenyltoluquinones (**66** and **67**) together with the already known merediterpenoid (3R)- and (3S)-tetraprenyltoluquinols (**68** and **69**), were isolated from

the hexane extract of *C. baccata*. These were the first compounds ever isolated from algae revealing activity against *Leishmania* parasites inducing cytoplasmic vacuolization and disruption of the mitochondrial membrane potential. The activity of this meroditerpenoids was evaluated against *L. infantum* promastigotes and amastigotes, being the compounds **68/69** more effective (IC_{50} = 25.0 ± 4.1 mM) in the inhibition of the intracellular infection (Bruno de Sousa et al., 2017). Structures of compounds **32–69** are showed in **Figure 2.4.**.

2.3.3. STEROLS AND STEROIDS

Several steroids (**70-88**) were identified from different *Cystoseira* species. Cholesterol (**70**) initially isolated in *C. compressa* (Kraan, 2012) was also found in *C. adriatica* (Kapetanović et al., 2005). Cholesterol derivatives **70-72** were isolated from *C. adriatica* (Kapetanović et al., 2005), and several others i.e., androst-5-en-3-ol (**73**), chol-5-en-3β-ol (**74**), 23,24-dinor-chol-5-en-3β-ol (**75**), 23,24-dinor-chol-5,20-dien-3β-ol (**76**), 24-isopropylcholesta-5,22-dien-3β-ol (**77**), 24-ethylcholesterol (**78**), isofucosterol (**79**) and stigmasterol (**80**) were reported from *C. crinita* (Milkova et al., 1997; Kamenarska et al., 2002) although no information about the bioactivity of these compounds was described. Compound **80**, simultaneously identified in *C. adriatica* (Kapetanović et al., 2005), displays anti-atherosclerosis, antiviral, anti-cancer and antiosteoarthritic activities (Li et al., 2015; Petrera et al., 2014; Kim et al., 2014; Gabay et al., 2010). The presence of saringasterol (**81**) was also confirmed in more than one *Cystoseira* species from the Adriatic (Kapetanović et al., 2005) and the Black sea (Milkova et al., 1997) regions. Fucosterol (**82**), the characteristic steroid of brown algae, identified for the first time in *C. compressa* (Kanias et al., 1992), was also detected in *C. barbata*, *C. crinita*, *C. nodicaulis*, *C. tamariscifolia*, *C. usneoides* (Milkova et al., 1997; Kamenarska et al., 2002; Mighri et al., 2009; Andrade et al., 2013) and *C. adriatica*, although in the last species this compound was detected at low concentration (Kapetanović et al., 2005). Several activities are reported for compound **82** such as radical scavenging, antioxidant, acetylcholinesterase AChE, BuChE, anti-diabetic, anti-cancer, anti-obesity, anti-inflammatory, anti-atopic, anti-photohaging, anti-osteoporotic, hepatoprotective, angiotensin-converting enzyme (ACE) inhibitors, cholesterol reducer, antifungal and antileishmanial (Hagiwara et al., 1986; Lee et al., 2003, 2004; Yoon et al, 2008; Kumar et al., 2010; Bang et al., 2011; Hoang, et al., 2012; Andrade et al., 2013; Jung et al., 2013a,b, 2014; Hwang, et al., 2014a,b; Ji et al., 2014; Becerra et al., 2015). 24-methylcholesta-5,24(28)-dien-3β-ol (**83**) usually known as chalinasterol was isolated from *C. adriatica* (Kapetanović et al., 2005) and brassicasterol (**84**), 24-nor-chol-5-en-3β-ol (**85**), 24-nor-chol-5,22-dien-3β-ol (**86**),

and pregn-5-en-3-ol (**87**) from *C. crinita* harvested in Turkey (Kamenarska et al., 2002). Saoussazine (**88**) was isolated in a specimen of *C. compressa* from Tunisia (Mighri et al., 2009).

Other steroids such as 3-keto-22-epi-28-nor-cathasterone (**89**) and cholest-4-ene-3,6-di-one (**90**) were identified in *C. myrica* collected in the Egyptian coast (Hamdy et al., 2009). These compounds showed activity against human liver and colon cancer cells, with compound **89** exhibiting a particularly higher cytotoxicity to liver cancer cells with selective activity for normal cells. Structures of steroids **70-90** are showed in **Figure 2.5.**.

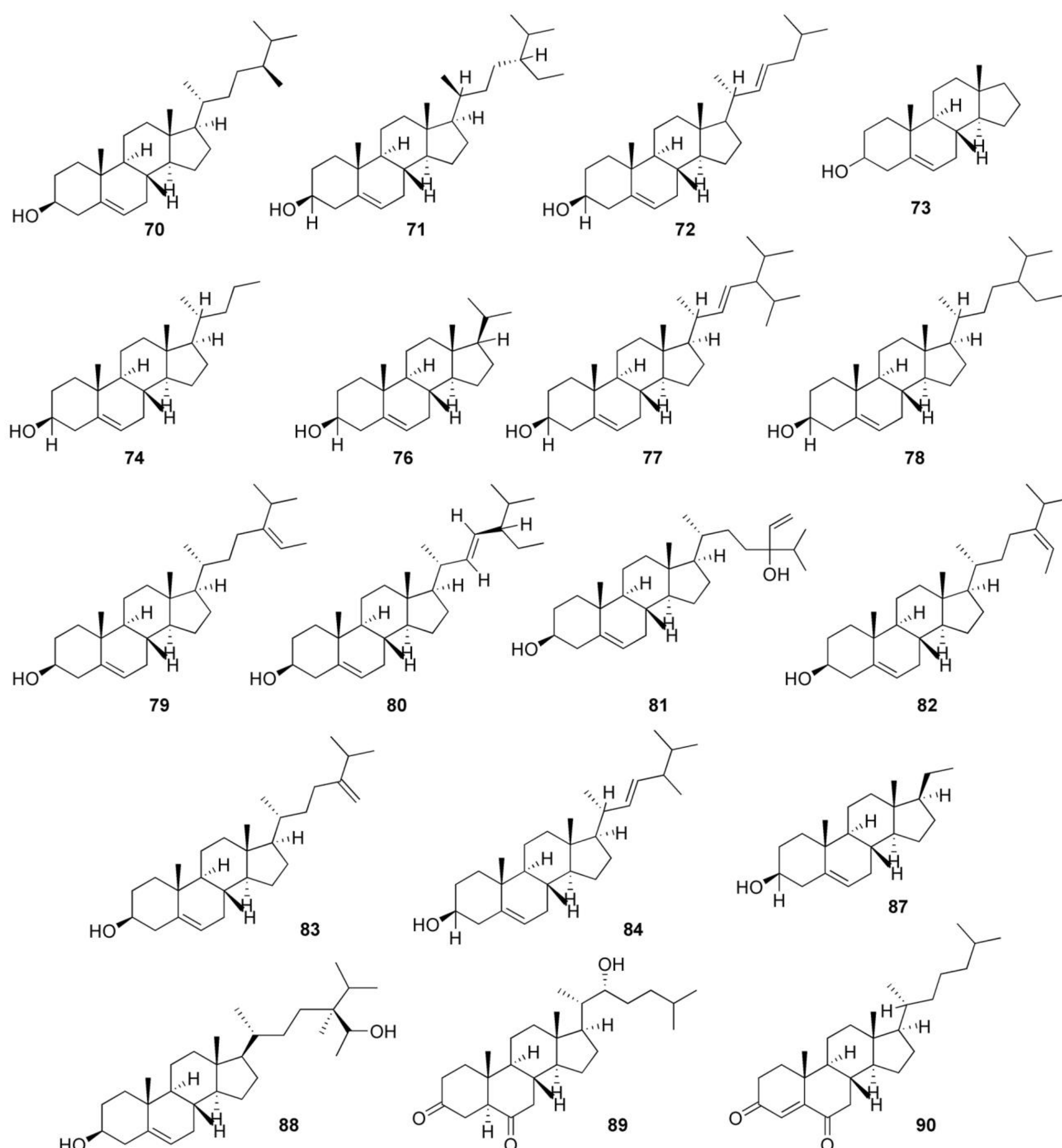

Figure 2.5. Structures of steroids **70-90** isolated from *Cystoseira* algae

2.3.4. PHLOROTANNINS AND PHENOLIC COMPOUNDS

The study of three *Cystoseira* species (*C. nodicaulis*, *C. tamariscifolia* and *C. usneoides*) by HPLC-DAD-ESI-MS^n, reported the occurrence of different phlorotannins belonging to eckol and fucophloroethol groups. Four of these phlorotannins, 7-phloroethol (**91**), fucophloroethol (**92**), fucodiphloroethol (**93**) and fucotriphloroethol (**94**) were identified in this species for the first time, revealing hyaluronidase inhibitory activity and radical scavenging (superoxide radical scavenging assay, as well as lipid peroxidation inhibition assay (Ferreres et al., 2012). Other study demonstrated that *C. humilis* produce compound **91** and *C. baccata* and *C. nodicaulis* present traces of fucols, phlorethols and fucophlorethols (Stiger-Pouvreau et al., 2014). The structures of compounds **91-99** are show in **Figure 2.6.**.

Lopes et al. (2012, 2013) evaluated, for the first time, the anti-bacterial and antifungal properties of phlorotannins rich extracts of *C. nodicaulis*, *C. tamariscifolia* and *C. usneoides*. The authors demonstrated that these algae are active against different species of the yeast *Candida* sp. (*C. albicans*, *C. dubliniensis, C. krusei*, *C. parapsilosis*) and the dermatophyte fungus *Tricophyton rubrum, T. mentagrophytes, Microsporum gypseum, M. canis,* and *Epidermophyton floccosum*. Otherwise, no effectiveness of these crude extracts was found against *Aspergillus* (*A. fumigatus*, *A. flavus* and *A. niger*). Antifungal activity of *C. nodicaulis* against *Candida* cells and of *C. usneoides* against dermatophyte fungi is related with a significantly reduction of the ergosterol

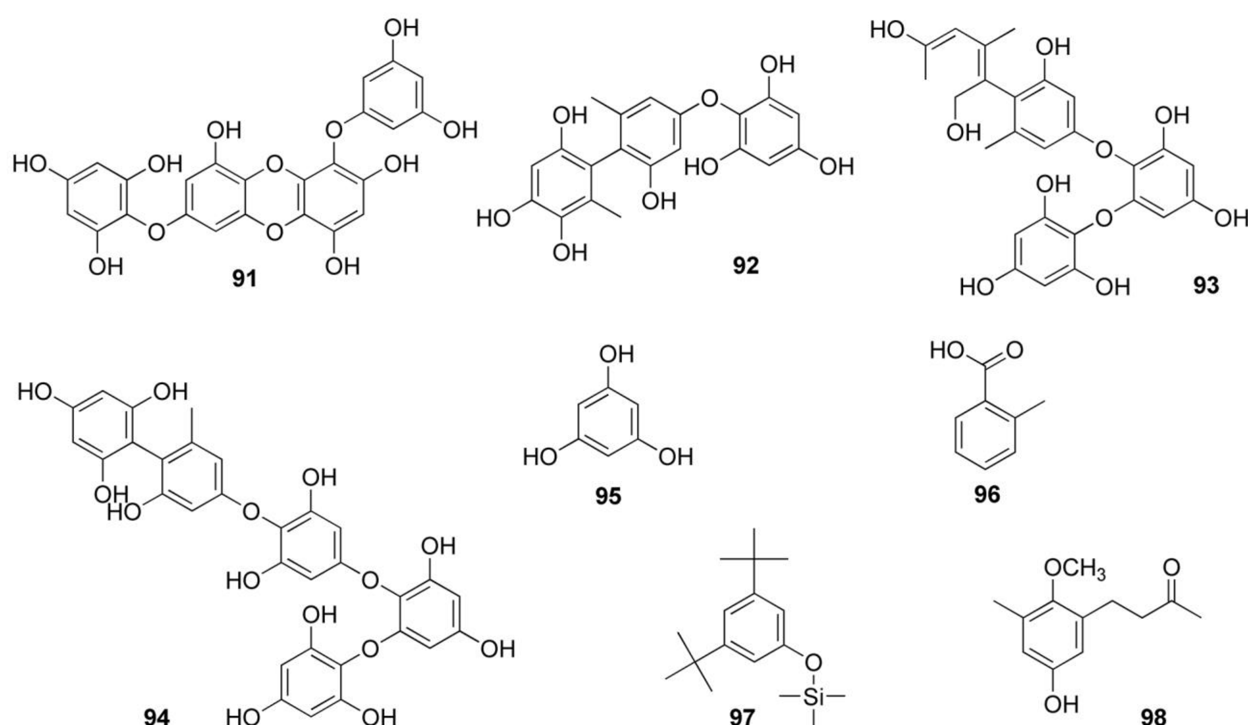

Figure 2.6. Structures of phlorotannins **91-98** identified in *Cystoseira algae.*

amount in the fungal cell membrane. Because of its fungistatic and fungicidal activity *C. nodicaulis*, is highlighted as very promising for the future development of antimycotic drugs. Additionally, the potential of *Cystoseira* phlorotannins against Gram-positive bacteria (*Staphylococcus aureus, S. epidermidis*, *Micrococcus luteus*, *Bacillus cereus*) and Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*) was demonstrated. In particular, phlorotannins obtained from *C. nodicaulis* were strongly active against *S. epidermidis* and *S. aureus*. Extracts enriched in these compounds showed also anti-inflammatory activity, inducing a marked decrease in nitric oxide (NO) production in LPS-stimulated macrophages, especially *C. tamariscifolia* extract, which led to a 75% decline of NO at 8.35 mg/mL with no toxicity to murine RAW 264.7 cells.

Phloroglucinol (**95**) isolated from *C. compressa* and *C. foeniculacea* revealed high antioxidant activity in comparison with the cpntent of this compound in other 12 brown algae species (Chkhikvishvili et al., 2000). High content of this compound were also detected in *C. baccata*, *C. humilis*, *C. nodicaulis*, *C. tamariscifolia* and *C. usneoides* (Andrade et al., 2013; Stiger-Pouvreau et al., 2014) and its antioxidant properties was confirmed (Andrade et al., 2013). Acetyl- and butyrylcholinesterase activities and inhibition of the α-glucosidase enzyme were also attributed to phloroglucinol (**95**) contents of *C. tamariscifolia* and *C. usneoides* (Andrade et al., 2013). Benzoic acid (**96**) was isolated from *C. abies-marina* (Gouveia et al., 2013b) and *C. crinita* (Kamenarska et al., 2002). Antioxidant, anti-inflammatory (Gouveia et al., 2013b) and antifungal (Hussain et al., 2014) activities were reported for compound **96**. Cytotoxicity against tumor HeLa and non-tumour Vero cells was also evaluated although without positive results (Gouveia et al., 2013b). Phenol-2,4 bis-(1,1-dimethylethyl) (**97**), identified in *C. barbata*, anti-intercellular bacteria communication and anti-biofilm activities against *S. marcescens* (Ozdemir et al., 2006) and the ketone (**98**) was isolated for the first time in *C. abies-marina* (Fernández et al., 2006). Moreover, and although without identifying specific compounds, phenolic compounds and phlorotannins-enriched fractions of *Cystoseira trinodis* exhibited prominent (DPPH) radical-scavenging activity (Sathya et al., 2013).

2.3.5. CARBOHYDRATES

Mannitol (**99**), responsible for osmoregulation and commonly found in macroalgae, was identified in *C. nodicaulis*, *C. tamariscifolia* (Andrade et al., 2013) and *C. usneoides* (Andrade et al., 2013; Bruno de Sousa et al., unpublished). Because of its hydrating and antioxidant properties, this carbohydrate is used in numerous cosmetic and pharmaceutical

applications (Iwamoto and Shiraiwa, 2005). Moreover, the radical scavenging, α-glucosidase inhibition, AChE and BuChE activities were also reported by Andrade et al. (2013). Compound 99 was also tested against *L. infantum* promastigotes without effectiveness (Bruno de Sousa et al., unpublished). Fucoidans (**100**) were detected together with uronic acid (**101**) in *C. sedoides*, *C. compressa* and *C. crinita* collected in Tunisia.

Compound **100** exhibited significant radical scavenging (DPPH) and anti-inflammatory activities, being found to be effective in inhibiting edema development. Moreover, compound **100**, isolated from *C. sedoides* and *C. compressa*, revealed gastro-protective activities (Ammar et al., 2015). The presence of the sugar acid **101** together with xylose (**102**), mannose (**103**), fucose (**104**), galactose (**105**) and glucose (**106**) was also detected in sulphated fucan-containing fractions of *C. indica* that showed strong antiviral activity against herpes simplex virus and absence of cytotoxicity against Vero cell cultures. Moreover, it was verified that these fractions did not display anticoagulant activity or an inactivating effect on virions (Mandal et al., 2007).

Fucans were isolated from *C. barbata* harvested in Tunisia. Sulphated polysaccharides, identified in *C. canariensis*, shown to bind to myostatin protein in serum, were deemed as interesting for the development of drugs for muscular related diseases (Ramazanov et al., 2003). Other sulphated polysaccharides (mainly 3-linked-α-l-fucopyranosyl backbone, acetylated and C-4 sulphated derivatives) revealed a wide range of biological activities such as antioxidant, ferric reducing potential, chelating activity and protection activity against hydroxyl radical-induced DNA breakage (Sellimi et al., 2014). Structures of compounds **99-106** are shown in **Figure 2.7.**.

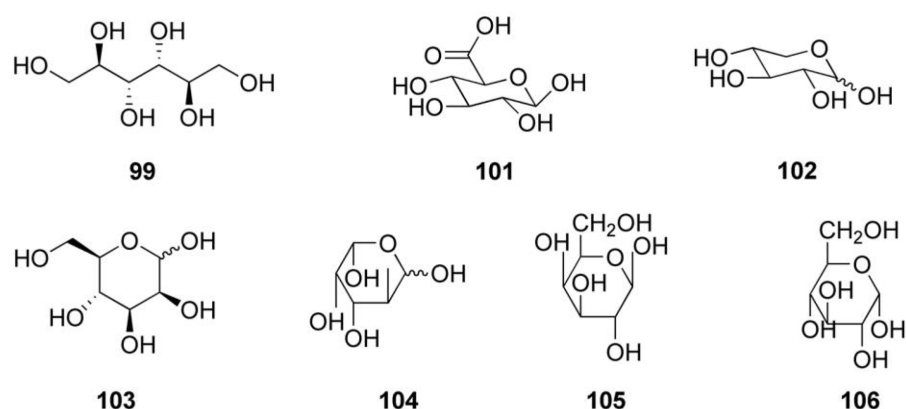

Figure 2.7. Structures of carbohydrates **99-106** isolated from *Cystoseira* algae

2.3.6. LIPIDS

Lipids fulfill some unique biological roles, as an important source of energy, and as constituents of cell membranes with active role in regulating trafficking cellular pathways, protein function and signal transduction (Ibarguren et al., 2014). In *Cystoseira* species, several types of lipids have been reported, including triacylglycerols and fatty acids.

2.3.6.1. Triacylglycerols

Triacylglycerols (TAGs) have an important role as intermediate compounds in several biosynthetic reactions. Eighteen molecular species of TAG (**107-125**) in the lipid fraction of *C. brachycarpa* have been identified by chromatography techniques coupled to mass spectrometry (Ragonese et al., 2014). TAGs are triesters that combine glycerol with three fatty acid molecules. In marine micro- and macroalgae, the chain lengths of fatty acid moieties in TAGs contain mostly 18 to 22 carbons, as is the case with eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids. Molecular species of *Cystoseira* TAGs are represented in **Figure 2.8.**).

2.3.6.2. Fatty Acids

Several saturated (SFA; **126-139**), monosaturated (MUFA; **140-144**) and polyunsaturated (PUFA; **146-158**) fatty acids were found in nine different *Cystoseira* species, namely *C. baccata*, *C. barbata*, *C. brachycarpa*, *C. compressa*, *C. crinita*, *C. humilis*, *C. nodicaulis*, *C. tamariscifolia*, *C. usneoides* (Kamenarska et al., 2002; Andrade et al., 2013; Ivanova et al., 2013; Panayotova and Stancheva, 2013; Ragonese et al., 2014; Vizetto-Duarte et al., 2015) as represented in **Figures 2.9.-2.11.** .

Caproic (**126**), heneicosylic (**136**), tricosylic (**138**), myristoleic (**140**), *cis*-10-heptadecenoic (**143**), erucic (**145**) and docosahexaenoic (**158**) acids were only reported in *C. barbata*. Although hexadeca,-4,7,10,13-tetraenoic (**147**) and docosapentaenoic (**157**) acids

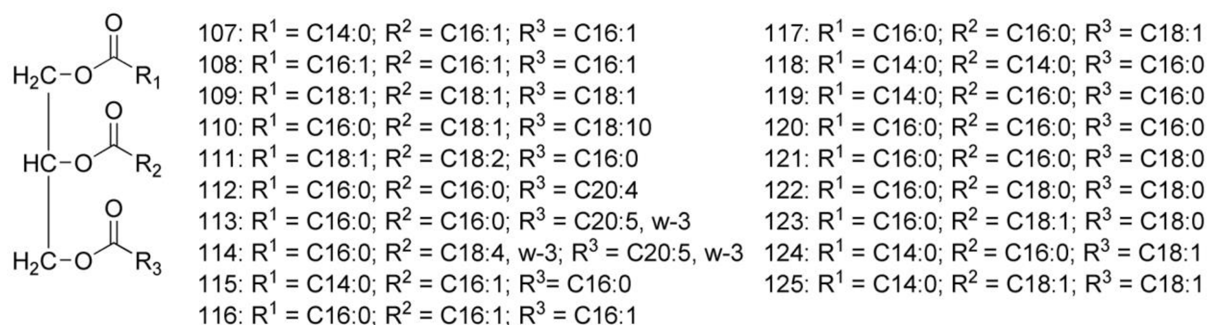

Figure 2.8. Structures of triacylglycerols **107-125** from *Cystoseira* algae

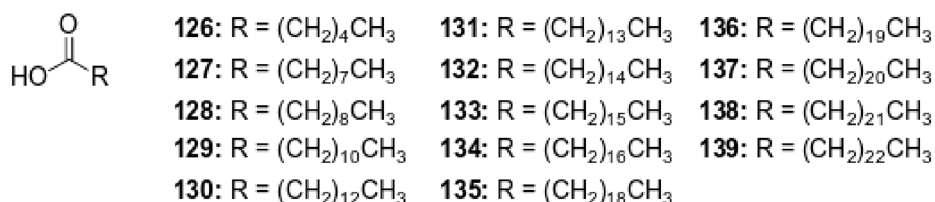

Figure 2.9. Structures of saturated fatty acids **126**-**139** identified in *Cystoseira* algae

was only reported in *C. crinita*, and the eicosatetraenoic acid (**154**) in *C. brachycarpa*. Beyond their energetic role, fatty acids are involved in several biological processes, including the regulation of membrane structure and function, of intracellular signaling pathways and of the bioactive lipid mediator production, gene expression and transcription factor activity. As a result, they are object of greater interest due to its influence on human health, well-being, and disease risk (Calder, 2015).

In addition to the recognized impact on cardiovascular diseases and its importance as endocrine and lipoprotein precursors, a wider range of therapeutic promising properties is identified in fatty acids (Radwan, 1991; Rocha Filho et al., 2011). In this work, we refer to it as cholinesterasic inhibitors, antioxidant, anti-inflammatory, immunomodulatory, anti-androgenic, anti-cancer, anti-diabetic, anti-atherosclerosis and hemorheologic agents (Andrade et al., 2013; Miyazawa et al., 2005; Ren et al., 2006; Gopalakrishna, 2011; Shultz et al., 1992; Ward and Singh, 2005; Kapoor and Huang, 2006; Shaikh and Edidin, 2008; Lavie et al., 2009; Huang, and Ebersole, 2010; Kawamura et al., 2011; Borow et al., 2015; Wei et al., 2016). These compounds are also useful in the treatment of premenstrual syndrome, hyperlipidemia, multiple sclerosis, rheumatoid arthritis, schizophrenia, skin ailments (Mcgregor et al., 1989; Vaddadi, 1992; Zurier et al., 1996; Kurabayashi et al., 2000; Emsley et al., 2003; Kawamura et al., 2011; Rocha Filho et al., 2011) as well as diet supplement for pregnant women and children (Ward and Singh, 2005) to ensure their correct development. Moreover, some of these compounds revealed activity against bacteria (Yff et al., 2002; Huang and Ebersole, 2010; Chen et al., 2011), fungi (Jung et al 2013c; Verma et al., 2014) and virus (Lee et al., 2009).

In what concerns cholinesterase (AChE) and butyrylcholinesterase (BuChE) and anti-radical activities (DPPH, nitric oxide, superoxide and hydroxyl), a relationship between chemical composition and biological activities of extracts suggest that the presence of fatty acids **130**, **142**, **155** and **156** contributes for these activities in the extracts of *C. tamariscifolia*, *C. nodicaulis*, and *C. usneoides* (Andrade et al., 2013). Anti-inflammatory, anti-cancer, anti-

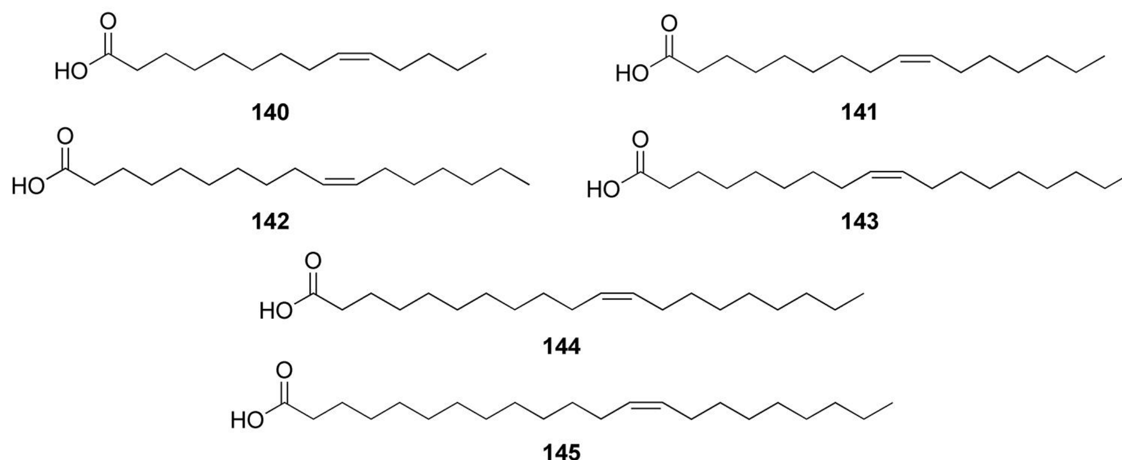

Figure 2.10. Structures of the monounsaturated fatty acids **140**-**145** identified in *Cystoseira* algae

diabetic, antiandrogenic, antifungal and anti-bacterial properties are also reported for **142** (Gopalakrishna, 2011).

Palmitic acid (**138)** was reported as an abundant SFA in various *Cystoseira* species (Kanias et al., 1992; Kamenarska et al., 2002; Ivanova et al., 2013; Andrade et al., 2013; Panayotova and Stancheva 2013; Ragonese et al, 2014; Vizzeto-Duarte et al., 2015). Antimicrobial activities such as antifungal, antiviral, anti-bacterial are reported for **138** (Yff et al., 2002; Lee et al., 2009; Jung et al., 2013c). Additionally, Vizzeto-Duarte et al. (2015) highlights *C. compressa*, *C. tamariscifolia* and *C. nodicaulis* for their low PUFA/SFA, low n-6 PUFA/n-3 PUFA ratios and also for its favorable unsaturation, atherogenicity and thrombogenicity indices, suggesting the potential application of these algae in the nutraceutical industry.

The n-3 PUFA **149**, **156** and **158** exhibited strong anti-bacterial activity against different oral pathogens (*Streptococcus mutans*, *Candida albicans*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, and *Porphyromonas gingivalis*), revealing the potential therapeutic effect for oral health improvement. γ-Linolenic acid (**150**), a metabolic precursor of **155** and prostaglandin E1, shows promising properties as an anti-inflammatory compound (Kapoor and Huang, 2006), and a biomolecule able to improve skin barrier function and mild atopic dermatitis (Kawamura et al., 2011), rheumatoid arthritis

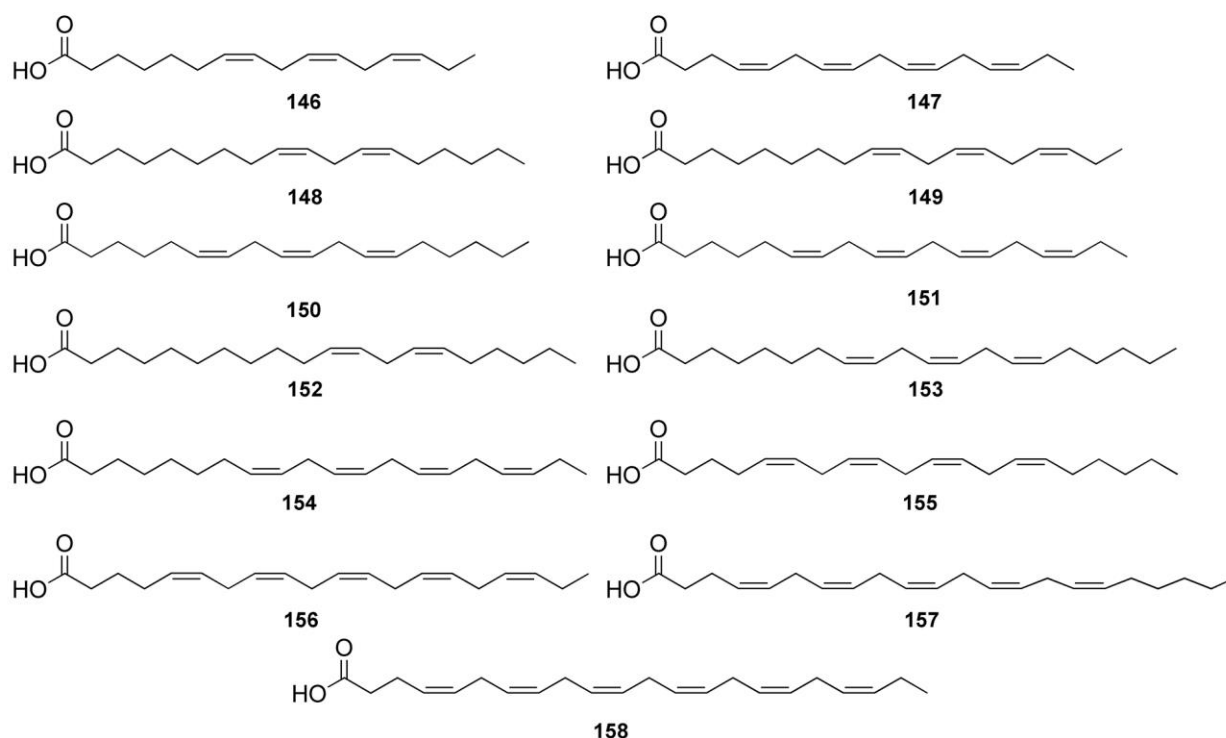

Figure 2.11. Structures of the polyunsaturated fatty acids **146**-**158** present in *Cystoseira* algae

(Zurier et al., 1996), multiple sclerosis (Mcgregor et al., 1989), schizophrenia, andpremenstrual symptoms (Rocha Filho et al., 2011). It is also included in infant diet supplements (Ward and Singh, 2005). Compound **155** is the most abundant omega-6 PUFA in humans. As a structural lipid, it is important for the correct development of the infants brain (Ward and Singh, 2005). Moreover, fatty acid **155** and other polyunsaturated derivatives are involved in the biosynthesis of eicosanoid hormones such as prostaglandins, thromboxanes and leukotrienes (Radwan, 1991) and a direct precursor of a number of eicosanoids regulating lipoprotein and hemorheology metabolisms, leucocyte function and platelet activation (Ward and Singh, 2005). Cardioprotective, anti-cancer, immunomodulatory, anti-inflammatory, atherosclerosis, hyperlipemia- and schizophrenia-preventive, antibacterial and antileishmanial properties are reported for compound **156**, which is also included in adult and pregnant women sussplements (Kurabayashi et al., 2000; Emsley et al., 2003; Ward and Singh, 2005; Shaikh and Edidin, 2008; Lavie et al., 2009; Vassalo et al., 2011; Borow et al., 2015). Moreover, compound **156** also plays an endocrine role as precursor of the prostaglandin-3, thromboxane-3 and leukotriene-5 eicosanoids (Radwan, 1991). As a major structural component of the brain, eye retina and heart tissue, **158** is essential for the proper development of these organs in infants and for cardiovascular health and for cancer

prevention, being recommended as diet supplement for pregnant women, infants and adults as well (Ward and Singh, 2005; Lavie et al., 2009). This compound is also of interest for the food industry as additive in the manufacture of cheese, yoghurt, breakfast cereals, spreads and dressings (Ward and Singh, 2005).

2.3.7. PIGMENTS AND VITAMINS

Two carotenoids, known by their antioxidant and anti-inflammatory properties, were found in *C. baccata*, β-carotene **159** and astaxanthin **160**. When evaluated for their antiproliferative effect on human T-cell leukemia cells these compounds revealed mild inhibitory activities (Panayotova and Stancheva, 2013), and compound **161** displayed antitumoral activity against colon cancer cells (Ishikawa et al., 2008).

Compound **160**, found in high amounts in *Cystoseira*, have several other applications, namely effects on cataracts and cardiovascular disease prevention, immune system boosting, anti-helycobacter pylori and liver function protection (reviewed in Higuera-Ciapara et al., 2006 and Chena and Kotanib, 2016). Fucoxanthin (**161**), another recognized algal carotenoid, identified in *C. brachycarpa* (Ragonese et al., 2014) has several potential applications requiring antioxidant, anti-inflammatory, anti-cancer, anti-obesity, anti-diabetic, antiangiogenic activities, protective effects on the liver, skin and eyes and antiparasitic activity against *Plasmodium falciparum* malaria parasites (reviewed in Peng et al., 2011). Moreover, other phytotoxic activities, as inhibition of seed germination (Islam et al., 2017) and inhibition of cyanobacterial growth (Xian et al., 2006), are reported for this compound. Chlorophyll *a* (**162**), with recognized antioxidant properties with interesting applications for food purposes, was isolated from *C. brachycarpa* (Ragonese et al., 2014). Structures of compounds **159-162** are presented in **Figure 2.12.**.

Three vitamins, α-tocopherol (**163**), retinol (**164**) and ergocalciferol (**165**), were identified in *C. barbata* (Panayotova and Stancheva, 2013). Beyond its accepted antioxidant activity (reactive oxygen species and reactive nitrogen species scavenging), compound **164** also displays activities in the regulation of cellular signalling and gene expression (Brigelius-Flohé, 2006; Zingg, 2007). Compound **164** is broadly recognized by its anti-aging effects, including induction of collagen synthesis in photoaged skin and the inhibition of UV-activated metalloproteinases (Kong et al., 2016). Currently recognized as a prohormone, compound **169** is able to inhibit leukemia cell growth (Chen et al., 2008), regulate parathyroid

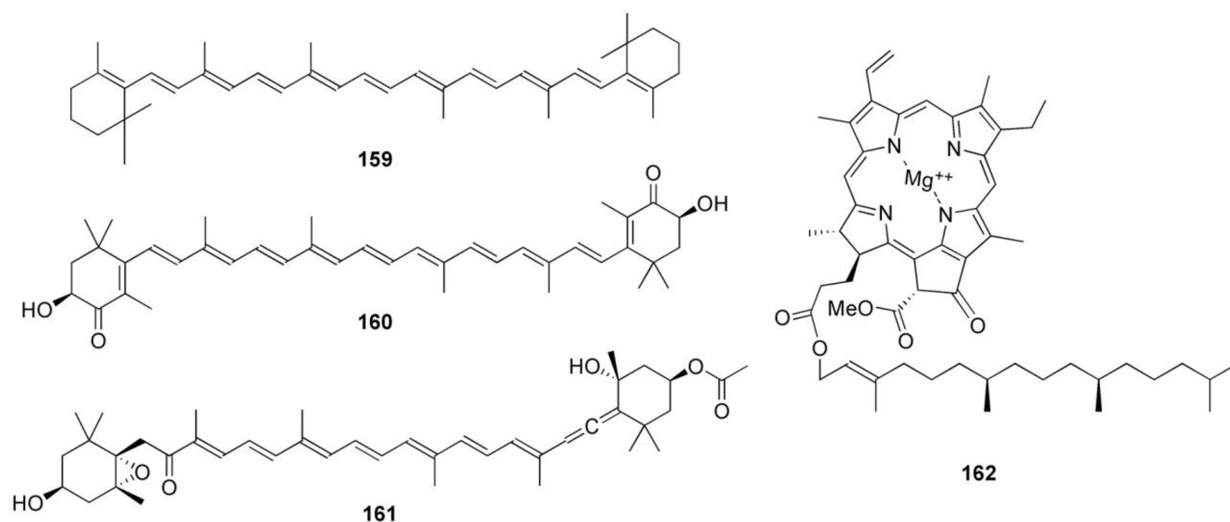

Figure 2.12. Structures of pigments **159**-**162** identified in *Cystoseira* algae

hormone levels (Thimachai et al., 2015) and the calcium and phosphate absorption (Sahay and Sahay 2012). Structures of compounds **163-165** are presented in **Figure 2.13**.

2.3.8. OTHERS

Other compounds (**166-214)** were identified in *Cystoseira* species (**Figure 2.14.**). *C. barbata* contains as volatile compounds mainly halogenated hydrocarbons, 3-bromo-2-chloro-1-propanol (**166**), 1-bromo-2-chloroethane (**167**), 1,1,2-trichloroethane (**168**), 1,1,2,2,-tetrachloroethane (**169**), hexachlorobutadiene (**170**), dimethylformamide (**171)** while the main volatile components of *C. crinita* appeared to be monoterpenoids, as for example compound **10**. As reported in literature (Milkova et al., 1997) the anti-bacterial, antifungic activity and toxicity against some crustaceans displayed by *C. barbata* extracts could be associated to the

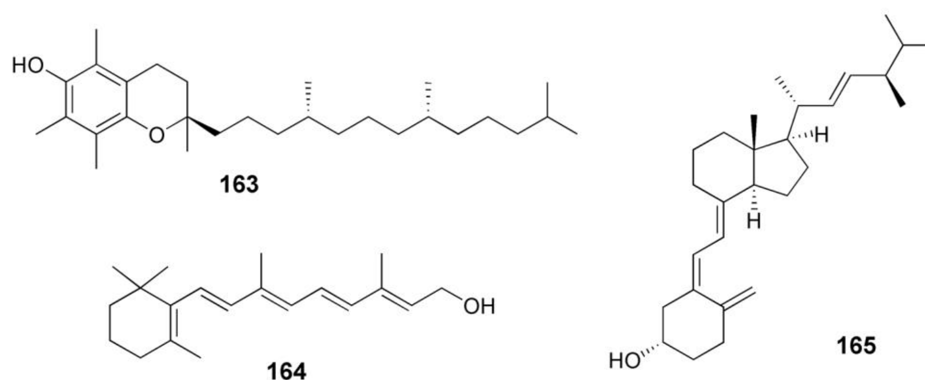

Figure 2.13. Structure of vitamins **163**-**165** present in *Cystoseira* algae composition.

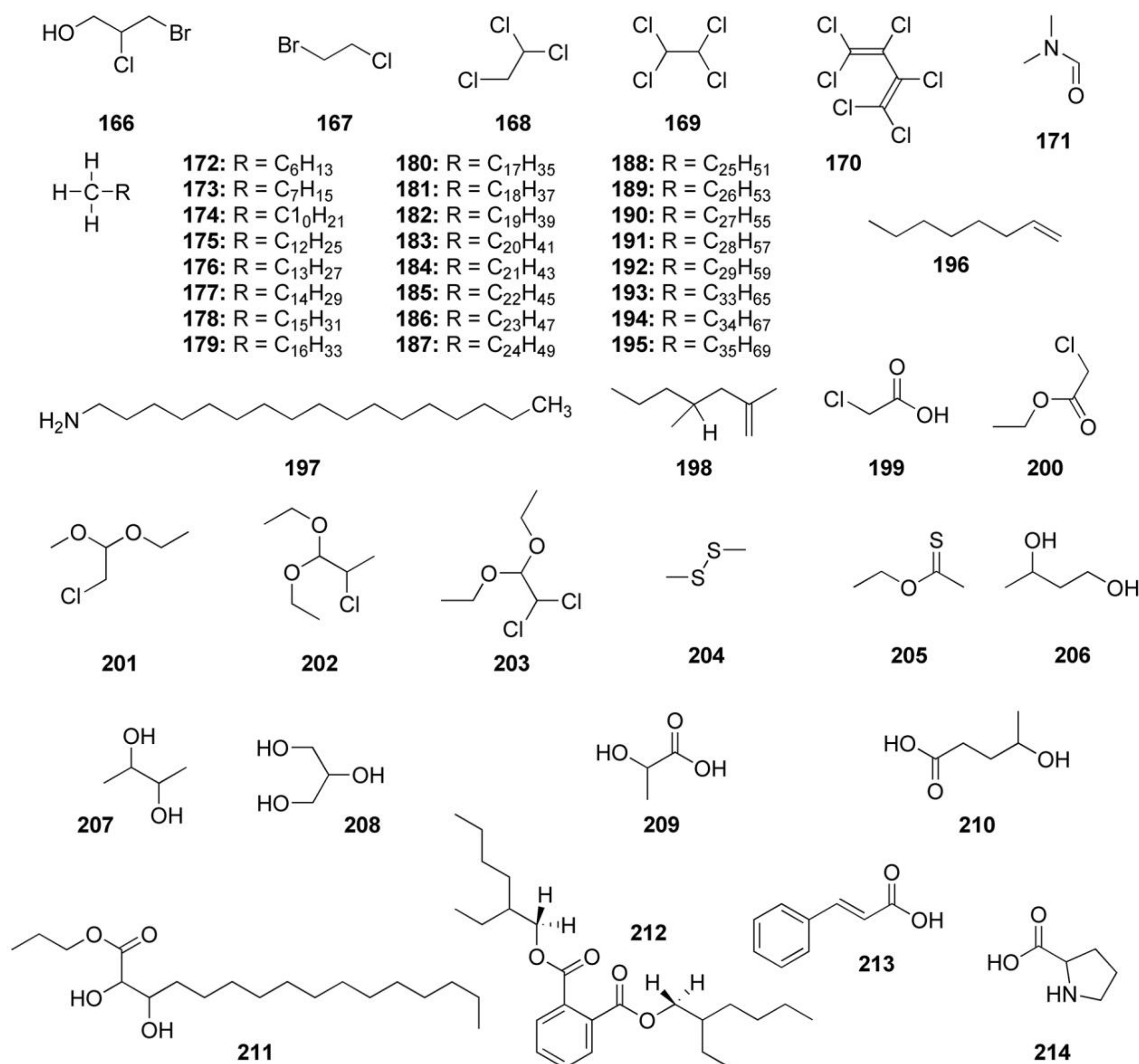

Figure 2.14. Structures of other different compounds **166-214** isolated from *Cystoseira* algae

presence of these halogenated hydrocarbons. In other study, twenty four acyclic alkanes (**172-195**) were identified in the volatile oil of *C. barbata* (Ozdemir et al., 2006).

The hydrocarbons **178-181** were also isolated from *C. crinita* (Kamenarska et al., 2002). Volatile fraction of *C. barbata*, was composed mainly of docosane (**184**), and tetratriacontane (**194**) followed by hexadecane (**178**), heptadecane (**179**), eicosane (**182**) and tricosane (**185**). Moreover three other components, 1-octene (**196**), 1-heptadecanamin (**197**) as well as 2,4-dimethyl-1-heptene (**198**)**,** were identified in *C. barbata* (Ozdemir et al., 2006). Antimicrobial activity is reported for the compound **197** (Sukatar, 2006). Halogenated and sulphur derivatives such as chloroacetic acid (**198**), chloroacetic ethyl ester (**200**), 1-chloro-2-ethoxy-2-methoxyethane (**201**), 1-chloro-2,2-diethoxyethane (**202**), 1,1-dichloro-2,2-diethoxyethane

(**203**), dimethyl disulfide (**204**) and thioacetic acid-O-ethyl ester (**205**) have been reported in the volatile fraction of the *C. crinita* from the eastern Mediterranean (Kamenarska et al., 2002). From *C. crinita*, other polar compounds were identified, namely the 1,3-butanediol (**206**), 2,3-butanediol (**207**), glycerol (**208**), 2-hydroxypropanoic (**209**) and 4-hydroxypentanoic (**210**) and 2,3-dihydroxy palmitic acid, propyl ester (**211**; Kamenarska et al., 2002). Furthermore, 2-ethylhexylphtalate (**212**) has been identified in *C. compressa* from Tunisia (Mighri et al., 2009) and cinnamic acid (**213**) from *C. crinita* (Kamenarska et al., 2002). The amino-acid proline (**214**), which has α-glucosidase inhibitory activity, acetyl- and butyrylcholinesterase activities, was isolated from *C. nodicaulis*, *C. tamariscifolia* and *C. nodicaulis* (Andrade et al., 2013).

2.4. CONCLUSIONS

Overall, the genus *Cystoseira* contains a wide variety of secondary metabolites, namely lipids, terpenoids, steroids, carbohydrates, phlorotannins, phenolic compounds, pigments and vitamins. Within these metabolites, 59 interesting biological properties are reported. The most commonly found are antioxidant, anti-inflammatory, cytotoxicity, anticancer, cholinesterase inhibition, anti-diabetic, and anti-herpetic activities. Antibacterial, antifungal and anti-parasitic activities as antimalarial and antileishmanial are also described, though with less detail. This comprehensive review shows that *Cystoseira* contain compounds with several biomedical potentialities, providing an extensive list of natural isolated structures that could be used as scaffolds to the design of novel and target-specific molecules for pharmacological purposes.

2.5. ACKNOWLEDGMENTS

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SCREENING FOR ANTILEISHMANIAL ACTIVITY IN IBERIAN MACROALGAE: SPECIAL EMPHASIS ON THE *CYSTOSEIRA* GENUS

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ANTILEISHMANIAL ACTIVITY IN IBERIAN MACROALGAE: EMPHASIS ON THE *CYSTOSEIRA* GENUS

Carolina Bruno de Sousa[1], João Henrique G. Lago[2], Jorge Macridachis[1], Marta Oliveira[1,3], Luis Brito[1], Catarina Vizetto-Duarte[1], Cláudia Florindo[4,5], Sarah Hendrickx[6], Louis Maes[6], Thiago R. Morais[2], Miriam Uemi[2], Luís Neto[7], Lídia Dionísio[7,8], Sofia Cortes[3], Luísa Barreira[1], Luísa Custódio[1], Fernando Albericio[9,10,11,12], Lenea Campino[3,13], João Varela[1,*]

[1] Centre of Marine Sciences, University of Algarve (UAlg), Campus de Gambelas, Faro, Portugal;

[2] Instituto de Ciências Ambientais, Químicas e Farmacêuticas, Universidade Federal de São Paulo, São Paulo, Brazil; [3] Global Health and Tropical Medicine Centre, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisboa, Portugal;

[4] Programa de Medicina Regenerativa, Departamento de Ciências Biomédicas e Medicina, UAlg, Faro, Portugal;

[5] Centro de Investigação Biomédica (CIB/CBMR) UAlg, Faro, Portugal;

[6] Department of Biomedical Sciences, Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, Antwerp University, Antwerp, Belgium;

[7] Faculdade de Ciências e Tecnologia. UAlg, Faro, Portugal;

[8] Centre for Mediterranean Bioresources and Food. UAlg, Faro, Portugal;

[10] University of Barcelona, Department of Organic Chemistry, Barcelona, Spain;

[11] Institute for Research in Biomedicine, Barcelona Science Park, Barcelona, Spain;

[12] CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, Barcelona Science Park, Barcelona, Spain;

[13] School of Chemistry, Yachay Tech, Yachay City of Knowledge, Urcuqui, Ecuador;

[14] Departamento de Ciências Biomédicas e Medicina, UAlg, Faro, Portugal.

* Correspondence: jvarela@ualg.pt; Tel.: +351-289-800-051

3.1. ABSTRACT

Forty-five extracts obtained from 15 macroalgae species from *Cystoseira* genus collected on the Iberian coast were screened for activity against *Leishmania (L.) infantum* parasites. Cytotoxicity against human macrophages was also determined. Seven extracts displayed IC_{50} values ranging from 29.8 to 101.8 µg/mL against promastigote forms. Hexane and CH_2Cl_2 extracts of *C. tamariscifolia* and the CH_2Cl_2 extract of *C. usneoides* were the most active with IC_{50} values ranging from 29.8 to 33.6 µg/mL. Additionally, the hexane extracts from *C. barbata* and *C. baccata* exhibited inhibitory activities against intracellular amastigote forms of *L. infantum* with IC_{50} values of 5.1 and 6.8 µg/mL, respectively. A preliminary identification of chemical composition of the active *Cystoseira* extracts was performed by nuclear magnetic resonance (NMR), gas chromatography/flame ionization detector (GC/FID), gas chromatography/low resolution electron ionization mass spectrometry (GC/LREIMS) and high resolution thin layer chromatography (HRTLC), revealing that *Cystoseira* extracts are composed of fatty acids, triacylglycerols, carotenoids, steroids, and meroterpenoids. These results suggest that Iberian *Cystoseira* macroalgae contain compounds with antileishmanial activity which could be explored as scaffolds to the development of novel sources of antiparasitic derivatives.

Keywords: L*eishmania (L.) infantum*; antileishmanial activity; macroalgae; *Cystoseira*.

3.2. INTRODUCTION

Leishmaniases are a group of vector-borne diseases resulting from the infection of mononuclear phagocytic cells by kinetoplastid parasites of the *Leishmania* genus, which are transmitted by several species of phlebotomine sand flies. With a worldwide growing annual incidence of about 2 million new human cases per year, these diseases are endemic in 98 countries, affecting more than 12 million people, particularly those living in the world's poorest areas, and causing chronic disability and poverty in low- and middle-income countries (Alvar et al., 2012). In the Mediterranean region, cutaneous and visceral clinical forms of human and canine leishmaniasis are caused by *L. (L.) infantum*, and are considered to be a severe public health issue (Campino et al., 2006). Presently, only a small number of drugs with limited effectiveness, due to growing parasite resistance, are available for controlling leishmaniases. In this context, the development of novel drugs continues to be important (Sundar and Chakravarty, 2015b). Plants are known to be a large source of bioactive compounds that currently continues to be explored (Al-Sokari et al., 2015) However, the marine environment has been recognized as another rich source of bioactive metabolites that could be used in antileishmanial therapy and control (Tempone et al., 2011).

Over the last few years, screening efforts have shown that a number of extracts from several seaweed species belonging to the Chlorophyta, Heterokontophyta and Rhodophyta phyla are toxic to *Leishmania* parasites (Freire-Pelegrin et al. 2008; Spavieri et al., 2010a; Süzgeç-Selçuk et al., 2010; Sabina et al., 2005; Orhan et al., 2006; Vonthron-Sénécheau et al., 2011; Bianco et al., 2013). These studies used different extracts of macroalgae from European, Asian, Middle-East and South American countries and all evaluated the effect of crude extracts against different *Leishmania* species, namely *L. donovani*, *L. major*, *L. amazonensis* and *L. mexicana* upon axenic promastigote and amastigote parasite forms. The most potent activities were found on ethanol (EtOH) extracts of *Laurencia pinnatifida* (Sabina et al., 2005) and *Ulva lactuca* (Orhan et al., 2006) and on the ethyl acetate (EtOAc) extract of the brown algae *Bifurcaria bifurcata* (Vonthron-Sénécheau et al., 2011). Among these studies, only four publications described the identification of promising molecules for future studies regarding the treatment of leishmaniases, namely the sesquiterpene elatol and obtusol isolated from *Laurencia dendroidea* (dos Santos et al., 2010; Machado et al., 2011) as well as the diterpenes 4-acetoxy-dolastane and dolabelladienetriol obtained from *Canistrocarpus cervicornis* and *Dictyota pfaffii,* respectively (dos Santos et al., 2011; Soares et al., 2012).

The diversity of secondary metabolites produced by *Cystoseira* algae (Amico, 1995) as well as the diversity of biological activities already reported by other authors (Spavieri et al., 2010a; Khanavi et al., 2010; Mhadhebi et al., 2011; Tajbakhsh et al., 2011; Ibraheem et al. 2012; Ghannadi et al., 2013) for this genus have led us to research whether these macroalgae could contain interesting cytotoxic compounds against *Leishmania* parasites. Members of this genus are known producers of different meroterpenoids and diterpenoids with antioxidant and cytotoxic activities. *C. barbata*, *C. baccata*, *C. crinita* and *C. tamariscifolia* extracts have been reported to be active against *L. donovani* (Spavieri et al., 2010a; Süzgeç-Selçuk et al., 2010). Although some reports have described the activity of other Sargassaceae algae against *L. major* and *L. mexicana*, none has reported cytotoxic bioactivities for *L. infantum* (Freire-Pelegrin et al., 2008; Vonthron-Sénécheau et al., 2011).

Taking into account the antiprotozoal potential of extracts from natural sources and the marine biodiversity of the Iberian coast, this study evaluated the *in vitro* antileishmanial activity and cytotoxicity of extracts of 15 macroalgal species collected on the Portuguese and Spanish coasts. Some of the algae tested were evaluated for the first time for this activity. In addition, the chemical composition of the bioactive extracts was tentatively determined by high field ^{1}H NMR, GC/FID and GC/HREIMS spectral analysis and/or HRTLC comparison with standard samples. Some of the evaluated extracts exhibited inhibitory activities against promastigotes and intracellular amastigotes, suggesting that Iberian *Cystoseira* contain compounds with antileishmanial activity.

3.3. EXPERIMENTAL SECTION

3.3.1. ALGAL MATERIAL

Samples from 15 different species belonging to the Rhodophyta, Chlorophyta and Heterokontophyta phyla were collected between July, 2010 and July, 2013 at different locations of the Portuguese and Spanish coasts (**Table 3.1.**). Samples were washed with seawater, and kept at +4 °C until they were washed with water to remove epiphytes at the laboratory. Biomass was freeze, dried, ground and stored at -20 °C. The 15 Voucher specimens are kept at Centre of Marine Sciences - MarBiotech herbarium - Faro, Portugal.

Table 3.1. Species, date of collection and collection site of the macroalgae

Phylum /Species	Date	Local	Country
Chlorophyta			
Cladophora albida (Nees) Kutzing	July 2010	Olhos de Água[1]	Portugal
Codium sp. Stackhouse	July 2010	Olhos de Água[1]	Portugal
Heterokontophyta			
Cladostephus spongiosus (Hudson) C. Agardh	July 2010	Olhos de Água[1]	Portugal
Cystoseira baccata (S. G. Gmelin) P. C. Silva	July 2012	Areosa[2]	Portugal
Cystoseira barbata (Stackhouse) C. Agardh	March 2013	Cadiz Bay[1]	Spain
Cystoseira humilis Schousboe ex Kützing	May 2012	Almograve[3]	Portugal
Cystoseira nodicaulis (Withering) M. Roberts	April 2013	Santa Mariña[2]	Spain
Cystoseira tamariscifolia (Hudson) Papenfuss	July 2012	Areosa[2]	Portugal
Cystoseira usneoides (L.) M. Roberts	September 2012	Olhos de Água[1]	Portugal
Halopteris scoparia (L.) Sauvageau	July 2010	Olhos de Água[1]	Portugal
Sargassum muticum (Yendo) Fensholt	July 2010	Olhos de Água[1]	Portugal
Taonia atomaria (Woodward) J. Agardh	July 2010	Olhos de Água[1]	Portugal
Rhodophyta			
Peyssonnelia squamaria (S. G. Gmelin) Decaisne	July 2013	Arrifes[1]	Portugal
Plocamium cartilagineum (L.) P. S. Dixon	July 2012	Olhos de Água[1]	Portugal
Scinaia furcellata (Turner) J. Agardh	July 2013	Olhos de Água[1]	Portugal

[1]Southern coast, [2]Northwestern coast and [3] Southwestern coast of the Iberian Peninsula.

3.3.2. PREPARATION OF THE EXTRACTS

Dried biomass was blended with hexane (1:10 w/v) by means of an IKA Ultra-Turrax disperser for 1 min for cell wall disruption, and after centrifugation (5000 × g, 10 min) the supernatants were recovered. The extraction was repeated three times. The residue was then sequentially extracted with CH_2Cl_2 and MeOH in a similar manner. All extracts were evaporated under reduced pressure at +40 °C and stored at +4 °C. For the bioactivity assays, extracts were dissolved in DMSO (dimethyl sulfoxide) at the concentration of 50 mg/mL.

3.3.3. ANTILEISHMANIAL AND CYTOTOXICITY ASSAYS

3.3.3.1. Cytotoxicity Assay.

Cytotoxicity of all extracts used for the antileishmanial assays was performed on human acute monocytic leukaemia cell line THP-1 (ATCC TIB-202). Cells were cultivated in RPMI-1640 medium supplemented with 10% heat-inactivated foetal bovine serum (FBS), L-glutamine (2 mM), penicillin (50 U/L) and streptomycin (0.05 mg/L) at +37 °C in humidified atmosphere with 5% CO_2. For the assay, 10^5 THP-1 cells per well were seeded onto the 96-

well plates. Extracts were added at concentrations ranging from 4 to 125 µg/mL for 48h, and cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay, as described elsewhere [51]. Cells used as negative control were treated with DMSO at the highest concentration used in test wells (0.5% v/v). Results were expressed in terms of cell viability (%) and half maximal inhibitory concentration values (IC_{50} – in µg/mL).

3.3.3.2. Antipromastigote Assay.

Promastigote forms of an *L. infantum* strain (MHOM/PT/88/IMT-151) were obtained from the cryobank of the 'Instituto de Higiene e Medicina Tropical' (Universidade Nova de Lisboa, Portugal) and maintained in RPMI-1640 medium supplemented with 10% FBS, L-glutamine, penicillin and streptomycin, at 24 °C. About 2 x 10^6 parasites per well were incubated in 96-well plates with the extracts at concentrations ranging from 4 to 125 µg/mL for 48h. Negative control cells were treated with DMSO at the highest concentration used in test wells (≤1% v/v). Amphotericin B (0.2 µg/mL), miltefosine (12.7 µg/mL) and pentamidine (0.5 µg/mL) were used as positive controls. The effect of the extracts on parasite viability was assessed by the MTT colorimetric assay. Results were expressed in terms of cell viability (%) and IC_{50} values (µg/mL).

3.3.3.3. Activity against intracellular amastigotes.

L. infantum intracellular amastigotes (MHOM/MA(BE)/67) were collected from the spleen of heavily infected donor hamsters and used to infect primary peritoneal mouse macrophages (PMM). PMM (3×10^4 per well) were seeded on 96-well plates, and incubated for two days for cell attachment. Then, 5×10^5 amastigotes were added to each well (infection ratio about 16 amastigotes per cell) and infected macrophages were further incubated at 37 °C for 2 h. The extracts at concentrations ranging from 0.25 to 64.0 µg/mL were added and the plates were further incubated at 37 °C and 5% CO_2. After 5 days, intracellular amastigote burdens were microscopically assessed upon Giemsa staining and the inhibitory concentration conferring a 50% reduction of the intracellular amastigote burden compared to the non-treated infected positive controls (CC_{50}) was determined. In addition, cytotoxicity of the extracts was evaluated on PMM cells and carried out as previously described (Mokrini et al., 2008). For the latter tests, the reference drug (miltefosine) was used as positive control.

3.3.3.4. Microscopic Analysis.

Leishmania promastigotes were incubated with the extracts (125 μg/mL) and with amphotericin (0.2 μg/mL) for 48h. After incubation and centrifugation, parasites were smeared on microscope slides, fixed with methanol and stained with Giemsa solution and observed using a Zeiss AXIOMAGER Z2 microscope, equipped with a cool SNApHQ2 camera and AxioVision software version 4.8 (Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

3.3.3.5. Apoptosis detection through Annexin V-FITC staining.

Promastigotes (4 x 10^6/mL) cultured in RPMI medium with DMSO (0.1%) were treated with *Cystoseira* extracts at IC_{50} concentrations for 48h. Negative and positive controls cells were treated with DMSO at the highest concentration used in test wells (≤ 1% v/v) and amphotericin B (0.6 μg/mL), respectively. Apoptotic effect of the tested extracts on L. infantum promastigotes was evaluated by flow cytometry using the Annexin V-FITC (fluorescein isothiocyanate) Apoptosis Kit - KA0714 (Abnova) in accordance with the manufacturer's recommendations. Briefly, treated and control promastigotes were washed with culture medium and resuspended in 500 μL binding buffer and stained with 5 μL of annexin V-FITC (10 mg/mL) and 5 μl of propidium iodide (PI; 50μg/ml) and incubate at room temperature for 5 min in the dark. Results were obtained by flow cytometry using a FACS Calibur Flow Cytometer (Becton-Dickinson, East Rutherford, NJ, USA) using the Cell Quest software (BD Biosciences, San Jose, CA, USA) for acquisition and result analysis. Ten thousand events were analysed and apoptosis evaluated based on the geometric mean of the fluorescence intensity detected in channels 1 (for annexin) and 2 (for PI) (Farias et al., 2013).

3.3.4. CHEMICAL CHARACTERIZATION OF *CYSTOSEIRA* EXTRACTS.

3.3.4.1. NMR analysis.

Hydrogen nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker Avance III 500 spectrometer, using 5 mm TXI probe, operated at 500.13 MHz for ^{1}H nucleus. Approximately 10 mg of each sample was dissolved in 0.6 mL of $CDCl_3$ with tetramethylsilane (TMS) as a standard reference with a chemical shift $\delta_H = 0.00$ ppm. Spectra were obtained at +25 °C, with 64 scans, 2 dummy scans, using a 90 degree high power pulse of 8.3 μs, a recycle delay of 1s, and 64 K data points covering a spectral width of 7684 Hz. All spectra were processed using Topspin 3.2 software.

3.3.4.2. HPTLC Analysis.

All studied crude extracts were submitted to comparative high resolution thin layer chromatography (HRTLC; PF_{254}, Merck) in SiO_2 (silicon dioxide) for investigating the presence of secondary metabolites. Different solvents were employed as the mobile phase and ceric sulphate/*p*-anysaldehyde as a post-derivatization agent. All planar chromatographic analyses were developed using different standard samples.

3.3.4.3. GC/FID and GC/LREIMS analysis

Gas chromatography with flame ionization detector (GC/FID) chromatograms were recorded on a Shimadzu GC-2010 gas chromatograph equipped with an FID-detector and an automatic injector (Shimadzu AOC-20i) using a RtX-5 capillary column (5% phenyl, 95% polydimethylsiloxane, 30 m × 0.32 mm × 0.25 μm film thickness; Restek, USA). These analyses were performed by injecting 1.0 μL of a 1.0 mg/mL solution of crude hexane extract in hexane in a split mode (1:30) employing helium as the carrier gas (1 mL/min) under the following conditions: injector and detector temperatures of +270 °C and 300 °C, respectively; oven programmed temperature from 120–290 °C at 8 °C/min, holding 20 min at 290 °C. Gas chromatography–low resolution electron ionization mass spectrometry (GC/LREIMS) analysis was conducted in a Shimadzu GC-17A chromatograph interfaced with a MS-QP-5050A mass spectrometer operating using ionization voltage of 70 eV and an ion source temperature of +350 °C with the same conditions described above. Helium was used as the carrier gas.

3.3.5. STATISTICAL ANALYSIS

The antileishmanial and cytotoxic assays were conducted in triplicate and the results were expressed as mean and standard error of the mean (SEM). The IC_{50} values were calculated using sigmoid regression on the logarithm of the concentration-response data in the GraphPad Prism V 5.0 software.

3.4. RESULTS

From the 45 extracts evaluated, seven were active against *L. infantum* promastigotes (**Table 3.2.**). The majority of the bioactive extracts belong to *Cystoseira* species. *C. tamariscifolia* hexane (IC_{50} = 31.2 μg/mL) and CH_2Cl_2 (IC_{50} = 29.8 μg/mL) extracts and *C. usneoides* CH_2Cl_2 (IC_{50} = 33.6 μg/mL) extract were the most active against this parasite form.

Table 3.2. Inhibitory concentrations of algal extracts against *L. infantum* and THP-1 cells

Species	Extract/ Compound	IC_{50} (μg/mL)[a] Promastigotes	CC_{50} (μg/mL)[b] Amastigotes	IC_{50} (μg/mL)[a] THP-1
Cladophora albida	Hexane	>125	-	>125
	CH_2Cl_2	>125	-	>125
	MeOH	>125	-	>125
Cladostephus spongiosus	Hexane	>125	-	>125
	CH_2Cl_2	>125	-	>125
	MeOH	>125	-	>125
Cystoseira baccata	Hexane	94.1 ± 1.5	5.1 ± 0.0	>125
	CH_2Cl_2	>125	-	76.2 ± 3.9
	MeOH	>125	-	>125
Cystoseira barbata	Hexane	78.7 ± 3.2	6.8 ± 0.0	79.5 ± 2.3
	CH_2Cl_2	>125	-	>125
	MeOH	>125	-	>125
Cystoseira humilis	Hexane	>125	-	>125
	CH_2Cl_2	>125	-	>125
	MeOH	>125	-	>125
Cystoseira tamariscifolia	Hexane	31.2 ± 0.9	-	30.9 ± 0.4
	CH_2Cl_2	29.8 ± 0.5	-	19.9 ± 0.5
	MeOH	>125	-	>125
Cystoseira nodicaulis	Hexane	>125	-	>125
	CH_2Cl_2	>125	-	>125
	MeOH	>125	-	>125
Cystoseira usneoides	Hexane	59.9 ± 0.2	-	16.6 ± 0.3
	CH_2Cl_2	33.6 ± 0.6	-	12.6 ± 0.4
	MeOH	>125	-	45.0 ± 0.4
Codium sp.	Hexane	>125	-	>125
	CH_2Cl_2	>125	-	>125
	MeOH	>125	-	>125
Dictyota spiralis	Hexane	48.3 ± 0.7	-	-
	CH_2Cl_2	>125	-	-
	MeOH	>125	-	-
Halopteris scoparia	Hexane	>125	-	>125
	CH_2Cl_2	>125	-	>125
	MeOH	>125	-	>125
Peysonnelia squamaria	Hexane	>125	-	>125
	CH_2Cl_2	>125	-	>125
	MeOH	>125	-	>125
Plocamium cartilagineo	Hexane	101.8±2.8	-	51.6±0.1
	CH_2Cl_2	>125	-	>125
	MeOH	>125	-	>125
Sargassum muticum	Hexane	>125	-	>125
	CH_2Cl_2	>125	-	>125
	MeOH	>125	-	>125
Scinaria funcellata	Hexane	>125	-	>125
	CH_2Cl_2	>125	-	>125
	MeOH	>125	-	>125
Taonia atomaria	Hexane	>125	-	>125
	CH_2Cl_2	>125	-	>125
	MeOH	>125	-	>125

Species	Extract/ Compound	IC_{50} (µg/mL)[a] Promastigotes	CC_{50} (µg/mL)[b] Amastigotes	IC_{50} (µg/mL)[a] THP-1
Positive controls	Amphotericin B	0.2 ± 0.0	-	-
	Miltefosine	12.7 ± 1.8	8.8 ± 0.0	8.8 ± 0.0
	Pentamidine	0.5 ± 0.1	-	-

[a]IC_{50} - Inhibitory concentration of extract / compound causing 50% reduction of the promastigote and human acute monocytic leukaemia cell line THP-1 cells growth; [b]CC_{50} - Inhibitory concentration of extract / compound causing 50% reduction of the intracellular amastigote burden compared to the non-treated infected controls. IC_{50} and CC_{50} values represent the mean ± standard error of the mean of three experiments performed in triplicate.

The effect of the extracts on promastigote forms was also analyzed by optical microscopy, revealing the occurrence of several morphological changes of the parasites (**Figure 3.1.**). The exposure of *L. infantum* promastigotes to extracts from *C. barbata* (**Figure 3.1.D**) resulted in motility loss, cell shrinkage, abnormal round cell shapes, vacuolated and slightly denser cytoplasm as well as reduction of flagellar length in parasite cells. In contrast, control cells and cells treated with the inactive hexane extract from *C. nodicaulis* (**Figure 3.1.C**) were indistinguishable regarding the typical elongated cell morphology, flagellar length and motility, similar to negative control - RPMI medium (**Figure 3.1.A**).

Phosphatidylserine externalization assay did not reveal an apoptotic effect of any *Cystoseira* extract on promastigotes upon a 48 h of treatment (data not shown). From the active *Cystoseira* extracts, those obtained in hexane from *C. baccata* and *C. barbata*

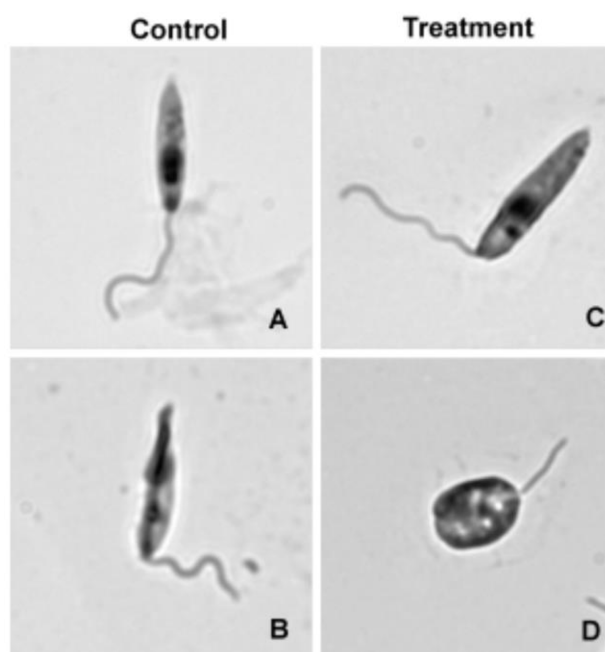

Figure 3.1. Effect of *C. nodicaulis* and *C. barbata* hexane extracts (125 µg/mL, 48h) on the morphology of *L. infantum* promastigotes. Control cells cultured in RPMI medium alone (A) treated with the control drug amphotericin B (0.2 µg/mL; B), and treated with *C. nodicaulis* (C) or *C. barbata* (D) extracts. Bright field images; the scale bar corresponds to 5 µm.

displayed the lowest toxicity against THP-1 mammalian cells (IC_{50} = 79.5 µg/mL and > 125 µg/mL, respectively - **Table 3.2.**). This lower toxicity allowed for the testing of the latter extracts on the intracellular model. When assayed towards the intracellular amastigote form of the parasite infecting the peritoneal mouse macrophages (PMM), hexane extracts from *C. baccata* and *C. barbata* revealed a CC_{50} of 6.8 and 5.1 µg/mL, respectively (**Table 3.2.**). Moreover, these extracts were as cytotoxic to PMM as the reference drug miltefosine (IC_{50} = 8.8 µg/mL).

The ^{1}H NMR spectral analysis of the *C. baccata*, *C. barbata*, *C. tamariscifolia*, *and C. usneoides* crude extracts (**ANNEXES 2-3**) revealed the occurrence of different classes of metabolites present in each studied extract (**Table 3.3.**). The chemical analysis of the bioactive hexane extracts suggest the presence of fatty acids, triacylglycerols, carotenoids, and steroids, except those from *C. barbata* and *C. usneoides*, which were also composed of meroterpenoids. In all obtained spectra, an intense singlet at δ_H 1.2, a deformed triplet at δ_H 0.8 and a multiplet at δ_H 5.3 were indicative of the presence of unsaturated fatty acids. Analysis of GC/FID using standard samples of arachidonic and eicosapentaenoic acids allowed the characterization of these fatty acids in the studied crude hexane extracts. Additionally, these spectra displayed singlets at δ_H 0.68, broad doublets at δ_H 5.4 and multiplets at δ_H 3.5, characteristics of steroids. Furthermore, the presence of singlets at range δ_H 0.9 - 1.8 associated to the unresolved signals at δ_H 5.9 - 6.4 suggested the presence of carotenoid derivatives in all studied hexane extracts, except for that of *C. usneoides*. The occurrence of steroids and carotenoids was confirmed by GC/LREIMS. Analysis of mass spectra of main peaks followed by comparison of obtained data with those available in the system indicated the presence of steroids cholesterol (*m/z* 386), sitosterol (*m/z* 414), stigmasterol (*m/z* 412) and fucosterol (*m/z* 412) as well as carotenoids β-carotene (*m/z* 536) and lutein (*m/z* 568). Particularly, in the hexane extracts of *C. barbata* and *C. usneoides*, overlapping peaks at δ_H 6.5 (m), 5.3 (m) and 3.2 (m) were observed. In addition, singlets at δ_H 3.7 (methoxyl group attached to the aromatic ring), 2.2 (methyl group at the aromatic ring) and 1.7-0.9 (methyl groups of the geranyl unit) characteristic of meroterpenoids were also detected. Analysis of crude extract by HRTLC (SiO_2 - Hex:EtOAc 4:1) using standard samples available in our laboratory allowed the identification of *E*- and *Z*-usneoidones as main derivatives.

Similarly to hexane extracts, the ^{1}H NMR spectra of CH_2Cl_2 extracts from *C. barbata* and *C. usneoides* showed multiplets at δ_H 6.5, 5.3 and 3.2 as well as singlets at δ_H 3.7, 2.2 and

Table 3.3. Class of metabolites found in *Cystoseira* extracts

Species	Extracts	Class of metabolites[1]	Species	Extracts	Class of metabolites[1]
C. baccata	Hexane	FA, CAR, ST	*C. tamariscifolia*	Hexane	FA, ST, CAR
	CH_2Cl_2	MT, FA		CH_2Cl_2	TAG, FA
C. barbata	Hexane	FA, CAR, ST, MT	*C. usneoides*	Hexane	MT, FA, ST
	CH_2Cl_2	MT, FA		CH_2Cl_2	MT, FA

[1]FA - fatty acids, TAG - triacylglycerols, CAR - carotenoids, ST - steroids, MT - meroterpenoids

1.7-0.9, assigned to hydrogens of meroterpenoid derivatives. These spectra also displayed intense peaks at δ_H 1.2 (s), 0.8 (br t) and 5.3 (m), indicating that meroterpenes are present in the crude extracts in a mixture with unsaturated fatty acids. Differently, the ^{1}H NMR spectra of CH_2Cl_2 extracts from *C. tamariscifolia* showed intense peaks related to fatty acids, suggesting that these compounds are the main metabolites. Additionally, the occurrence of triacylglycerol derivatives (esterified fatty acids) in this extract could be inferred due to the presence of characteristic peaks at δ_H 4.0 - 4.5 in the ^{1}H NMR spectra. In the case ^{1}H NMR spectrum of CH_2Cl_2 extract from *C. baccata* the presence of signals at δ_H 1.2 and 5.3 associated to less intense peaks at δ_H 6.4 (m), 5.3 (m), 3.7 (s), and 2.3 (s) was indicative of fatty acids and meroterpenoids. Despite of GC/LREIMS analyses having been conducted for these extracts, the obtained data did not allow the unequivocal identification of main derivatives.

3.5. DISCUSSION

In this work, the IC_{50} range on *Leishmania* promastigote forms of the *Cystoseira* active extracts was close to those obtained for crude extracts of other algae of the same phylum, namely *Dictyota caribaea* (Dictyotaceae), *Lobophora variegata* (Dictyotaceae), *Turbinaria turbinata* (Sargassaceae) and *Sargassum oligocystum* (Sargassaceae) (Freire-Pelegrin et al., 2008; Fouladvand et al., 2011). Moreover, the alterations observed in the promastigotes morphology (**Figure 3.1.**) were similar to those observed by other authors after treatment of different *Leishmania* species with extracts from some marine sponges (Kahla-Nakbi et al., 2010). Loss of motility and cellular vacuolization could be consequence of starvation processes caused by a deficient mitochondrial activity, autophagic mechanisms or cytoplasmic organelle disruption induced by the action of the extracts, as described by other authors (Lockshin and Zakeri, 2004; Monte Neto et al., 2011). Furthermore, phosphatidylserine externalization assay on *L. infantum* promastigotes upon a 48h-treatment

did not reveal an apoptotic effect of the tested extracts (data not shown). These results together with the extensive vacuolization observed by microscopy suggest that some crucial organelles, such as mitochondria, are compromised. Thus, the observed cytotoxic effect might not be associated with programmed cell death. As *Leishmania* parasites have a single mitochondrion, the role and stability of the membrane potential of this organelle are vital for their survival. Hence this organelle is usually considered as an indicator of cellular dysfunction and therefore an interesting target for chemotherapeutic studies (Souza et al., 2009).

Being an easier and affordable model, axenic forms of *Leishmania* are often used for the screening of drug candidates (Tempone et al., 2011). However, due the identification of differences in drug susceptibility of the different parasite forms, it is recommended that the most promising products be evaluated on intracellular amastigotes, the clinically relevant stage of the parasite that recreate the pathophysiological conditions of the disease (Cos et al., 2006). In this study, despite of the potent activity of *C. usneoides* and *C. tamariscifolia* extracts against promastigotes, their high toxicity against mammalian cells (**Table 3.2.**) prevented their use in intracellular assays. Therefore, hexane extracts from *C. baccata* and *C. barbata* were further tested for an inhibitory effect towards the intracellular amastigote. In fact, both extracts were more active on the intracellular form than on promastigotes, reinforcing the evidence that compounds with potential therapeutic interest against this parasite are present in these algae. The obtained results show higher activity ($IC_{50} \leq 6.8$ μg/mL) of the *C. baccata* and *C. barbata* hexane extracts compared to those already described for other macroalgae from the *Cystoseira* genus against intracellular *Leishmania* amastigotes. For example, Süzgeç-Selçuk et al. (2010) reported that the crude MeOH extracts of *C. barbata* and *C. crinita* displayed IC_{50} values within 23.5 and 70.0 μg/mL towards *L. donovani* intracellular amastigotes. Other examples reported in the literature are the $CHCl_3$/MeOH extracts of *C. baccata* and *C. tamariscifolia*, which exhibited activities towards *L. donovani* axenic amastigotes with IC_{50} values of 15.7 and 19.6 μg/mL (Spavieri et al., 2010a). Thus, the results obtained in this study compare favourably with those of similar reports published elsewhere.

Since *Cystoseira* extracts were the most promising, their chemical profiles were investigated (**Table 3.3.**). Previous studies focusing on different bioactivities from species of the *Cystoseira* genus identified diterpenoids, meroditerpenoids, phlorotannins, and sterols (Mokrini et al., 2008; Mighri et al., 2009). *C. baccata* reveal mainly the presence of meroditerpenoids (Cos et al., 2006), *C. barbata* halogenated hydrocarbons and sterols

(Milkova et al., 1997) and *C. tamariscifolia* phloroglucintriacetates, phlorotannins and meroditerpenoids (Bennamara et al., 1999; Lopes et al., 2012). However, none of these studies reported any antileishmanial activity

In this study, different analytical methods (NMR, HRTLC, GC/FID and GC/LREIMS) were used to give preliminary chemical evidence about the composition of active crude extracts. Thus, the obtained data revealed the presence of unsaturated fatty acids such as arachidonic and eicosapentaenoic (EPA) acids in the hexane extracts of the active *Cystoseira* species. This result is in agreement with other authors that have previously detected these compounds in different macroalgae of this genus (Vizetto-Duarte et al., 2015). Conjugated EPA was found to be effective against *L. donovani* promastigotes without affecting macrophages, probably by inhibiting the *L. donovani* topoisomerase (Vassallo et al., 2011). The triacylglycerols 1,3-dilinoleoyl-2-olein and 1,3-dioleoyl-2-linolein isolated from *Moringa stenopetala* revealed activity against promastigote (IC_{50} values of 0.08 and 242.5 µg/mL, respectively) and amastigote (IC_{50} values of 40.0 and 26.8 µg/mL, respectively) forms of *L. aethiopica* (Bekele et al., 2013). Moreover, a triacylglycerol obtained from *Theobroma glandiflorum* seeds induced a decrease in the size of cutaneous lesions in Golden hamsters infected with *L. amazonensis* identical to the one observed in the animals treated with kojic acid, a known antifungal agent (Rodrigues et al., 2014b).

In the present study, steroids such as cholesterol, sitosterol, stigmasterol and fucosterol were also found in the hexane extracts of all studied species and were already reported as main metabolites in nonpolar extracts of *C. adriatica* (Kapetanović et al., 2005). Fucosterol, isolated from the brown alga *Lessonia vadose,* was found to be particularly active against both *L. infantum* promastigotes (IC_{50} = 45 µM) and intracellular amastigotes (IC_{50} = 10 µM) (Becerra et al., 2015). In addition, sitosterol was one of the two main compounds found in the MeOH extract of the fungi *Lactarius pubescens*, which demonstrated activity against several *Leishmania* species (da Silva et al., 2014). Other authors have described that this sterol reduced the viability of *L. amazonensis* (Torres-Santos et al., 2004; Pulivarthi et al., 2015). Sitosterol, described as candidate for cancer chemotherapy, promotes significant the arrest of the second subphase of the cell cycle interphase (G2/M) and endoreduplication by favoring the microtubule polymerization by the Bcl-2 and PI3 K/Akt signaling pathways (Moon et al., 2008). A docking study also suggested that sitosterol displays a potent activity against the trypanothione reductase of *L. infantum*, an enzyme specific to the Kinetoplastida parasites (Gundampati et al., 2013). Thus, it is possible that the activity of evaluated hexane extracts

tested in this study against *Leishmania* parasites may be due to the presence of sitosterol and other related steroids.

Meroterpenoids are common among marine organisms (Menna et al., 2013). The presence of meroterpenoids *E*- and *Z*-usneoidones in the analyzed extracts was also previously reported in *Cystoseira* (Urones et al., 1992a). Potent activities of other meroterpenoids, namely prenylated hydroquinones isolated from the leaves of *Piper crassinervium* (Piperaceae), were described against the epimastigote forms of *Trypanosoma cruzi*, a trypanosomatid phylogenetically and biochemically closed to *Leishmania* (Lopes et al., 2008). Analysis of ^{1}H NMR spectra suggested the presence of meroterpenoids in the CH_2Cl_2 active extracts from *C. barbata, C. baccata* and *C. usneoides* but associated to the presence of fatty acids. Meroterpenoids are a class of secondary metabolites, mainly isolated from brown algae, which have already been described as having cytotoxic, antiviral, antibacterial and antioxidant properties (de los Reyes et al., 2013). Nonetheless, the antileishmanial effect of these compounds remains poorly studied. Meroterpenoids isolated from extracts of the marine sponge *Callyspongia* sp. inhibit *Leishmania* adenosine phosphoribosyltransferase, an important component of the purine salvage pathway essential for the parasites survival (Gray et al., 2006). As the presence of these metabolites were confirmed in the majority of the active *Cystoseira* extracts, further assessment of these metabolites as antileishmanial agents should be carried out in the near future.

3.6. CONCLUSIONS

Several macroalgae species have already been identified as sources of activity against protozoan agents responsible for neglected diseases, namely chagas disease, african trypanosomiasis and leishmaniasis (Torres et al., 2014). In this study, 15 marine macroalgae species from the Iberian coast were evaluated for their potential against *Leishmania infantum* parasites. Among the evaluated macroalgae, the *Cystoseira* genus stood out with four species (*C. baccata*, *C. barbata*, *C. nodicaulis* and *C. tamariscifolia*) displaying significant activity against this parasite. The activity of these species against promastigote forms was similar if not better than those described for other extracts of algae of the same phylum, suggesting the presence of compounds with therapeutic potential against *Leishmania* parasites. The observation of several morphological alterations and lack of externalization of phosphatidylserine in treated promastigotes indicate that these extracts might compromise the metabolism of vital organelles, such as mitochondria. Moreover, the chemical characterization of the *Cystoseira* active against the parasites revealed that hexane extracts are composed of

fatty acids, triacylglycerols derivatives, carotenoids, and steroids, whereas the CH_2Cl_2 extracts contain fatty acids and meroterpenoids. According to recent reports, the identified compounds might be involved in the antileishmanial activity here evaluated. Taken together, the results of this study indicate for the first time that the *Cystoseira* extracts might be used as source of compounds with activity agains *L. infantum*, which could be explored as scaffolds to the development of antiparasitic derivatives. In particular, the selective activity found in *C. baccata* and *C. barbata* against intracellular amastigotes suggests that they should be chosen for further study, since they exhibited higher antileishmanial activity as compared to results described in similar reports on bioactivities found in macroalgae.

3.7. ACKNOWLEDGMENTS

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CHAPTER IV

ANTILEISHMANIAL ACTIVITY OF MEROTERPENOIDS FROM THE MACROALGAE *CYSTOSEIRA BACCATA*

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ANTILEISHMANIAL ACTIVITY OF MERODITERPENOIDS FROM THE MACROALGAE *CYSTOSEIRA BACCATA*

Carolina Bruno-de-Sousa[a], Katkam N. Gangadhar[a,b], Thiago R. Morais[c], Geanne A. A. Conserva[c], Catarina Vizetto-Duarte[a], Hugo Pereira[a], Márcia D. Laurenti[d], Lenea Campino[e,f], Debora Levy[g], Miriam Uemi[c], Luísa Barreira[a], Luísa Custódio[a], Luiz Felipe D. Passero[d,h], João Henrique G. Lago[c,*], João Varela[a,*]

[a] Centro de Ciências do Mar, Universidade do Algarve, Campus de Gambelas, Faro, Portugal

[b] Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal

[c] Departamento de Ciências Exatas e da Terra, Instituto de Ciências Ambientais, Químicas e Farmacêuticas, Universidade Federal de São Paulo, Diadema, SP, Brazil

[d] Laboratório de Patologia das Moléstias Infecciosas (LIM-50), Departamento de Patologia, Faculdade de Medicina, Universidade de São Paulo, São Paulo, Brazil

[e] Global Health and Tropical Medicine Centre, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisboa, Portugal

[f] Departamento de Ciências Biomédicas e Medicina, Universidade do Algarve, Campus de Gambelas, Faro, Portugal

[g] Laboratório de Genética e Hematologia Molecular (LIM-31), Departamento de Clinica Médica, Faculdade de Medicina, Universidade de São Paulo, São Paulo, Brazil

[h] São Vicente Unit, Paulista Coastal Campus, Universidade Estadual Paulista Julio de Mesquita Filho, São Vicente, SP, Brazil

* Corresponding authors:

Centre of Marine Sciences, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal. Tel.: +351-289-800-051; Fax: +351-289-800-051. *E-mail address*: jvarela@ualg.pt (J. Varela).

Departamento de Ciências Exatas e da Terra, Instituto de Ciências Ambientais, Químicas e Farmacêuticas, Universidade Federal de São Paulo, 09972-270, Diadema, SP, Brazil. Tel.: +55-(11)-3091-6513; *E-mail address*: joao.lago@unifesp.br (J.H.G. Lago).

4.1. ABSTRACT

The development of novel drugs for the treatment of leishmaniases continues to be crucial to overcome the severe impacts of these diseases on human and animal health. Several bioactivities have been described in extracts from macroalgae belonging to the *Cystoseira* genus. However, none of the studies has reported the chemical compounds responsible for the antileishmanial activity observed upon incubation of the parasite with the aforementioned extracts. Thus, this work aimed to isolate and characterize the molecules present in a hexane extract of *Cystoseira baccata* that was found to be bioactive against *Leishmania infantum* in a previous screening effort. A bioactivity-guided fractionation of the *C. baccata* extract was carried out and the inhibitory potential of the isolated compounds was evaluated via the MTT assay against promastigotes and murine macrophages as well as direct counting against intracellular amastigotes. Moreover, the promastigote ultrastructure, DNA fragmentation and changes in the mitochondrial potential were assessed to unravel their mechanism of action. In this process, two antileishmanial merorditerpenoids, (3*R*)- and (3*S*)-tetraprenyltoluquinol (**1a/1b**) and (3*R*)- and (3*S*)-tetraprenyltoluquinone (**2a/2b**), were isolated. Compounds **1** and **2** inhibited the growth of the *L. infantum* promastigotes (IC_{50} = 44.9 ± 4.3 and 94.4 ± 10.1 µM, respectively), inducing cytoplasmic vacuolization and the presence of coiled multilamellar structures in mitochondria as well as an intense disruption of the mitochondrial membrane potential. Compound **1** decreased the intracellular infection index (IC_{50} = 25.0 ± 4.1 µM), while compound **2** eliminated 50% of the intracellular amastigotes at a concentration > 88.0 µM. This work identified compound **2** as a novel metabolite and compound **1** as a biochemical isolated from *Cystoseira* algae displaying antileishmanial activity. Compound **1** can thus be an interesting scaffold for the development of novel chemotherapeutic molecules for canine and human visceral leishmaniases studies. This work reinforces the evidence of the marine environment as source of novel molecules.

Keywords: *Leishmania infantum*; macroalgae; *Cystoseira baccata*; meroterpenoids; tetraprenyltoluquinol; tetraprenyltoluquinone.

4.2. INTRODUCTION

Leishmaniases are a group of infectious diseases caused by obligate intracellular protozoa of the *Leishmania* genus. Endemic in 98 tropical and subtropical countries and affecting 12 million people, leishmaniases may entail cutaneous, mucocutaneous and diffuse forms as well as the potentially fatal visceral form (Alvar et al., 2012). Visceral leishmaniasis causes considerable morbidity in 200-400 thousand individuals every year, with extreme suffering and financial loss, especially in the poorest populations of the Indian subcontinent (Mondal et al., 2014). Currently, leishmaniases are among the most neglected tropical diseases, facing problems of resistance of the parasite to the available therapeutic molecules. The need for the discovery and development of alternative drugs allowing more efficient and effective treatments is thus quite urgent (Freitas-Junior et al., 2012).

Nowadays, marine natural products are recognized as powerful reservoirs of novel, chemically diverse molecules with wide applicability to health sciences (Tempone et al., 2011). Occurring worldwide, mainly in the rocky substrates of the Mediterranean Sea and the adjoining Atlantic coasts, *Cystoseira* C. Agardh (1820) genus encompasses 39 species of brown macroalgae (Guiry and Guiry, 2016). Several bioactivities such as anti-inflammatory, antiproliferative, antioxidant (Mhadhebi et al., 2011), enzyme inhibitory (Ghannadi et al., 2013), cytotoxic (Khanavi et al., 2010), antifungal (Lopes et al., 2013), antiviral (Ibraheem et al., 2012), antibacterial (Tajbakhsh et al., 2011) and antiprotozoal (Spavieri et al., 2010a) have been detected in this algal genus. Despite the extensive chemical studies available for the *Cystoseira* genus, there have been only a few reports describing the antileishmanial potential effects of its crude extracts, and no information was found on the compounds responsible for the inhibitory effects on the *Leishmania* parasites (Amico, 1995; de los Reyes et al., 2013). As part of ongoing research on the identification of antileishmanial compounds from the *Cystoseira* genus, this work describes the bioactivity-guided fractionation of the hexane extract from *Cystoseira baccata* and the effect of the extract, fractions and isolated compounds on the promastigote and amastigote forms of *Leishmania infantum*.

4.3. MATERIAL AND METHODS

4.3.1. GENERAL EXPERIMENTAL PROCEDURES

Optical rotations were measured in a JASCO DIP-370 digital polarimeter (Na filter, λ = 588 nm). UV spectra were recorded using a UV/visible Shimadzu 1650-PC spectrophotometer. IR spectra were obtained with a Shimadzu IR Prestige-21

spectrophotometer. ^{1}H, ^{13}C, DEPT, COSY, HSQC, HMBC and NOESY NMR spectra were recorded in a Bruker Avance III 500 spectrometer, operating at 500 and 125 MHz, to ^{1}H and ^{13}C nuclei, respectively. $CDCl_3$ (Aldrich) was used as the solvent with TMS as the internal standard. HRESIMS spectra were measured with a Bruker Daltonics MicroTOF QII spectrometer while LRESIMS spectra were recorded on a VG Platform II spectrometer. Silica gel (Merck, 230–400 mesh) and Sephadex LH-20 (Amersham Biosciences) were used for column chromatographic separation, while silica gel 60 PF_{254} (Merck) was used for analytical (0.25 mm) and preparative TLC (1.0 mm).

4.3.2. ALGAL MATERIAL

Cystoseira baccata biomass was collected in July 2012 in Areosa, Viana do Castelo, Portugal (41º42'27.60''N, 8º51'44.90''W). After collection, biomass was cleaned and cryodesiccated. Voucher specimen (MB-1) was deposited within the Laboratory of the Marine Biotechnology Group - MarBiotech at the Centre of the Marine Sciences of the University of Algarve (Faro, Portugal).

4.3.3. EXTRACTION AND ISOLATION OF COMPOUNDS

Dried and powdered biomass (120 g) was exhaustively extracted with hexane in a Soxhlet apparatus. After evaporation of the solvent under reduced pressure, 1.3 g of crude extract were obtained. Part of this extract (0.6 g) was subject to column chromatography over SiO_2 eluted with hexane containing increasing amounts of EtOAc (up to 100%), followed with $CHCl_3$ containing increasing amounts of MeOH (up to 100%), generating 13 fractions (1-13). As fraction 10 (370.0 mg) displayed activity towards promastigote forms of *L. infantum*, it was fractionated over SiO_2 column, and eluted with hexane:EtOAc 1:1 yielding 6 sub-fractions (A-F). Bioactive sub-fraction E (195 mg) was purified in a Sephadex LH-20 column being eluted with hexane:CH_2Cl_2 1:4, CH_2Cl_2:Me_2CO 3:2 and 1:1 (Cardellina II, 1983) originating 4 groups (E1-E4). Bioactive group E4 (65.3 mg) was subjected to preparative TLC (hexane-EtOAc, 7:3, twice) to afford compounds **1a**/**1b** (23.2 mg; 0.30%) and **2a**/**2b** (2.5 mg; 0.04%) (**Figure.4.1.**).

3R – tetraprenyltoluquinol (**1a**) and *3S – tetraprenyltoluquinol* (**1b**). Yellowish oil; ^{1}H NMR and ^{13}C NMR (500 MHz, $CDCl_3$) data, see **Table 4.1.**; LRESIMS *m/z* 441 $[M+H]^+$ and 463 $[M + Na]^+$ (calcd for $C_{28}H_{41}O_4$, 441, and $C_{28}H_{40}O_4Na$, 463, respectively).

3R – tetraprenyltoluquinone (**2a**) and *3S – tetraprenyltoluquinone* (**2b**). Colourless oil; $[\alpha]_D^{25}$ = + 0.06 (*c* 0.15, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 352 (2.0), 248 (3.4) nm; IR (KBr) ν_{max} 3400, 1670, 1480, 1180, 1060 cm^{-1}; ^1H and ^{13}C NMR (500 MHz, $CDCl_3$), see **Table 4.1.** and **Figure. 4.2.**; HRESIMS (positive mode) *m/z* 455.2776 $[M+H]^+$ and 477.2604 $[M+Na]^+$ (calcd for $C_{28}H_{39}O_5$ and $C_{28}H_{38}O_5Na$, 455.2797 and 477.2616, respectively).

4.3.4. PARASITES, MAMMALIAN CELLS AND ANIMAL MAINTENANCE

L. infantum strain (MHOM/PT/88/IMT-151) promastigotes were obtained from the cryobank of the Instituto de Higiene e Medicina Tropical (Universidade Nova de Lisboa, Portugal) and cultivated in M199 medium supplemented with 10% foetal bovine serum (FBS), penicillin (10 U/L), streptomycin (0.01 mg/L) and 2% of human male urine at 25 ºC. Peritoneal macrophages from BALB/c mice were cultivated in RPMI-1640 medium supplemented with 10% FBS, L-glutamine (2 mM), penicillin (50 U/L) and streptomycin (0.05 mg/L) at 37 ºC in humidified atmosphere with 5% CO_2. BALB/c mice were obtained in the Animal Facility of the School of Medicine of São Paulo University – Brazil. These animals were maintained in accordance with the institutional guidelines regarding the welfare of experimental animals and with the approval of the Animal Ethics Committee of São Paulo University (322/12).

4.3.5. ACTIVITY AGAINST LEISHMANIA PROMASTIGOTES

For the determination of the antileishmanial activity, *L. infantum* promastigotes in stationary phase (2×10^6 parasites/mL) were incubated with the hexane extract at a concentration of 250 μg/mL for 24h on 96-well plates. Using the same methodology, the fractions obtained during the bioactivity-guided fractionation were tested at a concentration of 50 μg/mL. At a later stage, compounds **1** and **2** were added at concentrations ranging from 0.9 to 227.0 and 0.9 to 220.0 μM, respectively. Parasites treated with miltefosine at the half maximal inhibitory concentration (IC_{50} = 23.1 μM) were used as positive control. Promastigotes incubated with M199 medium were used as negative control. Parasite viability was determined by the MTT colorimetric assay (Dutta et al., 2005; Dal Picolo et al., 2014). Briefly, after incubation plates were centrifuged at 10 ºC, using an RCF of 1479 × *g* for 10 min, washed three times with PBS, and supernatants discarded. Afterwards, 50 μL of MTT (5 mg/mL in PBS) were added to each well and plates were re-incubated at 37 ºC for 2 h. Upon incubation, 50 μL of SDS were added to each well and plates were incubated for 18 h in order to dissolve the formazan crystals. Absorbance was measured at 590 nm using a Thermo

Scientifc Multiskan™ FC Microplate Photometer. Results were expressed in terms of parasite viability (%) relative to non-treated parasites and the half maximal inhibitory concentration (IC_{50}; µM).

4.3.6. ULTRASTRUCTURAL ALTERATIONS OF THE PROMASTIGOTES

L. infantum promastigotes in stationary phase (2 x 10^6 cells/mL) were incubated at 25 ºC for 24 h on 96-well plates with compounds **1** and **2** at their IC_{50} values, i.e. 44.9 µM of 94.4 µM, respectively. Non-treated promastigotes were used as negative control. After incubation, the plate was centrifuged at 1479 × *g* for 10 min at 4 ºC, and washed with PBS three times. Pellets were fixed in 0.1% tannic acid dissolved in 2.0% glutaraldehyde in a 0.15 M phosphate buffer pH 7.2 and incubated for 1h at 4°C. These were afterwards contrasted in 1% osmium tetroxide and a 0.5% uranyl acetate solution for 12 h; then the samples were embedded in araldite resin (Yamamoto et al., 2015). Ultrathin sections (70 nm), obtained with a ultramicrotome Reichert and double contrasted with 2% uranyl acetate and 0.5% lead citrate, were examined using a JEOL 1010 transmission electron microscope.

4.3.7. PROMASTIGOTES DNA INTEGRITY

To detect whether the compounds induced fragmentation on *L. infantum* nuclear DNA, promastigote forms in stationary phase of growth (2 x 10^8 cells) were incubated with IC_{50} concentrations of compounds **1** (44.9 µM), **2** (94.4 µM) and hydrogen peroxide (6.2 µM) as an inductor of DNA damage in parasites (Das et al., 2001b) for 24 h at 25 ºC. Non-treated cells were used as control. After incubation, plates were centrifuged at 1479 × *g* for 10 min at 4 ºC, and the supernatants discarded. Parasites pellets were extracted with a Macherey-Nagel nucleoSpin® Blood kit according with the manufacturer recommendations and ran on a 2% agarose gel, 100 V for 90 min.

4.3.8. PROMASTIGOTE TRANSMEMBRANE MITOCHONDRIAL POTENTIAL

In order to evaluate the influence of compound **1** on the promastigote mitochondrial membrane potential ($\Delta\Psi m$), parasites in the stationary phase (2×10^6 parasites/mL) were incubated with compound **1** and miltefosine at their IC_{50} values (44.9 and 23.1 µM, respectively) for 24h on 96-well plates. Mitochondrial membrane potential was evaluated using the widefield automated microscope Mitoscreen Kit (BD Biosciences) according to the manufacturer's recommendations (Levy et al., 2014; Yamamoto et al., 2015). Briefly, cells were incubated with working solution, containing the JC-1 (5,5,6,6-tetrachloro-1,1,3,3-

tetraethylbenzimidazolylcarbocyanine iodide) fluorochrome, for 15 min at 37 °C in an atmosphere of 5% CO_2. ΔΨm induces the uptake of JC-1 monomers into the functional mitochondria. Once inside the organelle, JC-1 monomers aggregate, exhibiting high levels of red fluorescence and ΔΨm is assessed through the determination of the presence of JC-1 fluorochrome inside the mitochondria. ImageXpress® Micro XLS Widefield High-Content Analysis System and transfluor MetaXpress software were used to determine the presence of J-aggregates in nine sites per well and three wells per treatment. ΔΨm was expressed as a percentage of J-aggregates per cell.

4.3.9. CYTOTOXICITY AGAINST MURINE MACROPHAGES

To determine the compounds toxicity *in vitro*, murine peritoneal macrophages were seeded in RPMI-1640 at a density of 10^6 cells/mL and incubated overnight at 37 ºC in humidified atmosphere with 5% CO_2, allowing the cells to adhere to the plate background. Compounds **1** and **2** were tested for 24h at concentrations ranging from 0.9 to 227.0 and 0.9 to 220.0 µM, respectively. Miltefosine control cells were incubated with RPMI-1640 medium at concentrations from 3.8 up to 490.7 µM. Cell viability was evaluated by the MTT colorimetric assay (Ferrari et al., 1990; Dal Picolo et al., 2014), as described above, for the determination of the activity against *Leishmania* promastigotes. Absorbance was measured at 590 nm using a Thermo Scientific Multiskan™ FC Microplate Photometer. Results were expressed in terms of the cytotoxic concentration causing a 50% decrease in cell viability (CC_{50}; µM) relative to non-treated cells (100 %).

4.3.10. ACTIVITY AGAINST *LEISHMANIA* INTRACELLULAR AMASTIGOTES AND NO PRODUCTION

Peritoneal macrophages of BALB/c mice were collected by intraperitoneal lavage, seeded on 24-well plates (10^5 cells/mL) and incubated at 37ºC with 5% CO_2 during 2h for cell attachment. Afterwards, *L. infantum* promastigotes in stationary phase were added to each well at an infection ratio of 10 promastigotes per cell, being further incubated at 37 ºC for 24h. Infected macrophages were treated with compounds **1** and **2** at concentrations ranging from 7 to 90 µM to determine the corresponding IC_{50}. Supernatants were collected for nitric oxide (NO) determination after 24h and intracellular amastigote burden was microscopically assessed upon Giemsa staining for determination of the infection index (% of infected macrophages × internalized amastigote forms / macrophage; Passero et al., 2015) and the inhibitory concentration allowing 50% reduction of the infection index (IC_{50}) was estimated.

Miltefosine was used as positive control. Culture supernatants of treated and control macrophages were used for NO determination that was performed using the Measure-iT^{TM} High-Sensitivity Nitrite Assay Kit in accordance with the manufacturer's recommendations (Life Technologies). The NO concentration was determined using a calibration curve prepared with several known concentrations (2.75, 5.5, 11, 22, 33, 44 and 55 μM) of nitrite as standard. Results were expressed as NO production (μM) and compared with untreated infected and non-infected macrophages. The selectivity index (SI) was obtained by calculating the ratio of the CC_{50} of the macrophage by the IC_{50} of the intracellular amastigotes.

4.3.11. STATISTICAL ANALYSIS

Bioassays results were expressed as mean ± standard error of the mean (SEM) of replicates samples from at least two independent assays. The IC_{50} values were calculated fitting the data as a non-linear regression using a dose-response inhibitory model, in the GraphPad Prism V 5.0 program. Student's *t*-test was used to determine whether differences between means were significant at different levels ($p < 0.05$ and $p < 0.01$).

4.4. RESULTS AND DISCUSSION

The hexane extract from the *C. baccata* was incubated with promastigote forms of *L. infantum* for 24h, and cell viability was determined by means of the MTT assay. As this extract decreased the viability of the parasite by 74% at a concentration of 250 μg/mL, it was selected for further study. Bioactivity-guided fractionation afforded compounds **1** and **2** (**Figure 4.1.**).

Compound **1** was obtained as an optically active oil $[\alpha]_D = + 17.8°$ ($CHCl_3$, *c* 2.7). Structural evidence was obtained by analysis of NMR (^1H, ^{13}C and DEPT 135°), HREIMS spectra and comparison with those data previously reported in the literature to (3*R*)-(**1a**) and (3*S*)-(**1b**) tetraprenyltoluquinol, previously isolated from *C. baccata* (Valls et al., 1993b). In addition, some corrections in the attributions of chemical shifts of C-18 and C-19 in ^{13}C NMR spectrum were carried out, based on the HMBC spectral analysis (**Table 4.1., ANNEX 4-10**).

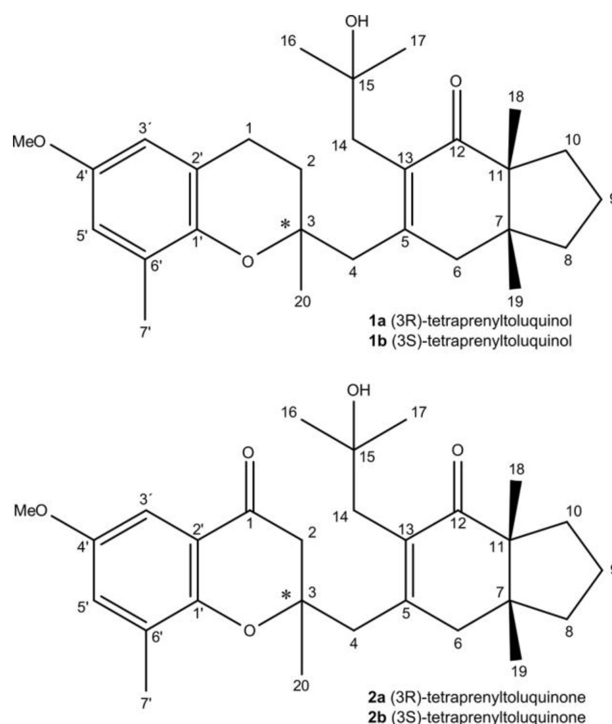

Figure 4.1. Structures of the tetraprenyltoluquinols (**1a-1b**) and tetraprenyltoluquinones (**2a-2b**) isolated from *C. baccata*

Compound **2,** also obtained as an optically active colourless oil $[\alpha]_D$ = + 0.06° ($CHCl_3$, *c* 0.15), appeared to be homogeneous on the TLC chromatograms, revealing that it is a mixture of closely related derivatives. The ^{1}H NMR spectrum of compound **2** revealed some similarities with compound **1** - two peaks assigned to hydrogens of aromatic ring at δ_H 7.15 (d, *J* = 3.0 Hz, H-3') and 7.00 (d, *J* = 3.0 Hz, H-5'), one methoxyl group at δ_H 3.78 (s) as well as five singlets assigned to methyl groups at δ_H 1.20 (H-20), 1.25/1.26 (H-17), 1.13/1.11 (H-16), 1.09/1.04 (H-18), and 0.91/0.83 (H-19). ^{13}C and DEPT 135° NMR spectra confirmed the presence of aromatic ring due the peaks at range δ_C 151.9 – 114.6 (C-1' – C-6'), and one methoxyl group at δ_C 55.7. Additionally, peaks assigned to a carbonyl group at δ_C 192.2/192.1 (C-1), to carbinolic carbons at δ_C 81.3/81.2 (C-3) and 71.0 (C-15) as well as an α,β-unsaturated carbonyl carbon at δ_C 153.3/154.3 (C-5), 133.5/134.0 (C-13) and 208.0/208.1 (C-12) were observed. Finally, HRESIMS showed the $[M+H]^+$ and $[M + Na]^+$ *quasi*-molecular ion peaks at *m/z* 455.2776 and 477.2604, respectively, indicating the molecular formula $C_{28}H_{38}O_5$. The connectivity between hydrogens and carbon atoms was revealed by analysis of the HMBC spectrum as showed in **Figure 4.2.** (**ANNEX 11-18**). The correlations between signals at δ_H 7.15 (H-3') and 2.56/2.57 (H-2) with δ_C 192.2/192.1 (C-1) as well as between δ_H

Compound	1a		1b		2a		2b	
Position	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	22.7, CH_2	2.79, (m)	22.6, CH_2	2.79, (m)	192.2, C	-	192.1, C	-
2	32.5, CH_2	1.80, (m)	33.6, CH_2	1.80, (m)	47.8, CH_2	2.56, (m)	48.6, CH_2	2.57, (m)
3	76.4, C	-	76.2, C	-	81.3, C	-	81.2, C	-
4	43.6, CH_2	2.66, (s)	45.2, CH_2	2.66, (s)	44.1, CH_2	2.70, (s)	44.8, CH_2	2.70, (s)
5	153.7, C	-	154.5, C	-	153.3, C	-	154.3, C	-
6	44.3, CH_2	2.57, (m) 2.60, (m)	44.7, CH_2	2.57, (m) 2.60, (m)	44.1, CH_2	2.60, (d, 4.0) 2.68, (d, 4.0)	44.1, CH_2	2.63, (d, 4.0) 2.68, (d, 4.0)
7	44.8, C	-	44.8, C	-	44.9, C	-	44.9, C	-
8	35.0, CH_2	1.54, (m) 1.73, (m)	35.0, CH_2	1.54, (m) 1.73, (m)	35.0, CH_2	1.54, (m) 1.73, (m)	35.0, CH_2	1.54, (m) 1.73, (m)
9	18.8, CH_2	1.74, (m)	18.8, CH_2	1.74, (m)	18.8, CH_2	1.74, (m)	18.8, CH_2	1.74, (m)
10	29.3, CH_2	1.46, (m)	29.3, CH_2	1.46, (m)	29.4, CH_2	1.40, (d, 13.0)	29.7, CH_2	1.42, (d, 13.0)
11	54.9, C	-	54.9, C	-	54.9, C	-	54.9, C	-
12	208.5, C	-	208.9, C	-	208.0, C	-	208.1, C	-
13	132.9, C	-	133.3, C	-	133.5, C	-	134.0, C	-
14	39.4, CH_2	2.45, (d, 15.0) 2.73, (d, 15.0)	39.9, CH_2	2.45, (d, 15.0) 2.73, (d, 15.0)	39.4, CH_2	2.54, (d, 15.0) 2.59, (d, 15.0)	39.6, CH_2	2.54, (d, 15.0) 2.59, (d, 15.0)
15	70.8, C	-	71.1, C	-	71.0, C	-	71.0, C	-
16	28.8, CH_3	1.12, (s)	28.8, CH_3	1.14, (s)	29.1, CH_3	1.11, (s)	29.1, CH_3	1.13, (s)
17	30.5, CH_3	1.24, (s)	31.6, CH_3	1.19, (s)	30.8, CH_3	1.25, (s)	31.3, CH_3	1.26, (s)
18	21.1, CH_3	1.09, (s)	21.1, CH_3	1.03, (s)	21.1, CH_3	1.04, (s)	21.1, CH_3	1.09, (s)
19	22.4, CH_3	0.91, (s)	22.5, CH_3	0.83, (s)	22.4, CH_3	0.83, (s)	22.5, CH_3	0.91, (s)
20	24.1, CH_3	1.28, (s)	24.5, CH_3	1.28, (s)	23.9, CH_3	1.20, (s)	24.2, CH_3	1.20, (s)
1'	145.2, C	-	145.3, C	-	167.8, C	-	167.8, C	-
2'	120.4, C	-	120.4, C	-	119.6, C	-	119.6, C	-
3'	111.1, CH	6.45, (d, 3.0)	111.2, CH	6.46, (d, 3.0)	114.6, CH	7.15, (d, 3.0)	114.6, CH	7.16, (d, 3.0)
4'	152.6, C	-	152.6, C	-	151.9, C	-	151.9, C	-
5'	115.2, CH	6.59, (d, 3.0)	115.3, CH	6.60, (d, 3.0)	104.5, CH	7.00, (d, 3.0)	104.5, C	7.01, (d, 3.0)
6'	127.0, C	-	127.2, C	-	126.5, C	-	126.5, C	-
Me-6'	16.6, CH_3	2.16, (s)	16.8, CH	2.17, (s)	16.2, CH_3	2.21, (s)	16.4, CH_3	2.23, (s)
OMe-4'	55.6, CH_3	3.73, (s)	55.6, CH	3.74, (s)	55.7, CH_3	3.78, (s)	55.7, CH_3	3.78, (s)

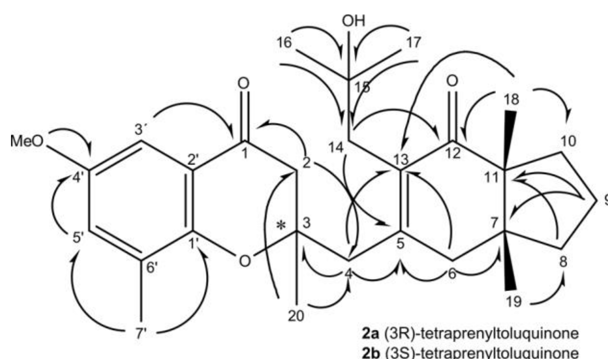

Figure 4.2. HMBC of the tetraprenyltoluquinones (**2a-2b**) isolated from *C. baccata*

2.70 (H-4) with δ_C 81.3/81.2 (C-3) and 133.5/134.0 (C-13) indicated that compound **2** ^1H and ^{13}C NMR data (500 and 125 MHz, $CDCl_3$, δ/ppm) for compounds **1** (**a**/**b**) and **2** (**a**/**b**) contained one additional carbonyl group at C-1. Based on these results, it was possible to identify **2** as epimers of (3*R*)-(**2a**) and (3*S*)-(**2b**) tetraprenyltoluquinones.

In vitro antiparasitic activity and cytotoxic studies of the compounds **1** and **2** were evaluated by the colorimetric MTT method against promastigote forms of *L. infantum* and murine macrophages, respectively (**Table 4.2.**). Compound **1** displayed an IC_{50} value of 44.9 ± 4.3 µM against promastigote forms of *L. infantum*. The cytotoxicity against mouse peritoneal macrophages (CC_{50} = 126.6 ± 21.1 µM) was similar to that of the reference drug, miltefosine (130.3 ± 17.2 µM). Compound **2** showed lower activity against the promastigote forms (IC_{50} = 94.4 ± 10.1 µM), and higher toxicity to the mouse peritoneal macrophages (CC_{50} = 84.5 ± 12.5 µM).

To assess the alterations induced by the compounds on the promastigotes forms of *L. infantum*, transmission electron microscopy images were acquired (**Figure 4. 3.**). Important changes were observed with both treatments, including loss of the typical fusiform shape (**Figure 4.3.A**). Ultrastructural analysis revealed morphologic changes in parasites treated with the IC_{50} concentrations of both compounds **1** (**Figures 4.3.B** and **4.3.C**) and **2** (**Figures 4.3.D** and **4.3.E**). Moreover, cellular vacuolization was observed, which might be a consequence of cytoplasmic organelle disruption (**Figures 4.3.B** and **4.3.D**). When treated with compound **1**, parasites presented coiled multilamellar structures within the mitochondria (**Figure 4.3.C**). These structures have been shown to be a consequence of starvation processes caused by deficient mitochondrial activity or autophagic mechanisms caused by the action of chemical compounds on these organelles (Lockshin and Zakeri, 2004). If left unchecked, both processes may result in the removal of the damaged organelles as well as cell death (Nishikawa et al., 2010). Previous studies have described similar structures in promastigotes of different *Leishmania* species treated with distinct natural products (Monte Neto et al., 2011). Compound **2** induced noticeable changes in the ultrastructure of the cell, in particular the occurrence of pyknotic nuclei, which was accompanied by the disappearance of the chromatin associated with the nuclear inner membrane (**Figure 4.3.D**).

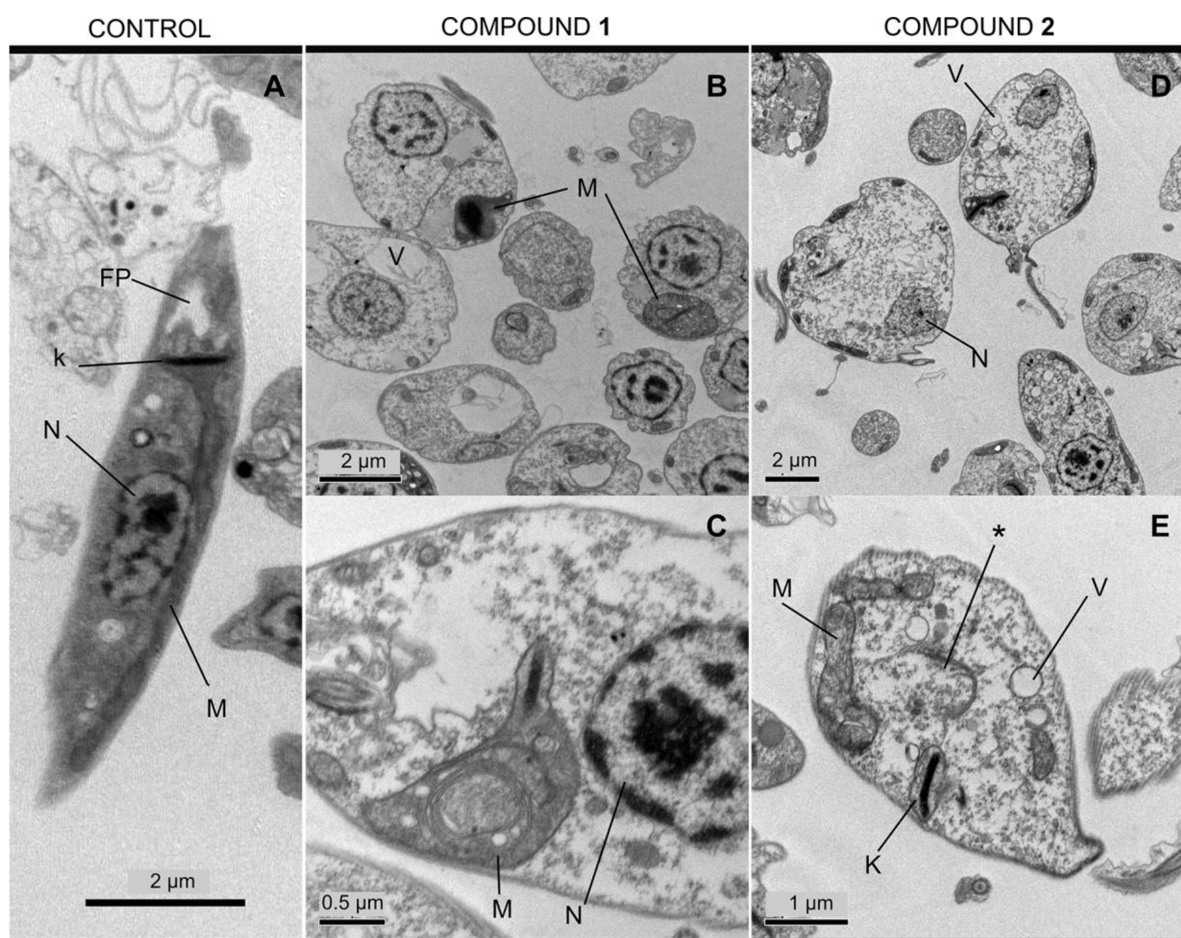

Figure 4.3. Effect of compounds **1** and **2** on the ultrastructure of *L. infantum* promastigotes. Parasites were treated with **1a/1b** (44.9 µM) and treated with compound **2a/2b** (94.4 µM). N – nucleus, FP - flagellar pocket, K – kinetoplast, M – mitochondrion, V – vacuole, * - disappearance of the chromatin associated with the nuclear inner membrane

Overall, these compounds seem to induce parasite death through different mechanisms. Other reports have shown that *Leishmania* apoptosis occurs in response to different drugs (Holzmuller et al., 2002). In order to evaluate if the alterations observed in the nuclei were associated with DNA fragmentation and consequently with programmed cell death, promastigote DNA was analysed through horizontal electrophoresis. This analysis did not reveal any fragmentation of the genomic DNA when promastigote forms of *L. infantum* were treated with the IC_{50} concentrations of compounds **1** and **2** (**Figure 4.5.A**), suggesting that the observed cytotoxic effect might not be associated with programmed cell death. Although chromatin condensation culminating in nucleolytic pyknosis is usually accompanied by macronuclear DNA digestion, generating oligonucleosomal fragments of low molecular weight (Kobayashi and Endoh, 2003), non-nucleolytic pyknotic processes have also been described previously (Burgoyne, 1999).

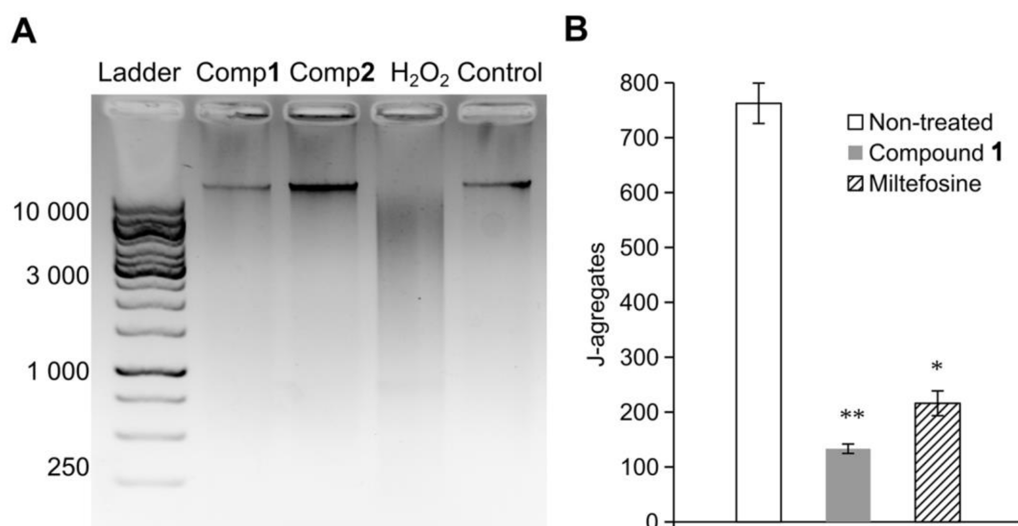

Figure 4.4. Effects of compounds **1** (Comp1) and **2** (Comp2) on the nuclear DNA fragmentation (A) and mitochondrial membrane potential (B) of *L. infantum* promastigotes. Parasites were treated with **1a/1b** (44.9 μM) and **2a/2b** (94.4 μM). Hydrogen peroxide (6.2 μM) (A) and miltefosine (23.1 μM) (B) and untreated parasites (A and B) were used as controls. *, $p < 0.05$; **, $p < 0.01$.

As *Leishmania* cells have a single mitochondrion, the proper functioning of mitochondria, including the stability of their membrane potential, is vital for the survival of the parasite. This organelle is usually considered as a good indicator of cellular dysfunction and therefore is an interesting target for chemotherapeutic studies (Souza et al., 2009). Because the variation of the mitochondrial membrane potential ($\Delta\Psi m$) in different *Leishmania* species exposed to various drugs has been reported (Britta et al., 2014) and that changes were observed in the morphology of the mitochondria of promastigotes treated with compound **1**, the $\Delta\Psi m$ in cells incubated with the latter chemical was evaluated. This was carried out in order to elucidate possible mechanisms of cell death induced by the compound displaying the most potent activity against *L. infantum* promastigotes. This parameter was determined by assessing the presence of JC-1 fluorochrome inside the mitochondria using a widefield automated microscope. $\Delta\Psi m$ induces the uptake of JC-1 monomers into the functional mitochondria. Once inside the organelle, JC-1 monomers aggregate, exhibiting high levels of red fluorescence. At the IC_{50}, compound **1** induced a significant ($p \leq 0.01$) decrease in fluorescence-emitting cells (133.3 ± 8.5 J-aggregates/well) as compared to non-treated (762.5 ± 36.7 J-aggregates/well) promastigotes (**Figure 4.5.B**), corresponding to a disruption of 83% of the $\Delta\Psi m$. This effect was higher than that observed with miltefosine (216.0 ± 22.6 J-aggregates/well), which disrupted the $\Delta\Psi m$ by only 72%. Interestingly, similar drops in $\Delta\Psi m$ coupled with changes in the mitochondrial ultrastructure have also been detected when using an iron chelator against *L. (V.) braziliensis* (Mesquita-Rodrigues et al., 2013).

When tested against *L. infantum*-infected macrophages the tetraprenyltoluquinol (**1**) applied at concentrations of 34 and 66 μM decreased the infection index by 64.5% and 66.3%, respectively, showing an IC_{50} of 25.0 ± 4.1 μM and a selectivity index of 5.04 against the peritoneal macrophages (**Figure 4.5A**; **Table 4.2**).

Only five compounds isolated from marine algae with antileishmanial activity have been reported previously (Machado et al., 2011; dos Santos et al., 2010, 2011; Soares et al., 2012). However, none of the studies was about *Cystoseira* macroalgae. Reported sesquiterpenes obtusol (IC_{50} = 9.4 μM; Machado et al., 2011) and elatol (IC_{50} = 13.5 μM and 0.45 μM) from the red alga *Laurencia dendroidea* (Machado et al., 2011; dos Santos et al., 2010) showed strong activity against *L. amazonensis* intracellular amastigotes. However, the triquinane sesquiterpene isolated from the same algae was significantly less effective (IC50 = 217.4 μM; Machado et al., 2011). In addition, 4-acetoxydolastane and dolabelladienetriol, isolated from the brown alga *Canistrocarpus cervicornis* (IC_{50} = 12.3 μM; dos Santos et al., 2011) and *Dictyota pfaffii* (IC_{50} = 44.0 μM; Soares et al., 2012), respectively, were also tested against the same species and form of *Leishmania*. Therefore, the activity of compound **1** was in the range of that reported for the aforementioned diterpenes.

Despite the lower activity of compound **2** against promastigotes (IC_{50} = 94.4 ± 10.1), it was higher than the effect reported for triquinane (IC_{50} = 195.5 μM) on promastigotes. However, similarly to what has been reported for triquinane (Machado et al., 2011), the treatment with the tetraprenyltoluquinone (**2**) did not decrease the infection index (**Figure. 4.5A**).

During the infection by *Leishmania*, NO is released by macrophages to eliminate intracellular amastigotes (reviewed by de Almeida et al., 2003). In addition, NO production can be triggered by natural compounds, including those from algae (Robertson et al., 2015). In the present study, infected peritoneal macrophages treated with compounds **1** and **2** produced low or undetectable amounts of NO as compared to controls. The NO released when the lowest concentrations (8.4 and 17 μM) were applied to the cells was residual, suggesting

Table 4.2. Effect of the compounds **1** and **2** against *L. infantum* promastigotes and intracellular amastigotes and mouse peritoneal macrophages

Compounds	Promastigotes[a]	Intracellular amastigotes[a]	Peritoneal macrophages[b]	SI[c]
1	44.9 ± 4.3	25.0 ± 4.1	126.6 ± 21.1	5.04
2	94.4 ± 10.1	> 88.0	84.5 ± 12.5	<0.96
Miltefosine	23.1 ± 0.0	20.3 ± 1.3	130.3 ± 17.2	6.42

[a]IC_{50} - Half maximal inhibitory concentration in μM; [b]CC_{50} - Cytotoxic concentration that causes the death of 50% of the viable cells in μM; [c]SI – Selectivity index concerning the activity against the intracellular amastigotes.

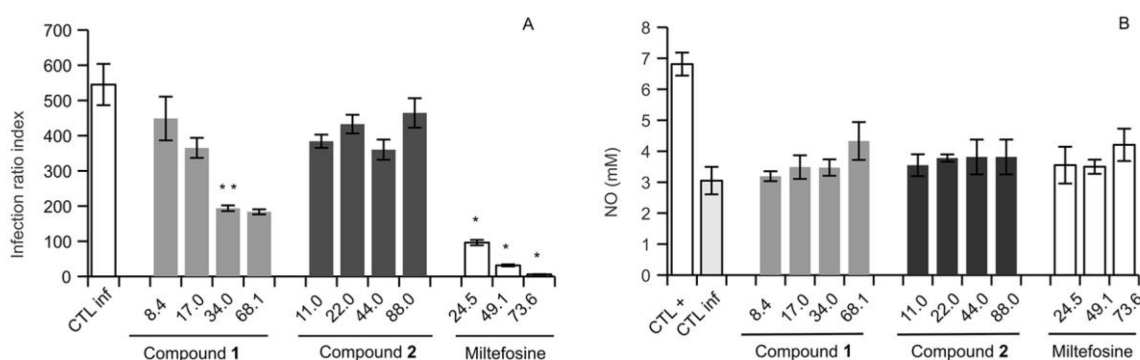

Figure 4.5. Effect of compounds **1** and **2** on the *L. infantum* intracellular amastigotes (A) and on the nitric oxide production (mM) of the infected mouse peritoneal macrophages (B) after a 24-h treatment with different concentrations (μM). Untreated non-infected macrophages (CTL+), untreated infected macrophages (CTL inf) and infected macrophages treated with a reference drug, miltefosine, were used as controls.

that the leishmanicidal effect observed for 1 was not related to NO production by the host macrophages (**Figure 4.5.B**) and that these compounds did not display an immunomodulatory effect. These results are in agreement with Machado et al. (2011) who observed that triquinane, elatol and obtusol did not promote enhanced NO levels, indicating that leishmanicidal effect of these compounds might be mediated by a mechanism that does not involve the release of this signalling molecule by the host cell.

In conclusion, this is the first report describing the identification of compounds from *Cystoseira* macroalgae displaying activity against *Leishmania* parasites. In addition, the isolation of tetraprenyltoluquinone (**2**) as a novel metabolite from algae of the *Cystoseira* genus is described. Concerning the particular chemical structure of these compounds, our data suggest that the presence of the carbonyl group in C-1 could play a role in the antileishmanial activity of the compounds **1** and **2**. Although not as active as miltefosine, tetraprenyltoluquinol (**1**) displayed significant antileishmanial activity and could be considered as an interesting scaffold for the development of novel chemotherapeutic molecules for canine and human visceral leishmaniases studies. Furthermore, this work reinforces the evidence of the marine environment as source of novel molecules.

4.5. ACKNOWLEDGMENTS

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CHAPTER V

A MT-BASED PHYLOGENY OF ATLANTIC-MEDITERRANEAN *CYSTOSEIRA* (FUCALES)

Research article *in preparation* to submit to *PLOS ONE*

A MT-BASED PHYLOGENY OF ATLANTIC-MEDITERRANEAN *CYSTOSEIRA* (FUCALES)

Carolina Bruno de Sousa[1], Cymon J. Cox[1], Luís Brito[1], Madalena Pavão[1], Ana Ferreira[2], Hugo Pereira[1], Lenea Campino[5], Ricardo Bermejo[3,4], Manuela Parente[6], João Varela[1,*]

[1] Centre of Marine Sciences, University of Algarve, 8005-139 Faro, Portugal. E-mail: carolbrunos@yahoo.com; cymon.cox@googlemail.com; lcbrito@gmail.com, madalenapavao@gmail.com, galvaohugo@gmail.com, jvarela@ualg.pt.

[2] Universidade dos Açores. Faculdade de Ciências e Tecnologia. Rua Mãe de Deus Apartado 1422 9501-855 Ponta Delgada Açores.

[3] Departamento de Biología. Área de Ecología. Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz. 11510 Puerto Real. Cádiz. Spain. E-mail: ricardo.bermejo@uca.es.

[4] Irish Seaweed Research Group & Earth and Ocean Sciences Department, Ryan Institute and School of Natural Sciences, National University of Ireland, Galway, Ireland.

[5] Global Health and Tropical Medicine, Instituto de Higiene e Medicina Tropical, IHMT, Universidade Nova de Lisboa, 1349-008 Lisboa, Portugal; lcampino@ihmt.unl.pt.

[6] CIBIO-Açores, Centro de Investigação em Biodiversidade e Recursos Genéticos, InBIO Laboratório Associado, Pólo dos Açores, Departamento de Biologia, Universidade dos Açores, 9501-801 Ponta Delgada, Portugal; nelaparente@hotmail.com.

* Corresponding author:
Centre of Marine Sciences, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal. Tel.: +351-289-800-051; Fax: +351-289-800-051. *E-mail address*: jvarela@ualg.pt (J. Varela).

5.1. ABSTRACT

Cystoseira C. Agardh is a common brown alga widely distributed throughout the Atlantic and Mediterranean whose taxonomical assignment of specimens is often hampered by intra- and interspecific morphological variability. In this study, three mitochondrial regions, namely cytochrome oxidase subunit 1 (COI), 23S rDNA (23S), and 23S-tRNA^{Val} intergenic spacer (mt-spacer) were used to analyse the phylogenetic relationships of 22 *Cystoseira* species (*n* = 93 samples). A total of 135 sequences (48 from COI, 43 from 23S and 44 from mt-spacer) were newly generated and analysed together with *Cystoseira* sequences (9 COI, 31 23S and 35 spacer) from other authors. Phylogenetic analysis of the three markers identified three well-resolved clades and corroborated the polyphyletic nature of the genus found in previous studies. Resolution of taxa within the three clades containing sequences of specimens classified as *Cystoseira* increased significantly when the inclusion of sequencing data from individuals of related genera was minimized. COI and mt-spacer markers resolved the phylogeny of some of the *Cystoseira* species, such as the *C. baccata, C. foeniculacea* and *C. usneoides*. Furthermore, trends between phylogeny, embryonic development and available chemotaxonomic classifications were identified, showing that phylogenetic, chemical and morphological data should be taken into account to study the evolutionary relationships among the algae currently classified as *Cystoseira*.

Keywords: Brown algae; *Cystoseira*; mitochondrial markers; phylogeny.

5.2. INTRODUCTION

Cystoseira (Fucales, Heteronkonphyta) brown algae are key elements of the marine seascape along warm-temperate North African and European coasts (Ballesteros et al., 2007; Thibaut et al., 2014; Bermejo et al., 2013, 2015). They form marine forests with a complex three-dimensional structure and provide a habitat for other algae, invertebrates and fish (Bellan and Bellan-Santini, 1972; Bulleri et al., 2002; Cheminée et al., 2013; Bermejo et al., 2016), playing a key role in the determination of biodiversity patterns and ecosystem functioning (Benedetti-Cecchi et al., 2001; Bulleri et al., 2002). Currently, many *Cystoseira* species are undergoing a strong demographic decline attributed to both local and global pressures (Thibaut et al., 2005; Mineur et al., 2015; Thibaut et al., 2015). Moreover, it has been suggested that this loss of biodiversity might be caused by the sensitivity of these species to increased water turbidity, eutrophication and pollution (Airoldi and Beck, 2007; Mangialajo et al., 2008; Sales et al., 2011), as consequence of the increasing anthropogenic activity near the Atlantic and Mediterranean coastal areas (Mineur et al., 2015; Thibaut et al., 2015). Because of the ecological importance of species assemblages dominated by *Cystoseira* and the deterioration of their populations during the past decades, the Mediterranean species of this genus are protected under the Barcelona Convention (Annex II, COM/2009/0585 FIN) and reforestation has been proposed as a management action to improve the conservation status of these species (Susini et al., 2007; Sales et al., 2011; Gianni et al, 2013).

The importance of the genus *Cystoseira* is further underscored by the observation that its members produce several potentially bioactive metabolites such as terpenoids, fatty acids, triacylglycerols, steroids, phlorotannins, and polysaccharides (Amico, 1995). Indeed, antioxidant, anti-inflammatory, antiproliferative, antifungal, antiviral, antibacterial and antiprotozoal activities have been reported to occur in *Cystoseira* algae with increasing frequency (Calvo et al., 1986; Spavieri et al., 2010a; Mhadhebi et al., 2011; Pujol et al., 2012; de los Reyes et al., 2016; Vizetto-Duarte et al., 2016a; Bruno de Sousa et al., 2017). This wide range of bioactivities detected in extracts of these algae might be explained by the bio- and chemical diversity of the genus (Amico, 1985b; Valls et al., 1993b).

The accuracy of the taxonomic identification of the biomass used for the isolation and identification of natural compounds is, however, an important issue concerning the reproducibility and reliability of the results as well as for the implementation of conservation measures for the target species (Leal et al., 2016). Taxonomic classification within the genus *Cystoseira* is challenging and controversial (Gómez-Garreta et al., 1994; Ballesteros and

Pinedo, 2004). Erroneous taxonomical assignments are frequent due to the wide morphological variability of *Cystoseira* individuals, in addition to there being many species that are still undergoing active speciation and hybridization (Roberts, 1978; Cormaci et al., 1992; Draisma et al., 2010). This has become especially apparent due to frequent conflits between classification of specimens based on morphology and molecular data. Chemotaxonomic classifications based on the presence or absence of specific chemicals (e.g. meroterpenoids) have also been attempted (Piatelli, 1990 cited by Amico, 1995; Valls et al., 1993b; Amico, 1995; Valls and Piovetti, 1995; Jégou et al., 2010). In addition, analysis of the global chemical profile and the lipophilic composition of five *Cystoseira* species from Brittany have been found to be in agreement with the phylogenetic relationships established by the ITS2 region (Jégou et al., 2010). However, congruence between morphology, chemistry and molecular taxonomy at the species level is yet to be achieved (Draisma et al., 2010), and the results obtained so far have not fully resolved the phylogeny of *Cystoseira* (Jégou et al., 2010).

Several authors have previoulsly attempted the elucidation of the relationships within this genus and with related genera using phylogenetic methods (Barceló-Martí et al., 2001; Draisma et al., 2010; Rožić et al. 2012). Analysis of Fucales (Phaeophyceae) Kylin based on large subunit (LSU) and small subunit (SSU) of the ribosomal DNA sequences led to the merging of the Cystoseiraceae and Sargassaceae families (Rousseau and de Reviers, 1999). The mitochondrial 23S ribosomal subunit (23S) proved to be useful for defining genera in the Fucales (Draisma et al., 2010) and in addition a set of 10 additional mitochondrial, plastid and nuclear markers has also been used to investigate the evolutionary history of brown algae at the ordinal level (Silberfeld et al., 2010). Other analysis including also organellar markers revealed that the genus *Cystoseira* was composed of at least six distinct evolutionary lineages, and clearly polyphyletic, although only 3 lineages (see below) were eventually classified as separated genera (Draisma et al., 2010). Based on morphologic, embryonic development characters and genetic data, several members of the genus were reclassified as belonging to the genera *Sirophysalis* (Tropical Indo-West-Pacific), *Polycladia* (eastern Indian Ocean) and *Stephanocystis* (N Pacific; Draisma et al., 2010). All other *Cystoseira* species, despite forming at least three separate NE Atlantic-endemic clades, retained the original classification. Currently, the genus *Cystoseira* encompasses approximately 40 species, the majority of which occurs in the Mediterranean and Atlantic-Mediterranean regions (García-Fernández and Bárbara, 2016; Guiry and Guiry, 2016). However, to date, full infrageneric resolution of the

genus and their position among related Sargassaceae genera has not been established. Therefore the taxonomy of the *Cystoseira* species is still unclear.

The mitochondrial gene coding for cytochrome oxidase subunit 1 (COI) is a well-known molecular tool used for the identification of different metazoan species (Arif and Khan, 2009; Hebert et al., 2003b; Sanaa, 2014). Although the COI gene was used in the study of red (Sherwood et al. 2010b) and brown algae (Mattio and Payri, 2010; McDevit and Saunders, 2009; Saunders and McDevit, 2013), the utility of this marker for the infrageneric identification of *Cystoseira* individuals has not been evaluated so far. With the purpose of improving the resolution of the *Cystoseira* species identification and clarify their phylogenetic relationships, a comprehensive study combining sequence information on the using cytochrome oxidase subunit 1 (COI), 23S rDNA (23S), and 23S-tRNA^{Val} intergenic spacer (mt-spacer) was undertaken. The results of this study confirm the polyphyly of the genus. Although the resolution of several *Cystoseira* species were improved with the addition of the protein-coding COI gene as suggested by previous authors (Draisma et al., 2010), other closely related taxa (e.g. *C. tamariscifolia* and *C. amentacea*) remained unresolved.

5.3. MATERIAL AND METHODS

5.3.1. SAMPLING

Overall, this study includes 93 samples of *Cystoseira* and 210 sequences belonging to 31 species of the Sargassaceae family (*Cystoseira*: 22 species; *Bifurcaria*: 1 species; *Polycladia*: 2 species; *Sirophysalis*: 1 species; *Stephanocystis*: 4 species and *Turbinaria*: 1 species; **ANNEX 19**).

Fifty-nine samples from 16 *Cystoseira* (55 individuals) species and *Bifurcaria bifurcata* (4 individuals) were collected along the Atlantic and Mediterranean coasts (**Figure 5.1.**), and mtDNA markers were specifically amplified. The samples, collected by the authors or kindly provided by expert coleagues, were morphologically classified using the taxonomic characteristics following Gómez-Garreta et al. (2001) and Guiry and Guiry (2016). After washed with tap water, a small portion of the biomass was separated, silica-dried and stored at room temperature for subsequent DNA extraction.

Additional sequences of *Cystoseira* (9 COI, 31 23S and 35 mt-spacer) and other Sargassaceae (3 COI, 8 23S and 8 mt-spacer) species deposited in the public GenBank database at the National Center for Biotechnology Information (NCBI) were included in the analyses (Benson

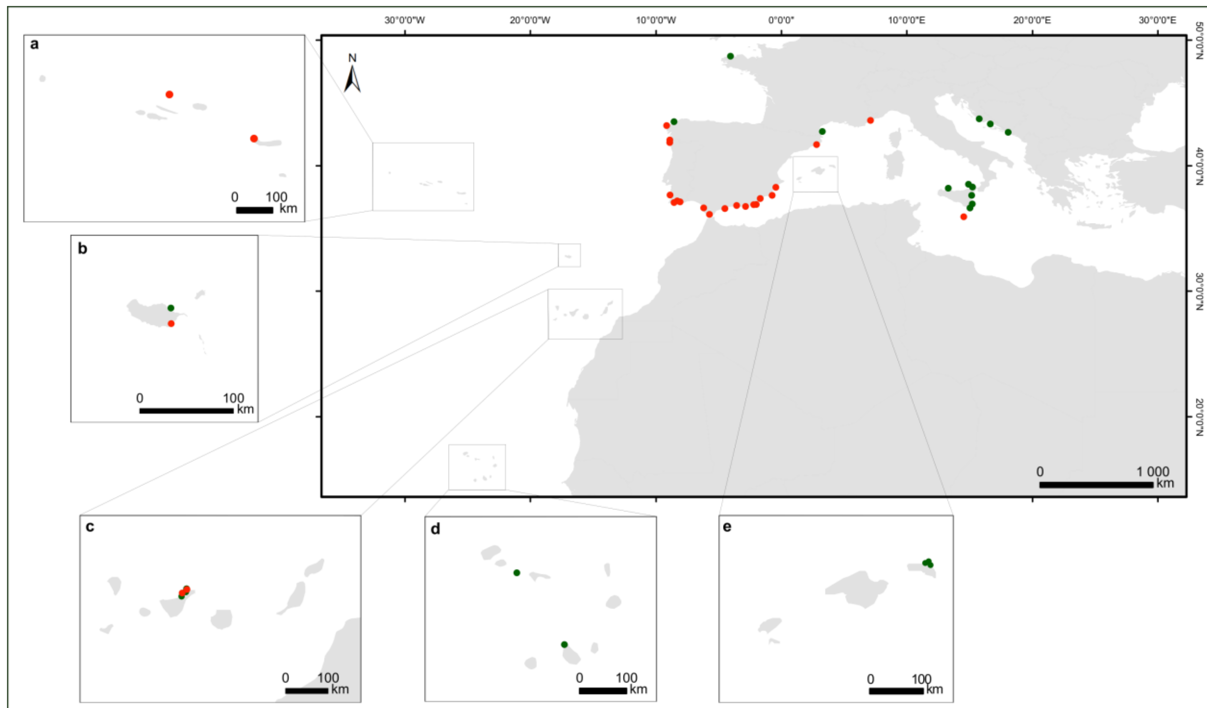

Figure 5.1. Geographical distribution of the *Cystoseira* samples used in this study. Symbols do not reflect the number of samples used for each location. Green dots represent GenBank sequences and the red dots data obtained from this study. The boxes show the archipelagos of (a) Madeira, (b) Azores, (c) Canary, (d) Cape Verde and (e) Balearics.

et al., 2013; **Figure 5.1.**). Similarly, sequences from 4 species of the Fucaceae family (4 COI, 4 23S and 4 IGS) were also obtained from GenBank and used as outgroups.

5.3.2. DNA EXTRACTION, AMPLIFICATION AND SEQUENCING

Genomic DNA was extracted from the silica gel-dried algal tissue using the method described by Doyle and Doyle (1987). The primers for amplification of the COI and 23S fragments were described by Lane et al. (2007) and Draisma et al. (2010), respectively. Moreover, primer pairs for amplification of the mt-spacer fragment were designed specifically for this study. Primer information, such as locus names, nucleotide sequences, and references are provided in **Table 5.1.**

Mt23S and mt-spacer were PCR-amplified in a final volume of 20.5 µL reactions containing 5 µL of genomic DNA (~10 ng/mL), 4 µL 5×PCR Buffer, 4 µL dNTP mix (1 mM of each dNTP), 2 µL 25 mM $MgCl_2$, 0.6 µL *Taq* DNA polymerase (GoTaq® DNA Polymerase, Promega), 0.5 µL of 10 µM 23S forward (mt23S-FB) and reverse (mt23S-RB) primers or 0.25 µL of 10µM mt-spacer forward (mt-spacer-F) and reverse (mt-spacer-R) primers. COI amplifications were perfomed in a 12-µL mix containing 2 µL of genomic DNA, 1.25 µL 5×PCR Buffer, 0.6 µL dNTP mix (1 mM of each dNTP), 1.25 µL 25 mM

Table 5.1. Molecular markers used in this study - locus name and target region, forward and reverse primer sequences, and references.

Target region	Primer	Sequence	References
23S	mt23S-FB mt23S-RB	5'-AGCGTAACAGCTCACTGACCTA-3' 5'-CTGTGGCGGTTTAAGGTACGGTT-3'	Draisma et al. (2010)
mt23S(partial)-IGS-tRNA^{Lys}-IGS-tRNA^{Val}	tRNALys-FW tRNALys-RV	5'-GGGGTGAAAAATATCACTTTGA-3' 5'-AACCCAAGACCCTCGGATTA-3'	This study
COI	GazF2 GazR2	5'-CCAACCAYAAAGATATWGGTAC-3' 5'-GGATGACCAAARAACCAAAA-3'	Lane et al. (2007)

$MgCl_2$, 0.1 μL *Taq* DNA polymerase, 0.25 μL of 10 μM COI forward (GazF2) and reverse (GazR2) primers. Amplifications were performed using an Applied Biosystems 2720 Thermal Cycler with the following conditions: 95 ºC for 6 min; 10 cycles of 95 ºC for 30 s, 64 ºC (decreasing 0.5 °C per cycle) for 30 s, 72 ºC for 60 s; 35 cycles of 95 ºC for 30 s, 59 ºC for 30 s, 72 ºC for 60 s; and a final elongation step of 10 min at 72 ºC for the 23S and mt-spacer fragments; for COI, samples were incubated at 95 ºC for 2 minutes; 5 cycles of 95 ºC for 30 s, 45 ºC for 30 s and 72 ºC for 1 min; 35 cycles of 95 ºC for 30 s, 46.5 ºC for 30s and 72 ºC for 1 min; and a 72 ºC elongation step for 7 min. PCR amplicons were screened for specific fragment size on 2 % agarose gel electrophoresis and subsequently purified using a E.Z.N.A.® MicroElute Cycle-Pure Kit (Omega Bio-Tek, USA) purification kit. Amplified fragments were sequenced using the Sanger method at the Molecular Biology Core Laboratory, Centre of Marine Sciences (Algarve University, Faro), in an 3130XL Genetic Analyzer (Applied Biosystems) using PCR primers in cycle sequencing reactions.

5.3.3. SEQUENCE VALIDATION AND GENETIC DIVERSITY

New sequences were compared with GenBank data using the basic local alignment search tool BLASTn (Altschul et al., 1990) to confirm the proximate identity of their biological source (i.e. that they were from Sargassaceae). GenBank accession numbers of the sequences are indicated in **ANNEX 19**.

Sequences were also organized in two datasets: one including only sequences from individuals of the *Cystoseira* genus, and the second comprising the same data plus those from the Sargassaceae and Fucales.

The 23S and mt-spacer sequences were aligned with the CLC Sequence Viewer V.7.6.1 (Quiagen), using the default settings. For COI, sequences were aligned with transAlign software (Bininda-Emonds, 2005) using ClustalW multiple sequence alignment (Higgins et al., 1996). Alignments were further inspected with CLC Sequence Viewer V.7.6.1 and

manually improved before a final curation step with Gblocks v.0.91b software (Talavera and Castresana, 2007) available at the Phylogeny.fr web service (Dereeper et al., 2008). Gap positions within the final blocks option were allowed and a maximum of 8 contiguous non-conserved positions were considered with a minimum block length of 5 nucleotides (nt). The concatenated matrix was obtained using Seaview v.4.5.3 (Gouy et al., 2010).

The number of polymorphic and phylogenetically informative sites of the aligned sequences was estimated for each marker using DnaSP v.5.10.1 software (Librado and Rozas, 2009). Haplotype identification was carried out for each mitochondrial marker using the same software and the respective Median-Joining (MJ) network of haplotypes was constructed using NETWORK version 4.5.10 software (Bandelt et al., 1999). Genetic distance analyses between *Cystoseira* sequences for each species were perfomed using MEGA5 software (Tamura et al., 2011). The pairwise distances for intra- and interspecific frequencies were estimated using the Kimura 2-parameter model (Kimura, 1980). The rate variation among sites was modeled with a gamma distribution (shape parameter = 6). All ambiguous positions were removed for each sequence pair.

5.3.4. PHYLOGENETIC RELATIONSHIPS

Phylogenetic analysis was carried out using Maximum likelihood (ML) and Bayesian inference (BI). The substitution models that best fit the data were selected using MrModeltest2 v.2.3 (Nylander, 2004) and PAUP* v.4.0b10 (Swofford, 2003) by applying the Akaike information criterion (AIC; Akaike 1974). The substitution models selected were: GTR+I+Γ_4 [general time-reversible (GTR) model with a proportion of invariant sites (I) and among-site rate variation modelled by a discrete gamma distribution with 4 categories (Γ_4)] for 23S, HKY+I+G [Hasegawa-Kishino-Yano model (HKY)] for COI and GTR+Γ_4 for the mt-spacer.

ML analysis was performed using RaxML v.7.0.4 (Stamakis, 2006) with 400 bootstrap replicates, assuming the best-fitting models. Posterior probabilities were determined by Markov Chain Monte Carlo (MCMC) sampling in MrBayes v.3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). MrBayes analyses were also conducted using the best-fitting models, using 6 chains for 10,000,000 generations, sampling every 1,000th generation, and default settings for the remaining options. Convergence of the MCMC and burn-in were determined through the analysis of the generations *vs.* log probability plot using the trace analysis tool TRACER v1.6 (Rambaut A., Suchard M.A., Xie D. and

Drummond A.J., 2014; http://beast.bio.ed.ac.uk/Tracer). The initial burn-in step discarded 20% of the samples.

After inferring the phylogeny for each marker (COI, 23S, mt-spacer) topological congruence between gene trees was visually assessed. Subsequently, the sequences obtained for the 3 markers were concatenated and analyzed by ML and BI as described before. ML and BI best consensus trees for each marker dataset (COI, 23S, mt-spacer, and concatenated COI-23S-mt-spacer) were generated and edited with the graphical viewer FigTree v.1.3.1 (Rambaut and Drummond, 2009).

5.4. RESULTS

5.4.1. ALIGNMENT CHARACTERIZATION

Overall, sequences from 92 *Cystoseira* samples belonging to 22 species from the Atlantic (Macaronesian and Iberian Peninsula south and west coasts) and the Mediterranean (Adriatic, Alboran, Balearic and Tyrrhenian seas) regions were included in this study (**Figure 5.1.**). Among these, the 55 *Cystoseira* samples collected for this study generated 135 new sequences representing a sequencing success of 87.3 % (48 sequences), 78.2 % (43 sequences) and 80.0 % (44 sequences) for COI, 23S and mt-spacer loci, respectively.

The conjoint analysis of *Cystoseira* sequences obtained during this study and from GenBank (56 COI, 74 23S and 79 mt-spacer sequences) resulted in alignments with 656, 391, 258 nt for COI, 23S and mt-spacer, respectively. Upon phylogenetic analysis, three lineages (Cystoseira-I, -II, -III) with support values close to the maximum (BS = 100; PP = 1) were identified (**Figures 5.2.-5.4.** and **ANNEXES 20-27**). Detailed information of the alignment results obtained for each marker and phylogenetic group is shown in **Table 5.2.**. Longer alignment lengths and higher number of conserved positions were observed for COI (656 nt; 86.1 %) and 23S (391 nt; 81.7 %) loci, and the lowest for mt-spacer (258 nt; 52.7 %).

Concatenation of the 3 loci (COI-23S-mt-spacer) consisted of a 1305-nt alignment with 78% of conserved positions. Depending upon the marker considered, 15.6-24.0% of polymorphic sites (PS) and 14.3-22.5% of parsimony informative (PI) sites were identified (**Table 5.2.**). Group Cystoseira-II showed the highest number of variable PS (7.9-13.2%) and PI (5.6-11.2%) for all loci, except for the 23S marker, where 6.4 % of PS were found (**Table 5.3.**). Group Cystoseira-III showed the lowest PS (2.6-7.0 %) and PI (2.3-6.2 %) values for 23S and mt-spacer loci, respectively.

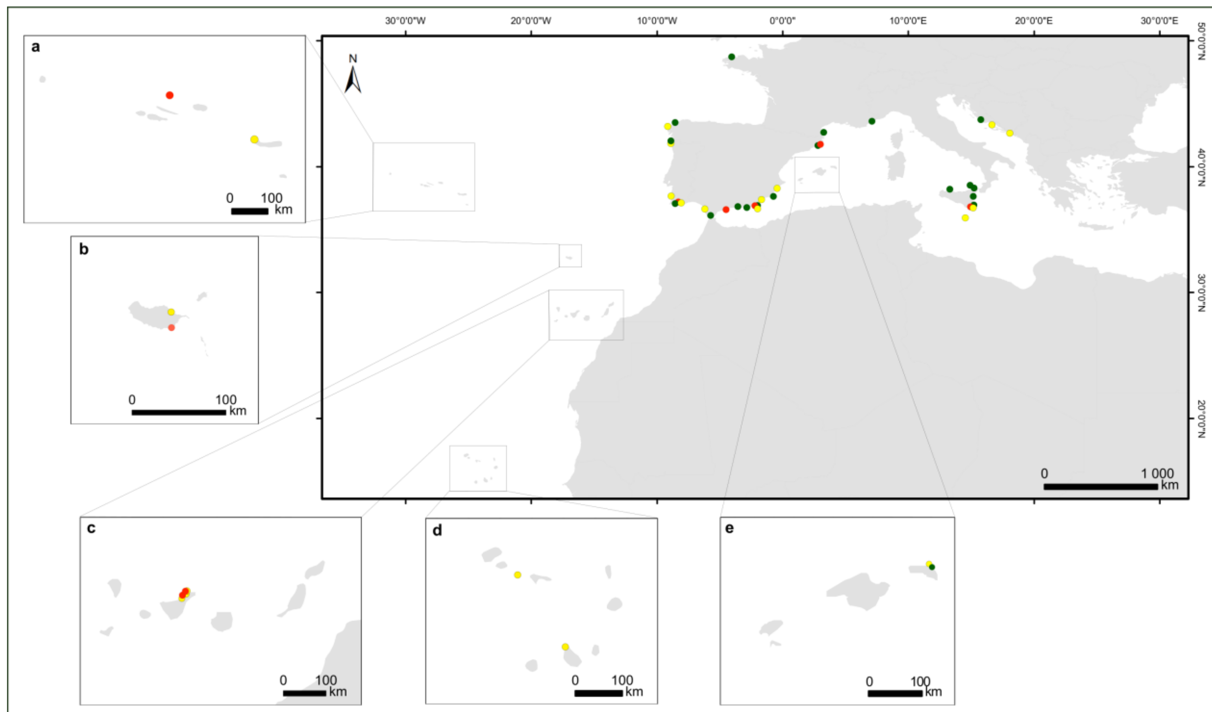

Figure 5.2. *Cystoseira* groups defined by the phylogenetic analysis. Green dots represent the species belonging to the Group I (*Cystoseira tamariscifolia*, *C. amentacea* and *C. amentacea* var. *stricta*, *C. funkii*, *C. mediterranea*, *C. brachycarpa* var. *brachycarpa*, *C. crinita*, *C. barbatula*, *C. zosteroides*, *Cystoseira* RB105 and *Cystoseira* sp. 1); yellow dots represent the species belonging to the Group II (*C. mauritanica*, *C. barbata* f. *repens*, *C. spinosa* and *C. spinosa* var. *tenuior*, *C. barbata*, *C. nodicaulis*, *C. granulata*, *C. elegans*, *C. squarrosa*, *C. usneoides*, *C. baccata*, *C. abies-marina*, *C. sonderi*, *Cystoseira* sp. 2 and *Cystoseira* sp. MP14); red dots represent the species belonging to the Group III (*C. compressa* and *C. compressa* var. *pustulata*, *C. humilis*, *C. humilis* var. *myriophyoides* and *C. foeniculacea*, *Cystoseira* sp. MP1, *Cystoseira* sp. MP2 and *Cystoseira* sp. MP31).

Interspecific evolutionary divergence of *Cystoseira*, when considered only the species that have information for all the 3 markers, ranged from 0 to 4.4 % in COI, 0-10.7 % in 23S and 0-10.9 % in the mt-spacer (**Table 5.4.** and **ANNEXES 28-30**). Highest amount of interspecific variation was observed in the Cystoseira-III (0-10.9 %), whereas Cystoseira-I species showed the lowest range of distances (0-1.1 %). Concerning intraspecific variation, lower values were observed, with a maximum of 3.9 % for the mt-spacer loci, followed by 23S (2.2 %) and COI (0.0 %). When considering all the samples included in the phylogenetic analysis, the inter- and intraspecific divergences were slightly higher (0.0-13.8%; 0.0-7.6%), a result of the higher heterogeneity of the species included. COI marker showed maximum interspecific genetic variability (0-4.4 %) and low intraspecific genetic variability (0.0-0.6 %).

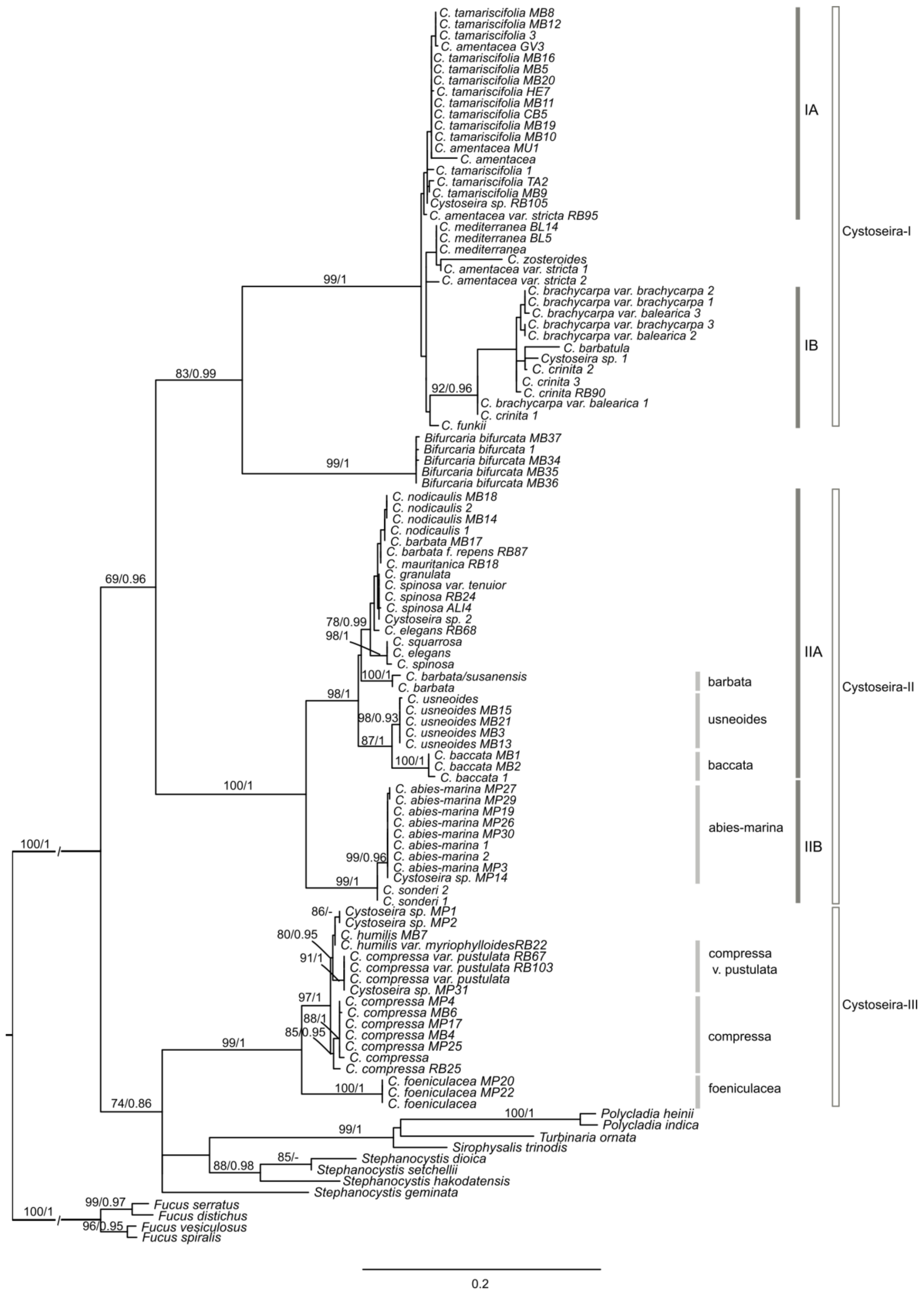

Figure 5.3. Maximum likelihood phylogenetic tree obtained with RAXML and based on the concatenated COI-23S-IGS sequences of samples from the Sargassaceae family. Values on the branches represent maximum likelihood bootstrap support values (≥ 75) on the left, and Bayesian posterior probabilities (≥ 90%) on the right.

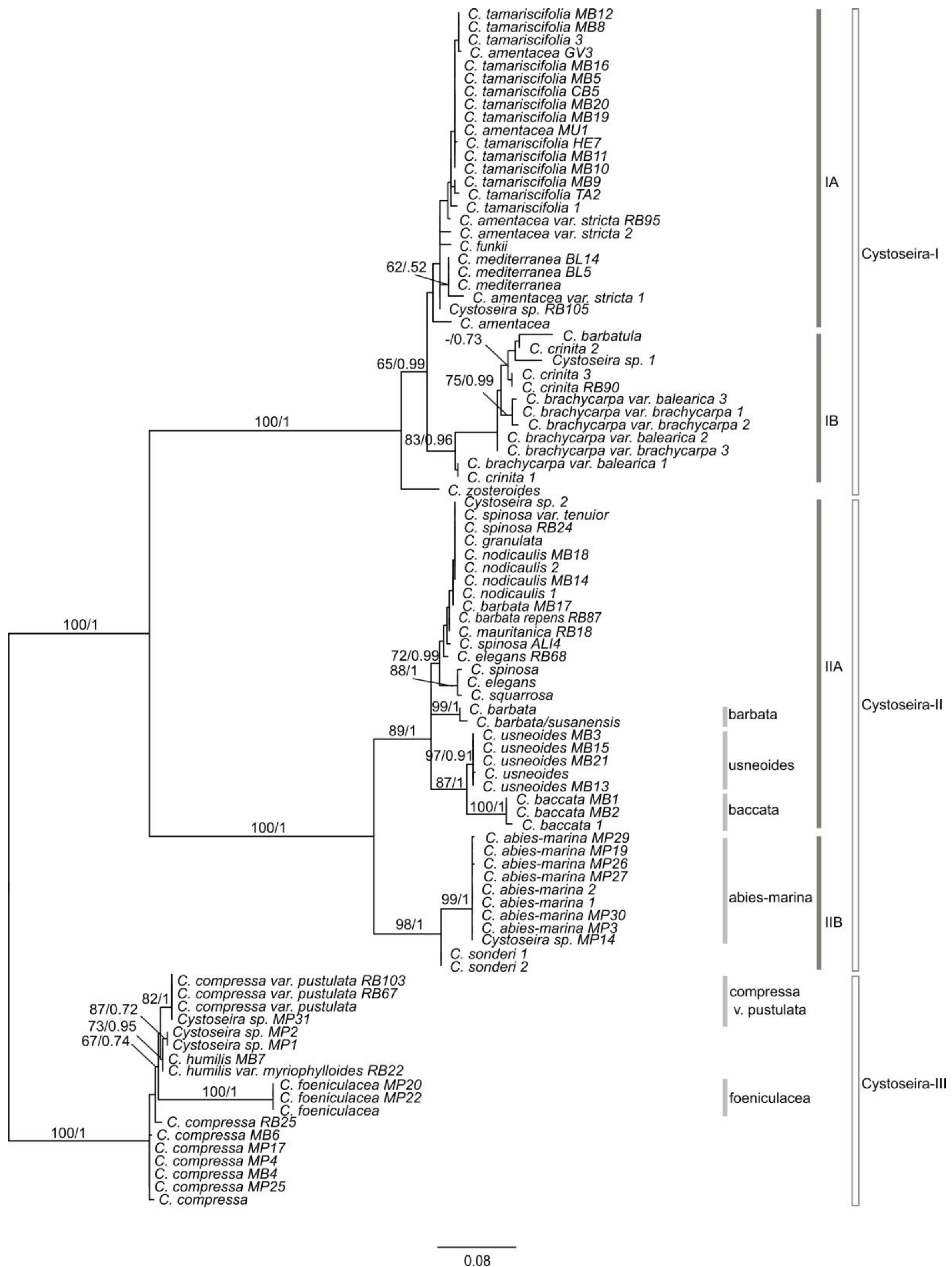

Figure 5.4. Maximum likelihood phylogenetic tree obtained with RAXML and based on the concatenated COI-23S-mt-spacer sequences of samples from the *Cystoseira* genus. Values on the branches represent maximum likelihood bootstrap support values (≥ 75) on the left, and Bayesian posterior probabilities (≥ 90%) on the right.

Table 5.2. Number of *Cystoseira* species and samples included in this study. Alignment characteristics (with gaps) are also shown for each marker and phylogenetic group.

Parameters	All species	Group I[1]	Group II[2]	Group III[3]
COI				
Species	13	3	7	3
Number of samples (sequences)	58	18	25	15
Alignment length (nt)	656	656	656	656
Conserved sites[a]	565 (86.1%)	648 (98.7%)	604 (92.0%)	535 (81.6%)
Polymorphic sites	114 (17.4%)	8 (1.2%)	52 (7.9%)	30 (4.6%)
Singleton variable sites	4 (0.6%)	1 (0.2%)	4 (0.6%)	2 (0.3%)
Parsimony informative sites	110 (16.7%)	7 (1.1%)	48 (7.3%)	28 (4.3%)
23S				
Species	20	8	9	3
Number of samples (sequences)	73	31	29	13
Alignment length (nt)	391	391	391	391
Conserved sites[a]	317 (81.7%)	335 (85.7%)	331 (84.7%)	352 (90.0%)
Polymorphic sites	61 (15.6%)	25 (6.4%)	22 (5.6%)	10 (2.6%)
Singleton variable sites	5 (1.3%)	10 (2.6 %)	0 (0%)	1 (0.3%)
Parsimony informative sites	56 (14.3%)	15 (3.8%)	22 (5.6%)	9 (2.3%)
IGS				
Species	21	7	11	3
Number of samples (sequences)	79	35	33	11
Alignment length (nt)	258	258	258	258
Conserved sites[a]	136 (52.7%)	183 (70.9%)	141 (54.6%)	168 (65.1%)
Polymorphic sites	62 (24.0 %)	25 (9.7%)	34 (13.2%)	18 (7.0%)
Singleton variable sites	4 (1.6%)	7 (2.7%)	5 (1.9%)	2 (0.8%)
Parsimony informative sites	58 (22.5%)	18 (7.0%)	29 (11.2%)	16 (6.2%)

[1]Group I - available individuals belonging to *Cystoseira tamariscifolia*, *C. amentacea* and *C. amentacea* var. *stricta*, *C. funkii*, *C. mediterranea*, *C. brachycarpa* var. *brachycarpa*, *C. crinita*, *C. barbatula*, *C. zosteroides*, and *Cystoseira sp.* 1; [2]Group II - available species belonging to *C. mauritanica*, *C. barbata* var. *repens*, *C. spinosa* and *C. spinosa* var. *tenuior*, *C. barbata*, *C. nodicaulis*, *C.granulata*, *C. elegans*, *C. squarrosa*, *C. usneoides*, *C. baccata*, *C. abies marina*, *C. sonderi*, *Cystoseira sp. 2* and *Cystoseira sp.* MP14; [3]Group III - available species belonging to *C. compressa* and *C. compressa* var. *pustulata*, *C. humilis*, *C. humilis* var. *myriophyoides* and *C. foeniculacea*, *Cystoseira sp.* MP1, *Cystoseira sp.* MP2 and *Cystoseira sp.* MP31. [a]Percentage calculated relative to the alignment length.

A total of 16 COI, 26 23S and 37 mt-spacer haplotypes were identified, in 58, 73 and 79 *Cystoseira* sp. individuals, respectively. Several haplotypes were exclusive to each *Cystoseira* group, and the Median-Joining analysis revealed three cleary independent networks correspondent to Cystoseira-I, -II and –III groups for each marker (**ANNEXES 31-33**). Only 21 haplotypes out of the 79 found were shared among at least two species of the same group. Among all, the Cystoseira-I species were those with the highest number of shared haplotypes (*n*= 11), followed by the Cystoseira-II species (*n*= 5), and Cystoseira-III with only 5 shared haplotypes per locus that are spread within the sub-groups .

Table 5.3. Comparison of the different *Cystoseira* phylogenetic groups defined in this study with the groups identified by other authors based on genetic, chemical and morphological traits.

Reference	This study	Draisma et al. (2010)	Amico (1995)[2]	Valls et al. (1993b)[3]	Piatelli (1990)[4]	Amico et al. (1985b)[5]	da Colombo et al. (1982)[6]
Type of data	Phylogeny		Chemistry			Morphology	
Species[1]	COI, 23S, IGS	23S	Lipophylic, diterpenoid and meroditerpenoid content			Anatomic traits	Embryo germination
C. amentacea	Cystoseira-IA	Cystoseira-5	VI	IIIB / IIIC	VII	I	I
C. funkii	Cystoseira-IA	Cystoseira-5	-	-	-	-	-
C. mediterranea	Cystoseira-IA	Cystoseira-5	VII	IIIB / IIIC	VII	I	I
C. tamariscifolia	Cystoseira-IA	Cystoseira-5	VII	IIIB / IIIC	VII	I	I
C. barbatula	Cystoseira-IB	Cystoseira-5	III	IIIA	III	-	-
C. brachycarpa	Cystoseira-IB	Cystoseira-5	II	II	II	II	I
C. crinita	Cystoseira-IB	Cystoseira-5	III	IIIA	III	II	I
C. zosteroides	Cystoseira-IC	Cystoseira-5	IV	IIIB	IV	III	I
C. baccata	Cystoseira-IIA	Cystoseira-6	V	IIIB	-	IV*	II
C. barbata	Cystoseira-IIA	Cystoseira-6	I	I	III	II	I
C. elegans	Cystoseira-IIA	Cystoseira-6	V	IIIA / IIIB	V	III	I
C. granulata	Cystoseira-IIA	-	-	-	-	-	-
C. mauritanica	Cystoseira-IIA	-	-	-	-	III	-
C. nodicaulis	Cystoseira-IIA	-	-	-	-	III	I
C. spinosa	Cystoseira-IIA	Cystoseira-6	V	IIIB	V	III	I
C. squarrosa	Cystoseira-IIA	-	IV	-	IV	III	-
C. usneoides	Cystoseira-IIA	Cystoseira-6	IV	-	-	III	-
C. abies-marina	Cystoseira-IIB	Cystoseira-6	-	-	-	II	-
C. sonderi	Cystoseira-IIB	-	-	-	-	-	-
C. compressa	Cystoseira-IIIA	Cystoseira-4	I	I	I	IV	III
C. humilis	Cystoseira-IIIA	Cystoseira-4	I	I	I	IV	III
C. foeniculacea	Cystoseira-IIIB	Cystoseira-4	-	IIIA	III	IV	III

[1] Conspecifity of taxa used by different authors (Guiry and Guiry, 2016): *C. amentacea = C. stricta; C. brachycarpa = C. balearica = C. caespitosa; C. barbata= C. susanensis; C. nodicaulis = C. granulata; C. spinosa = C. jabukae; C. squarrosa = C. spinosa* var. *squarrosa; C. foeniculacea = C. Ergovicii*; [2] Chemical groups based on the meroditerpenoids composition: Group I = no lipophilic secondary metabolites; Group II = linear diterpenoids; Group III = linear meroditerpenoids; Group IV = tetrahydrofurans, furans and pyran ring; Group V = cyclic meroditerpenoids; Group VI = Bicyclo[3.2.0]heptane ring system; Group VII = Rearranged meroditerpenoids; [3] Valls et al.'s chemical groups: Group I - No diterpenoids; Group II - Linear diterpenoids; Group III – Meroditerpenoids: III.A - Linear meroditerpenoids; III.B - Cyclic rneroditerpenoids; III.C - Rearranged meroditerpenoids; [4] Piatelli (1990)'s chemical groups on the chemical composition (Valls and Piovetti, 1995): Group I - no lipophilic secondary metabolites; Group II - linear diterpenoids; Group III - open-chain meroditerpenoids; Group IV - tetrahydrofurans and furans; Group V - cyclopentane ring; Group VI – bicyclo[4.3.0]nonane ring system; Group VII – bicyclo[3.2.0]heptane ring system; [5] Morphological groups based on the receptacle, conceptacle and axis characteristics: Group I = *C. ericaefolia* (*C. amentacea, C. mediterranea, C. tamariscifolia*); Group II = *C. crinito-selaginoides* (*C. abies-marina*, *C. barbata*, *C. brachycarpa*, *C. crinita*); Group III = *C. spinifero-opuntioides* (*C. elegans*, *C. mauritanica*, *C. nodicaulis*, *C. spinosa*, *C.squarrosa*, *C. zosteroides*); Group IV (*VI) = *C. discors-abratanifolioides* (*C. baccata*, *C. compressa*, *C. foeniculacea*, *C. humilis*), [6] Da Colombo et al (1982) identified morphological groups based on the embryo characteristics (see Amico et al., 1985b): Group I – Spherical embryo germination and 4 primary rhizoids; Group II - Spherical embryo germination and 4 primary rhizoids and different segmentation sequence; Group III – Ovoid embryo germination with 8 primary rhizoids.

Table 5.4. Evolutionary divergence between COI, 23S and mt-spacer *Cystoseira* sequences

Markers - Group	All *Cystoseira* samples		*Cystoseira* with information of the 3 markers*	
	Interspecific	Intraspecific	Interspecific	Intraspecific
COI - I	0.0 - 1.1	0.0 - 0.3	0.0 - 1.1	0.0
COI - II	0.0 - 6.8	0.0 - 0.5	0.0 - 3.8	0.0 - 0.5
COI - III	0.0 - 4.4	0.0 - 1.0	0.0 - 4.4	0.0 - 0.6
23S - I	0.0 - 4.9	0.0 - 2.2	0.0 - 2.3	0.0 - 2.2
23S - II	0.0 - 3.1	0.0 - 1.6	0.0 - 3.1	0.0 - 1.6
23S - III	0.0 - 11.0	0.0 - 0.3	0.0 - 10.7	0.0 - 0.3
mt-spacer - I	0.0 - 9.6	0.0 - 7.6	0.0 - 4.4	0.0 - 2.6
mt-spacer - II	0.0 - 13.8	0.0 - 3.9	0.0 - 8.9	0.0 - 3.9
mt-spacer - III	0.0 - 11.4	0.0 - 1.5	0.0 - 10.9	0.0 - 0.8

* samples without species identification were excluded

5.4.1. PHYLOGENETIC ANALYSIS

Maximum likelihood and Bayesian inference analyses of the Sargassaceae (**Figures 5.3.**) and Cystoseira-only (**Figures 5.4.**) concatenated datasets confirm the subdivision of *Cystoseira* in 3 well-suported clades (Cystoseira-I-III; **Figures 5.3., 5.4.** and **ANNEXES 20-25**). This subdivision was congruent among analyses using single mitochondrial markers (**ANNEXES 22-27**). Overall, the Cystoseira-III group, which includes *C. discors-abratanifolioides* species (*C. compressa, C. foeniculacea, C. humilis*; Amico et al., 1985b), clearly branched off Cystoseira-I (*C. amentacea*, *C. barbatula*, *C. brachycarpa*, *C. crinita*, *C. funkii*, *C. mediterranea*, *C. tamariscifolia*, *C. zosteroides*) and -II (*C. abies-marina*, *C. baccata*, *C. barbata*, *C. elegans*, *C. mauritanica*, *C. nodicaulis*, *C. sonderi*, *C. spinosa*, *C. squarrosa*, *C. usneoides*; **Table 5.3**). However, these results suggest that Cystoseira-I and -II are more closely related as compared to Cystoseira-III, sharing a common branch with maximum support (BS = 100; PP = 1; **Figure 5.4.**). Nonetheless Cystoseira-I and -II are paraphyletic when *Bifurcaria* is included in the analysis, as was observed with the Cystoseira-III taxa that clustered together with other genera from the Indio-Pacific region previously classified as *Cystoseira* (BS = 74; PP = 0.86), such as *Polycladia*, *Sirophysalis* and *Stephanocystis* (Draisma et al., 2010).

Cystoseira-I could be divided into two subgroups Cystoseira-IA and -IB (**Figures 5.3.** and **5.4.**). Cystoseira-IA (*C. amentacea*, *C. funkii*, *C. mediterranea*, *C. tamariscifolia*) formed a well-supported cluster (BS = 96; PP = 1) using mt-spacer sequences (**ANNEXES 26-27**), although without significant statistical support in the 23S analysis (**ANNEXES 24-25**). Within this group, *C. mediterranea* formed a cluster that was ML-supported in the COI tree

(BS = 99; PP = 0.93; **ANNEXES 22-23),** while *C. tamariscifolia* and *C. amentacea* remained unresolved. Subgroup Cystoseira-IB (*C. barbatula*, *C. brachycarpa*, *C. crinita*) was significantly supported in the concatenated datasets analysis (BS = 92; PP = 0.96; **Figure 5.3-5.4.**); however, in the 23S tree, support was highly significant (BS = 99; PP = 1; **ANNEXES 24-25**). This result suggests that *C. crinita*, *C. barbatula* and *C. brachycarpa* are indeed closely related. In addition, Cystoseira-I taxa clustered together with a well-supported *Bifurcaria bifurcata* cluster (BS = 94; PP = 1; **Figures 5.3** and **ANNEX 20**), confirming that they are sister taxa.

Cystoseira-II branched into two well-supported subgroups, Cystoseira-IIA (BS = 100; PP = 1) and Cystoseira-IIB (BS = 98/99; PP = 1; **Figures 5.3-5.4**). This high support is mainly due to the inclusion of the COI and mt-spacer markers (**ANNEXES 22-23)**. Analysis of the concatenated dataset showed that Cystoseira-IIA (*C. baccata*, *C. barbata*, *C. elegans*, *C. mauritanica*, C. nodicaulis, *C. spinosa*, *C. squarrosa*, *C. usneoides*) encompassed two well-resolved species, namely *C. usneoides* (BS = 98/97; PP = 0.93/0.91) and *C. baccata* (BS = 100; PP = 1) (**Figure 5.3-5.4.**). Maximum support of the *C. baccata* clade was also obtained in the COI tree (**ANNEXES 22-23**), whereas in the 23S tree the branch support values were lower (BS = 89; PP = 0.92; **ANNEXES 24-25**). *C. usneoides* cluster was supported by the ML analysis using the COI (BS = 96; PP = 0.54; **ANNEXES 22-23**) and 23S (BS = 94; PP = 0.92; **ANNEXES 24-25**) loci. In addition, Cystoseira-IIA included an unresolved heterogeneous set of species (**Figure 5.3-5.4.**) although the COI locus allowed the resolution of a *C. nodicaulis* cluster (BS = 86; PP = 0.99; **ANNEXES 22-23**). However, the presence of a well-supported heterogeneous cluster (BS = 98/88, PP = 1) encompassing 3 sequences acquired from the GenBank and classified as *C. spinosa*, *C. elegans*, *C. squarrosa* from the Adriatic and nearby Sicily Mediterranean coasts was not in agreement with the results of sequences of the same species obtained in the Spanish south Mediterranean coast (**ANNEX 19**). Sister to Cystoseira-IIA, Cystoseira-IIB contained *C. abies-marina* and *C. sonderi* and formed a well-supported cluster (BS = 99/98; PP = 1; **Figures 5.3-5.4.**) although this topology was not detected in the 23S analysis (**ANNEXES 24-25**).

Within the Cystoseira-III group, *C. foeniculacea* formed a clade with maximum support (BS = 100; PP = 1), sister to *C. compressa* and *C. humilis* as defined by all markers (**Figure 5.3.-5.4.** and **ANNEXES 20-21**). Although without significant support values (BS = 80/67, PP = 0.95/0.74 in **Figure 5.3.** and **5.4.**, respectively), *C. compressa* branched off *C. compressa* var. *pustulata* and *C. humilis.* These results are in agreement with some authors

(Gómez-Garreta et al., 2001; Giaccone and Bruni, 1973) that consider *C. compressa* var. *pustulata* a synonym of *C. humilis* var. *humilis*. These relations are better defined in the COI trees (**ANNEXES 22-23**) that suggest the occurrence of 3 independent clades: *C. compressa* (BS = 90, PP = 0.9), *C. humilis* (BS = 94, PP = 0.95) and *C. compressa* var. *pustulata* (BS = 96, PP = 1). Notwithstanding, the importance of COI to clarify the infrageneric phylogeny and improve the identification of *Cystoseira* samples is highlighted by *Cystoseira* sp. MP2 and *Cystoseira* sp. MP31. Even though these individuals were classified tentatively as belonging to other taxa, the trees based on COI sequencing data strongly suggested that they should be classified as *C. humilis* and *C. compressa* var. *pustulata*, respectively (**Figures 5.3.-5.4.** and **ANNEXES 22-23**).

5.5. DISCUSSION

The present study represents a comprehensive survey of the diversity of the genus *Cystoseira*, based on 92 samples from 22 different *Cystoseira* species and other Cystoseiracea. To the best of our knowledge, this is the first study using COI, 23S and mt-spacer sequences to investigate the phylogeny of the genus *Cystoseira*. In particular, this work contributed with 48 COI, 43 23S and 44 mt-spacer sequences from a wide geographic area (**Figures 5.1.-5.2.**), enlarging significantly the number of available sequences in GenBank. Additionally, emphasis was given to the *C. ericaefoli*a group (*C. tamariscifolia*, *C. amentacea* and *C. mediterranea*), whose phylogeny is still poorly clarified.

Compared to previous studies (Draisma et al., 2010; Silberfeld et al., 2010), *Cystoseira* sequences used in the present work had a relatively low number of phylogenetically informative sites (16.7% PI sites for COI, 14.3 % for 23S and 22.5% for mt-spacer). This might be explained by our focus on *Cystoseira* and the limited use of sequences of related genera in order to minimize the number of gaps in alignments of highly variable regions, such as the mt-spacer. Analyses of the interspecific divergence show results similar to values described for other algae (Saunders, 2005; Kucera and Saunders, 2008).

Fucales seem to have low zygote dispersal (Clayton, 1990; Guern, 1962) and, as a result, it is predicted that macrophytes belonging to this order show low intra-population genetic diversity, but larger differentiation among diferent regional populations (Coleman and Brawley, 2005; Susini et al., 2007). The inclusion of a wider array of closely related genera suggests, however, that Cystoseira-I and Cystoseira-III maintain more phylogenetic closely relations with species of other genera (*Bifurcaria*, *Policladia*, *Stephanocystis* and

Syrophysalis) than with the *Cystoseira* of the other groups (**Figures 5.3.**), making this genus, as noted by Draisma (2010), polyphyletic. Therefore, our results suggest that Atlantic-Mediterranean *Cystoseira*, currently defined, are not a natural group from an evolutionary point of view correspondig a distinct groups that should be taxonomically separated in 3 different genera.

The comparison of our results with those of other studies, including genetic, chemical and morphological information (Colombo et al., 1982 cited by Amico et al., 1985b; Amico et al., 1985b; Piatelli, 1990 cited by Valls and Piovetti, 1995; Valls et al., 1993b; Amico, 1995; Draisma et al., 2010; **Table 5.3.**), led to identification of similarities between species of these groups. Phylogenetic results corroborate the polyphyletic nature of the *Cystoseira* genus described previously (Rousseau and de Rivers, 1999; Draisma et al., 2010). A direct correspondence between our classification and that proposed by Draisma et al. (2010) was found, namely Cystoseira-I, Cystoseira-II and Cystoseira-III map to Cystoseira-5, Cystoseira-6, and Cystoseira-4, respectively. Concerning the morphology of some reproductive and support structures (receptacle, conceptacle and axis) described by Amico et al. (1985b), our group Cystoseira-I matches their Groups I and II. Moreover, Amico's Group III, known as *C. spinifero-opuntioides*, corresponded to the Cystoseira-II taxa of the present work. The only exception was *C. zosteroides*, which branches off early in trees either obtained in this study (**Figures 5.4.** and **ANNEXES 24-27**) or as described by Draisma et al. (2010), though often without statistical support. Amico's Group IV (*C. discors-abratanifolioides*) seems, however, to be heterogeneous, containing Cystoseira-II (*C. baccata*) and Cystoseira-III (*C. compressa*, *C. humilis* and *C. foeniculacea*) algae. Group III of da Colombo and colleagues (1982), based on criteria related to embryo germination (Amico et al., 1985b), matches Cystoseira-III taxa.

Although Draisma et al. (2010) discarded any connection between phylogeny and the published chemotaxonomic classifications, careful comparison between all traits can detect some trends. For example, linear diterpenoids and rearranged meroterpenoids (Piatelli, 1990 cyted by Valls and Piovetti, 1995; Valls et al., 1993b; Amico, 1995) are exclusive to Cystoseira-I taxa, which have been identified as the most developed group of species (Valls et al., 1993b), in agreement with the results obtained in this phylogenetic study. Unlike Cystoseira-I and –II algae, all Cystoseira-III taxa lack diterpenoids and lipophilic secondary metabolites, being thus defined not by presence of a given class of chemicals, but by its absence. Similar trends can even be observed at the sub-group level (**Table 5.3.**). For example, Cystoseira-IA and -IB taxa match chemical Groups VI/VII and Groups II/III as

described by Amico (1995), respectively. Another example would be the fact that Cystoseira II-B algae are restricted to Amico's chemical Groups I, IV and V. Interestingly, only *C. zosteroides*, which branches early off the remaining Cystoseira-I taxa, shares a similar chemical profile to Cystoseira II-A algae, namely *C. squarrosa* and *C. usneoides.* Taken together, these results suggest that there might be a closer relationship between phylogenetic, chemical and morphological classifications than previously thought.

The mt-spacer locus described as having high resolving power for *Fucus* spp. (Coyer et al., 2006) was considered to be useful only at a generic level for Sargassaceae (Draisma et al., 2010) and insufficiently informative to differentiate between the closely related *C. spinosa* and *C. squarrosa* species (Rožić et al., 2012). Despite these arguments and the high variability of mt-spacer, that can generate large gaps if the choice of taxa to include in the alignment is too divergent, *C. barbata*, *C. baccata* and *C. abies-marina* (Cystoseira-II), and *C. foeniculacea* (Cystoseira-III) were resolved from their closest relatives with significant support in mt-spacer trees.

Another question addressed by the present work is the difficulty to distinguish the closely related species *C. tamariscifolia*, *C. amentacea and C. mediterranea* only based on morphological criteria alone. Morphological plasticity, crypticism and seasonal variability in the appearance of these species often hinders and, in some cases, even prevents the accurate, unambiguous taxonomical assignment of the samples (Ballesteros and Catalán, 1981; Gómez-Garreta et al., 1994; Ballesteros and Pinedo, 2004). Thus, this reinforces the need for novel tools able to differentiate these species, especially in places where they coexist (Ballesteros and Catalán, 1981). Although the analyses using the 3 markers under study did not support the resolution of *C. tamariscifolia* from *C. amentacea* and, the COI trees show a well-supported cluster of *C. mediterranea.* Moderately high interspecific divergences with low intraspecific variations, as verified in the studied *Cystoseira* COI sequences, are considered to be prerequisites of a marker to be considered a suitable DNA barcode (Saunders and Kucera, 2010), thereby these results suggest that the COI could be useful to differentiate *Cystoseira* species, and in particular *C. mediterranea* from *C. tamariscifolia* and *C. amentacea*.

Even though other mitochondrial markers have been used to analyse the phylogeny of brown algae, the results of this study are consistent with those of Silberfeld et al. (2010), and also with those of Draisma et al. (2010) and Rožić et al. (2012) that studied 23S, mt-spacer and/or *psb*A loci. In certain cases, individual markers were shown not to be sufficiently informative to infer relationships between species (Draisma et al., 2010; Rožić et al., 2012).

Therefore, multi-gene datasets have been used to try to achieve greater phylogenetic resolution (Rousseau and de Reviers, 1999; Lane and Mayes, 2006; Draisma et al., 2010; Silberfeld et al., 2010; Vaidya et al., 2011; Lam et al., 2016). The phylogenetic trees obtained from the combined datasets used in this work (only *Cystoseira* samples, and *Cystoseira* together with other Sargassaceae) were congruent with previous phylogenies of Fucales (Philips et al., 2005; Cho et al. 2006; Harvey and Goff, 2006; Draisma et al., 2010; Rožić et al., 2012). Even though COI, 23S and mt-spacer markers resolved several taxa, the polyphyletic nature of the genus *Cystoseira* is a clear obstacle for further taxonomic resolution. As shown by Rousseau and de Reviers (1999) and Draisma et al. (2010), the Sargassaceae family includes a few polyphyletic genera, such as *Cystoseira*, *Sargassum* and *Bifurcaria*, and consequently there is still much to define within this family.

In spite of the current limitations, the comparative phylogenetic analysis of several Sargassaceae with three genetic markers and the divergence analysis enabled the authors to assign previously unidentified samples (*Cystoseira* sp. 1, *Cystoseira* sp. 2, *Cystoseira* sp. MP1, *Cystoseira* sp. MP14, *Cystoseira* sp. MP2, *Cystoseira* sp. MP31) to their respective taxa at the species level. In particular, we were able to classify the following samples: *Cystoseira* sp. 1 as *C. crinita* (Cystoseira-I); *Cystoseira* sp. 2 as *C. spinosa, Cystoseira* sp. MP14 as *C. abies-marina* (Cystoseira-II); and *Cystoseira* sp. MP31 as *C. compressa* var. *pustulata*, *Cystoseira* sp. MP1 and *Cystoseira* sp. MP2 as *C. humilis* (Cystoseira-III).

5.6. CONCLUSIONS

Comprising 22 different *Cystoseira* species and infra-generic taxa currently accepted, this work shows that the identification of the *Cystoseira* species using molecular markers is more effective when only closely related species are chosen in order to minimize the number and extension of gaps in the alignment of highly variable regions. The combined use of genetic markers with more conserved evolutionary signals allowed for a better resolution of the taxonomic relationships within this group of species. Gien the high variability of mt-spacer, this marker can be used in combination with COI to distinguish the majority of the *Cystoseira* species, resolving the phylogeny of several species of different groups, namely *C. barbata* and *C. baccata* (Cystoseira-II), and *C. foeniculacea* (Cystoseira-III). Even though there are quite a few exceptions, our results and the chemotaxonomic classifications suggest that the relationships of the phylogenetic, chemical and morphological classifications should not be promptly discarded. Moreover, our results suggest that European *Cystoseira*, as

currently defined, should be split into 3 separate genera, to reflect their different evolutionary histories, relationships with other genera, and genetic divergence.

Overall, these results strongly suggest that a combined effort should be carried out to further elucidate the observed relationships between taxonomy, chemical profiles, anatomical traits and phylogeny in order to clarify the phylogenetic relations of the studied three groups of *Cystoseira*.

5.7. ACKNOWLEDGMENTS

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CHAPTER VI

FINAL CONSIDERATIONS AND FUTURE PERSPECTIVES

6.1. FINAL CONSIDERATIONS

This work constitutes a pioneer study about the antileishmanial potentialities of the algae belonging to *Cystoseira* genus. In this context, our main aim was to identify algae metabolites active against *Leishmania* parasites. The decision to focus on *Cystoseira* species was taken after an initial screening of 15 red, green, and brown algae species that strongly indicated that algae from this genus contained compounds with antileishmanial potentialities (Chapter III). The comprehensive review carried out showed that *Cystoseira* contain compounds with several biomedical potentialities; however, the antileishmanial activity had never been described before for these species (Chapter II). Our work allowed to isolate two meroditerpenoid coumpounds from *C. baccata* with antileishmanial activity: one with an unknown tetraprenyltoluquinone structure, and the other previously isolated tetraprenyltoluquinol active against *Leishmania* intracellular amastigotes (Chapter IV). Considering that currently only 11 algal compounds are reported as active against these parasites (dos Santos et al., 2010; dos Santos et al., 2011; Machado et al., 2011; Soares et al. 2012), our results are extremely useful. Being the identification of the biomass origin a primordial issue in drug screening studies, the controversial taxonomical assignment of *Cystoseira* specimens raised questions concerning the accuracy of the identification of the samples used in this study. In this context, this project also evaluated the usefulness of mitochondrial markers for identification of *Cystoseira* species, complementing the previously published information on the genetics of these species, namely with major contributions for the knowledge of the COI region (Chapter V).

The focus of this study on the search for natural products active against *Leishmania* parasites is justified by the imperative need for molecules that overcome or inhibit the development of these parasites, in order to develop novel and effective antileishmanial therapies. Parasite resistance, limited therapeutic indexes, and considerable toxicities of the existing antileishmanial drugs have been entailing new challenges to the fight against leishmaniasis (Croft et al., 2006). In this sense, WHO (2013b) pointed out the need to reinforce the natural products drug research, traditionally recognized as having a large medical importance worldwide. Research on marine natural products (NPs) is nowadays accepted as a promising route to discover novel therapies against several diseases that needs to be enlarged in order to increase the chances of finding a candidate with strong and effective properties, in particular against leishmaniasis. In this context, our work constitutes a relevant contribution in line with the current needs and recommended priorities, providing natural

isolated structures that could be used as scaffolds for the design of new and target-specific bioactive molecules.

Afecting 12 million people in 98 developing and developed countries (Alvar et al., 2012), leishmaniasis is in active geographical expansion with 1.3 million human cases registered annually worldwide over the past two decades, being strongly associated with poverty, migration phenomena, and climatic and environmental changes (WHO, 2015a). In the Old World, *L. infantum* is the causative agent of visceral (VL), cutaneous (CL) and canine (CanL) leishmaniases, and the most relevant *Leishmania* species due to the fact that VL and CanL are potentially fatal if left untreated (Assimina et al., 2008; CFSPH, 2009). Endemic in all the southern European countries, infection by *L. infantum* has an annual burden in the Mediterranean Region of about 900 VL (Alvar et al., 2012) and more than 2.5 million CanL cases (Moreno and Alvar, 2002). With dogs as major host, and an increasing risk of infection in Europe (Ready, 2010, Mansueto et al., 2014) this zoonosis constitutes a serious and challenging public health and veterinary issue.

Algae contain hundreds of metabolites, and the search for the highly desired active NPs is a complex and long process (Bucar et al., 2013). The structure of the natural bioactive compounds is often complex, hindering its identification as well as isolation, and insufficient yields and purity can also become a limitation (Cechinel and Yunes, 1998). Therefore, the extraction and separation processes are challenging essential tasks for the success of the NPs isolation. Extract composition and bioactivity are strongly influenced by the polarity of the solvents and the solubility of the present molecules. In our case, antileishmanial activity was mostly detected in the hexane and CH_2Cl_2 extracts (Chapter III), corroborating published data which suggests that, in general, the active anti-protozoal compounds were found in non-polar fractions of the studied marine seaweeds (Vonthron-Sénécheau et al., 2011). Nonetheless, it is known that several other factors influence the ability to extract compounds of interest from a given sample (biomass amount; matrix solubility; stability of the interest compounds; solvents toxicity, volatility, viscosity and purity; artefacts presence; Bucar et al., 2013), and our review confirmed that antileishmanial activity can be found in extracts obtained with different solvents (**ANNEX 1**).

Concerning the drug screening for anti-leishmanial purposes, bio-guided fractionation assays are usually used in order to achieve an efficient isolation of the antileishmanial activity (Tempone et al., 2011). Therefore, dried biomass samples, collected at various sites of the Portuguese and Spanish coast, and morphologically identified, were efficiently extracted using

different organic solvents of increasing polarity (Hexane, CH_2Cl_2 and MEOH) using *Soxhlet* extractors. Forty-five extracts from 15 marine algae species were obtained and submitted to bio-guided fractionation for evaluation of its antileishmanial and cytotoxic activities. Fractionation assays were sequentially repeated until attainment of less complex fractions and isolation of the purified compounds, using liquid-solid (silica and Sephadex) and thin layer chromatography techniques (Chapters III and IV). The random screening of organic extracts to identify the molecules responsible for a biomedical activity requires fast, simple, and reproducible bioassays that allow to quantify the activity of interest (Sereno et al., 2007).

Bioassays for evaluation of the NPs efficiency can involve the manipulation of the different *Leishmania* forms: promastigotes and amastigotes (Tempone et al., 2011). The use of axenic promastigotes is widely used by many authors as an easier, faster, and low-cost alternative for preliminary screenings (Tempone et al., 2011), and were therefore chosen to evaluate the efficacy of the crude extracts/fractions on reducing the viability of *L. infantum* in the early stages of the fractionation process. The use of the MTT method and the axenic promastigote model allowed to determine the antileishmanial effect and the cytotoxicity against mammalian cells of a large number of extracts and fractions in order to select the less-toxic and most active algal fraction. As a result, seven of the 45 studied extracts displayed considerable activities against *Leishmania* promastigote forms (IC_{50} 29.8 to 101.8 μg/mL). Among these, *Cystoseira* genus stood out, with four species (*C. baccata*, *C. barbata*, *C. nodicaulis* and *C. tamariscifolia*) displaying significant activity against this parasite form, being the hexane and CH_2Cl_2 extracts of *C. tamariscifolia,* and the CH_2Cl_2 extract of *C. usneoides* the most active (IC_{50} 29.8 to 33.6 μg/mL) and similar to those described in literature for other algae (Freire-Pelegrin et al., 2008; Fouladvand et al., 2011; Chapter III).

Because of the intracellular requirement of *Leishmania* amastigotes infection, the *in vitro* amastigote–macrophage model is considered the gold standard to determine the drug sensitivity profile of an antileishmanial compound (Croft et al., 2011; Tempone et al., 2011). For these assays, macrophage susceptibility to the studied drug has to be firstly assessed, in order to find the most selective fractions/compounds, which ones are toxic to the parasite, and which are less or non-toxic to macrophages. In our case, the high toxicity revealed by the *C. usneoides* and *C. tamariscifolia* extracts against human acute monocytic leukaemia THP-1 cells prevented their use in the intracellular assays, even displaying potent anti-promastigote activities; on the other hand, the selectivity exhibited by the *C. baccata* and *C. barbata* hexane extracts allowed to proceed with the study of its effect towards the intracellular

amastigote model (Chapter III). In fact, these extracts were more active above the parasite intracellular form ($IC_{50} \leq 6.8$ µg/mL) than on promastigotes ($IC_{50} \geq 78.7$ µg/mL), proving the existence of differences in drug susceptibility of both parasite forms, and furthermore reinforcing the importance of using the clinically relevant stage of the parasite for the selection of the active and less toxic compounds.

The composition of the *Cystoseira* crude extracts that displayed antileishmanial activity was also investigated. This preliminary characterization showed that hexane extracts are composed of fatty acids, triacylglycerols derivatives, carotenoids, and steroids, whereas the CH_2Cl_2 extracts contain fatty acids and meroterpenoids, complementing the information published, as can be verified in the data compiled in Chapter II. According to recent reports, those compounds might be involved in the antileishmanial activity here evaluated. In particular, unsaturated fatty acids, such as the eicosapentaenoic acid; steroids, such as fucosterol and sitosterol; and different meroterpenoids, such as the ones detected in our *Cystoseira* extracts, were recently found to be responsible for reducing the viability of viscerotropic and dermotropic *Leishmania* species, as discussed in Chapter IV. These results suggest that identified metabolites may be related with the antileishmanial activity of the extracts here studied. However, a more detailed characterization of these metabolites should be carried out in the near future, in order to clarify their potentialities as antileishmanial agents.

Among all the other extracts presenting antileishmanial activity, *C. baccata* hexane extract was selected because of its lower toxicity against the mammalian cells, and the lower complex profile of its fractions. Bioactivity-guided fractionation of this extract allowed to isolate two meroditerpenoids with antileishmanial activity: (3*R*)- and (3*S*)-tetraprenyltoluquinol; and (3*R*)- and (3*S*)-tetraprenyltoluquinone. In practice, our work led to the identification of 4 compounds, since each of the mentioned meroditerpenoids was isolated as a mixture of two inseparable epimers (Chapter IV). Elucidation of the isolated compounds constituted a major challenge for us, in that it required the decoding of the information obtained from the different NMR (^{1}H, ^{13}C, DEPT 135º, HMBC, HSQC), UV and mass spectrometry spectra (**ANNEXES 2-18**). Tetraprenyltoluquinol was previously isolated from *C. baccata* by Valls et al. (1993b); however, due to the use of the additional HMBC spectral analysis, our study introduced some corrections to the structural elucidation, thus complementing the information already published. Concerning the tetraprenyltoluquinone, a comprehensive literature review allowed to conclude that it corresponds to an unidentified structure so far, being therefore a novel metabolite, here described for the first time.

Both isolated meroditerpenoids inhibited the growth of *L. infantum* parasites. The tetraprenyltoluquinol was shown to be the most effective in reducing the promastigote viability and intracellular amastigote infection index (IC_{50} ~ 44.9 and ~ 25.0 µM, respectively) followed by the tetraprenyltoluquinone (IC_{50} ~ 94.4 µM and > 88.0 µM, respectively). Assessment of its effect on mouse peritoneal macrophages showed that tetraprenyltoluquinol has a similar cytotoxicity (CC_{50} ~ 126.6 µM) to that of the reference drug miltefosine, largely used in the treatment of the VL and CL. Moreover, the tetraprenyltoluquinone showed to exert a higher toxic effect (CC_{50} ~ 84.5 µM), without compromising its intracellular evaluation (Chapter IV). These results clearly suggest that these compounds, in particular the tetraprenyltoluquinol, may be interesting as scaffolds for the development of novel chemotherapeutic molecules, namely for canine, and human visceral leishmaniases studies.

This study also provides relevant information for the identification of the mechanism of action of the *Cystoseira* extracts and the isolated compounds. Several morphological alterations and lack of externalization of phosphatidylserine were observed in promastigotes treated with the achieved active extracts, indicating that the observed toxic effect compromised the metabolism of vital organelles, such as mitochondria (Chapter III). These results were corroborated by the electron microscopy data that proved the mitochondrial commitment of the promastigotes treated with the isolated compounds (Chapter IV). Published data indicate that cytoplasmic vacuolization and the presence of coiled multilamellar structures observed in the mitochondria, as well as the disruption of the mitochondrial membrane potential could be a consequence of starvation processes of a deficient mitochondrial activity, autophagic mechanisms or cytoplasmic organelle disruption (Lockshin and Zakeri, 2004; Monte Neto et al., 2011) induced by the tested compounds. These results are relevant, since this organelle is usually considered an indicator of cellular dysfunction and is therefore a very interesting target for chemotherapeutic studies (Souza et al., 2009), particularly against *Leishmania* parasites that only have a single mitochondrion, vital for their survival.

Taken together, our results about the pharmaceutical potencialities of macroalgae indicate, for the first time, that *Cystoseira* extracts might be used as a source of compounds active against *L. infantum* which could be explored as scaffolds for the development of novel antileishmanial drug leads. The selective activity identified in *C. baccata* and *C. barbata* against intracellular amastigotes suggests that they should be chosen for further studying,

since they exhibited higher antileishmanial activity as compared to results described in similar reports on bioactivities found in macroalgae. In the context of the isolation of NPs, we can say that this study reinforces the evidence of the marine environment as source of novel molecules, and that *Cystoseira* algae are in fact a source of products potentially useful to fight against parasitic diseases such as Leishmaniasis.

Concerning, the usefulness of the mitochondrial markers for the correct identification of the *Cystoseira* samples, phylogenetic and population genetics approaches were followed to investigate the genetic relationships between different Atlantic-Mediterranean *Cystoseira* species.

Informational variability of a chosen marker influences its usefulness. In this study, three mitochondrial regions (COI, 23S and mt-spacer) with distinct evolutionary signals were chosen based on previous studies that generated *Cystoseira* sequences available at the GenBank database. Using the combined information of these genetic markers, the relationships between 22 Atlantic-Mediterranean *Cystoseira* species (n = 92), were analysed using Maximum likelihood and Bayesian inference methods as well as population genetics. The dataset comprised 55 samples specifically collected for this study, including those used in the chemical study, and generated 135 new sequences for the 3 markers (48 COI, 43 23S and 44 mt-spacer); other GenBank sequences were included, resulting in a global dataset of 210 *Cystoseira* sequences (58 COI, 73 23S and 79 IGS).

Phylogenetic and population genetics results were concordant indicating that Atlantic-Mediterranean *Cystoseira* species are clearly divided in three distinct *Cystoseira* natural groups (Cystoseira-I, -II and –III). The results are in agreement with the polyphyletic nature of *Cystoseira* previous described by Draisma et al. (2010). Moreover, the observation that Cystoseira-I, and –III are more closely related with other Sargassaceae genera, namely the *Bifurcaria*, and the Indo-Pacific *Policladia*, *Stephanocystis* and *Syrophysalis* than between what is observed the three *Cystoseira* groups is an indicator that the currently defined *Cystoseira* genus should be revised and probably divided into distinct genera.

Given the high variability of the mt-spacer, this marker allowed to distinguish the majority of the *Cystoseira* species and helped resolving the phylogeny of several species of different groups, namely *C. barbata* and *C. baccata* (Cystoseira-II), and *C. foeniculacea* (Cystoseira-III). Concerning the COI region, moderately high interspecific divergences and low intraspecific variations were observed; therefore, our results suggested its usefulness in differentiating among *Cystoseira* species, namely the highly similar species *C. mediterranea* from the *C. tamariscifolia* and *C. amentacea*.

Concluding, the attained results on *Cystoseira* genetics allowed to confirm the identification of the samples used in the chemical studies, validating the suitability of the studied mitochondrial markers for the identification of several *Cystoseira* species within each group (Cystoseira-I, -II and –III) and providing a reliable method structure for the study of the taxonomy, evolution and speciation history of this genus. Furthermore, the comparison between phylogenetic, chemotaxonomic and morphological classifications, previous published, showed congruent results between the different methodologies suggesting that these approaches should not be promptly discarded.

6.2. FUTURE PERSPECTIVES

This study constitutes a preliminary effort on the research of *Cystoseira* natural products for antileishmanial purposes, and we are certain that future investigations can further complement the knowledge here acquired.

The effect of the tetraprenyltoluquinol isolated in this study on *Leishmania* parasites justifies its further investigation, namely evaluating its *in vivo* antileishmanial efficacy on a mouse model; and clarifying what mechanisms are beyond the mitochondrial commitment and exploring other possible mechanisms of action. Moreover, the effect of tetraprenyltoluquinol should be evaluated against other *Leishmania* species, since different species reveal different drug responses and sensitivities. Regarding these issues, collaborations have been established, allowing to proceed with the testing of these molecules in other *Leishmania* species from both the Old and the New Worlds. Additionally, it would be interesting to expand the knowledge about the bioactive potentialities of this compound by testing it against other parasites such as the protozoan *Trypanossoma* species, responsible for the American and the African trypanosomiases.

All the active extracts obtained in this study should continue to be explored so as to find more compounds potentially active against *Leishmania* or other parasitic diseases. Particularly the extracts of *C. nodicaulis* and *C. tamariscifolia*, which have been discarded because of their low selectivity, may show pleasant surprises upon further fractionation, since the extracts are complex mixtures of compounds with different activities that mask each other. Moreover, other non-fully explored *C. baccata* and *C. barbata* fractions can be further investigated in order to isolate and identify more bioactive compound(s). For the chemical investigation a collaboration was established with a research group on the organic chemistry of NPs which will allow to continue the work of isolation and structural elucidation of coumpounds that could reinforce the contribute of this work for the fight against *Leishmaniasis*.

Concerning the identification of the *Cystoseira* samples, our results suggest that a combined phylogenetic/taxonomic/chemical approach should be carried out to further elucidate the phylogenetic relationships of the studied *Cystoseira* species. In particular, a more extensive sampling, to include specimens of all the studied species from different geographic origins, would allow to complement the genetic information obtained in this study with sequences of all the species for the three markers used here. Moreover, ecological data should also be investigated once these species have a large geographical intraspecific variability. In this sense, collaborations were arranged regarding expertise in DNA barcoding, morphological identification of *Cystoseira* specimens, biogeography and population genetics that could support future studies.

Finally, despite the potentialities displayed by COI, 23S and mt-spacer regions for the identification of the different *Cystoseira* species, we are certain that further studies using nuclear markers, such as the nuclear internal transcribed spacer (ITS), would lead to a more comprehensive perspective on the genetic relationships and evolutionary history of the *Cystoseira*.

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ANNEXES

ANNEX 1. Antileishmanial activities identify in marine algae extracts

Phylum Family	Algal species	Crude extract	IC_{50} (µg/mL)	Tested organism	Parasite form	References (a-p)
Chlorophyta						
Acrochaetiaceae	*Avrainvillea cf digitata*	DCM:H_2O	>100	*L. mexicana*	P[1]	d
Anadyomenaceae	*Anadyomene saldanhae*	Me_2CO	50/87.9*	*L. braziliensis*	P[1]	n
Caulerpaceae	*Caulerpa faridii*	EtOH	34.0	*L. major*	P[1]	a, o
	Caulerpa cupressoides	ME_2CO	50/51.7*	*L. braziliensis*	P[1]	n
	Caulerpa racemosa	EtOH	37.6	*L. major*	P[1]	a, o
		MeOH	22.7	*L. donovani*	AA[3]	l
	Cauluerpa sertularioides	H_2O hot H_2O cold	85.0 125.0	*L. major*	P[2]	h
Cladophoraceae	*Cladophora glomerata*	EtOH	39.2	*L. donovani*	AA[3]	c
	Cladophora rupestris	CF:MeOH	20.2	*L. donovani*	AA[3]	k
Codiaceae	*Codium bursa*	MeOH	31.71	*L. donovani*	AA[3]	l
	Codium elongatum	-	na*	*L. donovani*	-	b
	Codium flabellatum	EtOH	34.4	*L. major*	P[1]	a, o
	Codium fragile	CF:MeOH	16.6	*L. donovani*	AA[3]	k
	Codium iyengarii	EtOH	60.4	*L. major*	P[1]	a, o
	Codium tomentosum	EtOAc EtOH	9.7/0.0* 9.7/29.0*	*L. donovani*	AA3	m
Halimedaceae	*Halimeda incrassata*	DCM:H_2O	>100	*L. mexicana*	P[1]	d
	Halimeda tuna	DCM:H_2O	>100	*L. mexicana*	P[1]	d
Udoteaceae	*Penicillus dumetosus*	DCM:H_2O	>100	*L. mexicana*	P[1]	d
	Penicillus lamourouxii	DCM:H_2O	>100	*L. mexicana*	P[1]	d
	Rhipocephalus phoenix f. brevifolius	DCM:H_2O	>100	*L. mexicana*	P[1]	d
	Udotea conglutinata	DCM:H_2O	>100	*L. mexicana*	P[1]	d
	Udotea flabellum	DCM:H_2O	>100	*L. mexicana*	P[1]	d
Ulvaceae	*Ulva clathrata*	EtOAc EtOH	9.7/7.0* 9.7/0.0*	*L. donovani*	AA[3]	m
	Ulva fasciata	EtOH	50.0	*L. major*	P[1]	a, o
	Ulva intestinalis	CF:MeOH	14.9	*L. donovani*	AA[3]	k
	Ulva lactuca	EtOAc EtOH	9.7/11.0* 9.7/26.0*	*L. donovani*	AA[3]	m
		CF:MeOH	12.0	*L. donovani*	AA[3]	k
		EtOH	5.9	*L. donovani*	AA[3]	c
		EtOH	64.8	*L. major*	P[1]	a, o
	Ulva rigida	EtOH	65.7	*L. major*	P[1]	a
Heterokontophyta						
Acinetosporaceae	*Pylaiella littoralis*	CF:MeOH	47.1	*L. donovani*	AA[3]	j
Chordaceae	*Chorda filum*	CF:MeOH	21.1	*L. donovani*	AA[3]	j
Chordariaceae	*Leathesia difformis*	CF:MeOH	77.4	*L. donovani*	AA[3]	j
Dictyotaceae	*Canistrocarpus cervicornis*	ME_2CO	50/85.8*	*L. braziliensis*	P1	n
	Dictyopteris polypodioides	EtOAc	10.8	*L. donovani*	AA3	m
	Dictyota sp.	DCM:MeOH	50/93.3*	*L. braziliensis*	P[1]	n
	Dictyota dichotoma	EtOAc	8.8	*L. donovani*	AA[3]	m
		CF:MeOH	42.4	*L. donovani*	AA[3]	j

Phylum Family	Algal species	Crude extract	IC_{50} (µg/mL)	Tested organism	Parasite form	References (a-p)
	Dictyota caribaea	DCM:H_2O	24.4	*L. mexicana*	P^1	d
	Lobophora variegata	DCM:H_2O	49.9	*L. mexicana*	P^1	d
	Padina sp.	ME_2CO	50/80.9*	*L. braziliensis*	P^1	n
	Padina perindusiata	DCM:H_2O	>100	*L. mexicana*	P^1	d
Fucaceae	*Ascophyllum nodosum*	CF:MeOH	66.3	*L. donovani*	AA^3	j
	Fucus ceranoides	CF:MeOH	25.3	*L. donovani*	AA^3	j
	Fucus serratus	EtOAc EtOH	9.7/14.0* 9.7/15.0*	*L. donovani*	AA^3	m
		CF:MeOH	34.1	*L. donovani*	AA^3	j
	Fucus spiralis	CF:MeOH	34.3	*L. donovani*	AA^3	j
	Fucus vesiculosus	CF:MeOH	33.0	*L. donovani*	AA^3	j
	Pelvetia canaliculata	CF:MeOH	35.7	*L. donovani*	AA^3	j
		EtOAc EtOH	9.7/37.0* 9.7/32.0*	*L. donovani*	AA^3	m
Fucophyceae	*Dictyota dichotoma*	EtOH	52.0	*L. donovani*	AA^3	c
Himanthaliaceae	*Himanthalia elongata*	CF:MeOH	64.7	*L. donovani*	AA^3	j
		EtOAc EtOH	9.7/43.0* 9.7/40.0*	*L. donovani*	AA^3	m
Laminariaceae	*Laminaria digitata*	CF:MeOH	34.5	*L. donovani*	AA^3	j
		EtOAc EtOH	9.7/8.0* 9.7/11.0*	*L. donovani*	AA^3	m
Phaeophyceae	*Turbinaria turbinata*	DCM:H_2O	10.9	*L. mexicana*	P^1	d
Phyllariaceae	*Saccorhiza polyschides*	CF:MeOH	31.8	*L. donovani*	AA^3	j
Sargassaceae	*Bifurcaria bifurcata*	CF:MeOH	6.4	*L. donovani*	AA^3	j
		EtOAc EtOH	3.8 9.7/40.0*	*L. donovani*	AA^3	m
	Cystoseira baccata	CF:MeOH	15.7	*L. donovani*	AA^3	j
	Cystoseira barbata	MeOH	23.5[a] 69.9[b]	*L. donovani*	AA^3	l
	Cystoseira crinita	MeOH	28.2	*L. donovani*	AA^3	l
	Cystoseira tamariscifolia	CF:MeOH	19.6	*L. donovani*	AA^3	j
		HEX ETH CF H_2O	n.d >100 >100 n.d	*L. infantum*	P^2	o
	Halidrys siliquosa	CF:MeOH	8.6	*L. donovani*	AA^3	j
	Sargassum fluitans	DCM:H_2O	>100	*L. mexicana*	P^1	d
	Sargassum muticum	EtOAc EtOH	9.7/48.0* 9.7/37.0*	*L. donovani*	AA^3	m
		CF:MeOH	34.7	*L. donovani*	AA^3	j
	Sargassum natans	EtOH	90.9	*L. donovani*	AA^3	c
	Sargassum oligocystum	H_2O hot H_2O cold	78.0 105.0	*L. major*	P^2	h
		H_2O hot H_2O cold	78.0 105.0	*L. major*	P^2	h
Scytosiphonaceae	*Colpomenia peregrina*	CF:MeOH	29.1	*L. donovani*	AA^3	j
	Scytosiphon lomentaria	CF:MeOH	34.3	*L. donovani*	AA^3	j
Stypocaulaceae	*Halopteris scoparia*	EtOH	>100	*L. donovani*	AA^3	c
Stypocaulaceae	*Stypocaulon scoparium*	CF:MeOH	30.4	*L. donovani*	AA^3	j

Phylum Family	Algal species	Crude extract	IC_{50} (µg/mL)	Tested organism	Parasite form	References (a-p)
Rhodophyta						
Acrotylaceae	*Claviclonium ovatum*	CF:MeOH	61.2	*L. donovani*	AA[3]	f
Bangiaceae	*Porphyra leucosticta*	CF:MeOH	90/0.0*	*L. donovani*	AA[3]	f
	Porphyra linearis	CF:MeOH	55.5	*L. donovani*	AA[3]	f
Bonnemaisoniaceae	*Asparagopsis armata*	HEX EtOH EtOAc MeOH	480.43 149.50 ≥500.0 ≥500.0	*L. infantum*	P[2]	p
		HEX DCM EtOH-Hex:EtOAc EtOH-EtOAc EtOH-EtOAc:MeOH EtOH-MeOH EtOH-H_2O	>40.0 >40.0 10.0 19.0 na na na	*L. donovani*	P[2]	e
	Asparagopsis taxiformis	HEX DCM EtOH-Hex:EtOAc EtOH-EtOAc	17.0 16.0 14.0 20.0	*L. donovani*	P[2]	e
Bornetieae	*Bornetia secundiflora*	HEX EtOH EtOAc MeOH	≥500.0 188.3 150.7 ≥500.0	*L. infantum*	P[2]	p
Ceramiaceae	*Centroceras clavulatum*	EtOH	57.9	*L. major*	P[1]	a
		EtOH	57.9	*L. major*	P[1]	i
	Ceramium nitens	DCM:H_2O	>100.0	*L. mexicana*	P[1]	d
	Ceramium rubrum	MeOH	16.8	*L. donovani*	AA[3]	l
	Ceramium virgatum	CF:MeOH	25.6	*L. donovani*	AA[3]	f
	Halurus flosculosus	EtOAc	9.7/49.0*	*L. donovani*	AA[3]	m
Champiaceae	*Champia salicornioides*	DCM:H_2O	>100	*L. mexicana*	P[1]	d
	Chylocladia verticillata	CF:MeOH	47.3	*L. donovani*	AA[3]	f
Corallinaceae	*Corallina granifera*	MeOH	35.02	*L. donovani*	AA[3]	l
	Corallina officinalis	CF:MeOH	22.7	*L. donovani*	AA[3]	f
	Jania capillacea	DCM:H_2O	>100.0	*L. mexicana*	P[1]	d
		HEX EtOH EtOAc MeOH	≥500.0 ≥500.0 ≥500.0 197.1	*L. infantum*	P[2]	p
	Jania rubens	CF:MeOH	60.7	*L. donovani*	AA[3]	f
		MeOH	28.0	*L. donovani*	AM[3]	l
Cystocloniaceae	*Calliblepharis jubata*	CF:MeOH	49.8	*L. donovani*	AA[3]	f
		EtOAc EtOH	9.7/40.0* 9.7/34.0*	*L. donovani*	AA[3]	m
	Cystoclonium purpureum	CF:MeOH	67.3	*L. donovani*	AA[3]	f
Dasyaceae	*Dasya pedicellata*	MeOH	23.0	*L. donovani*	AA[3]	l
	Heterosiphonia gibbesii	DCM:H_2O	>100.0	*L. mexicana*	P[1]	d
Delesseriaceae	*Cryptopleura ramosa*	CF:MeOH	85.6	*L. donovani*	AA[3]	f

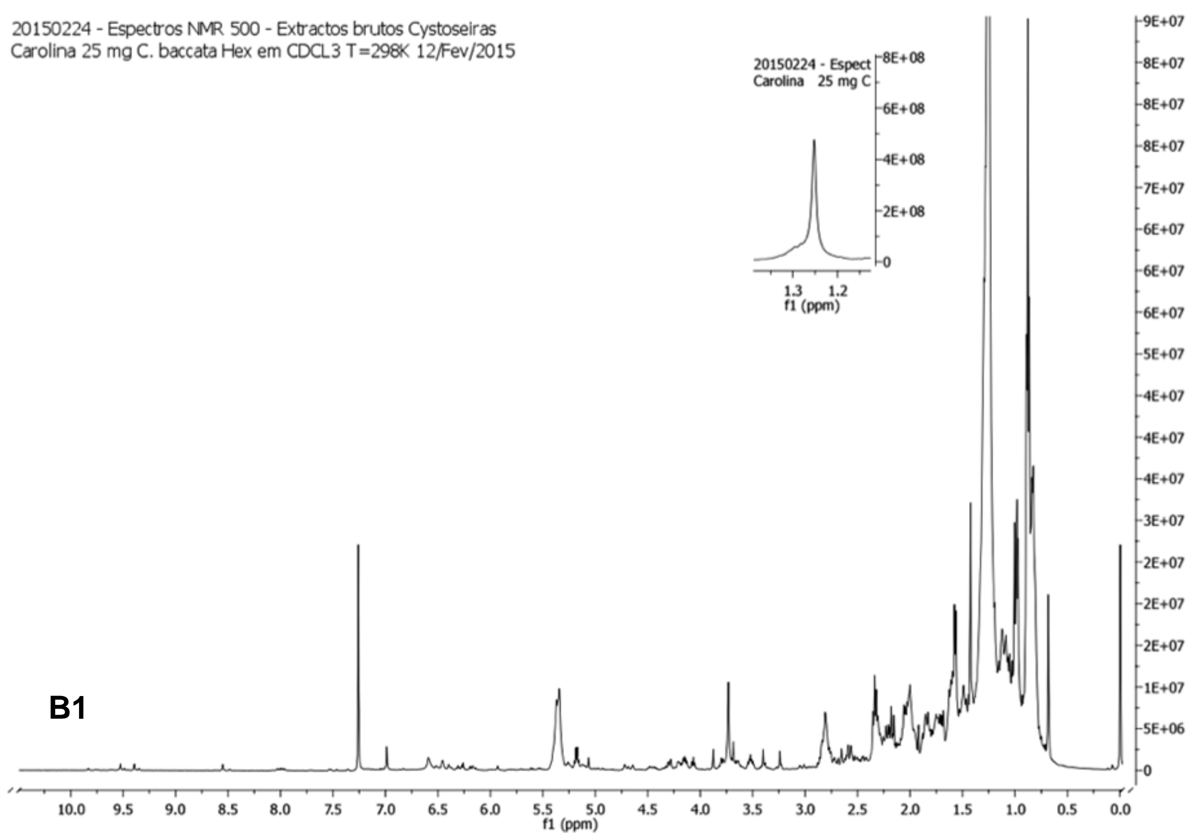
Phylum Family	Algal species	Crude extract	IC_{50} (µg/mL)	Tested organism	Parasite form	References (a-p)
Dumontiaceae	*Dilsea carnosa*	EtOAc EtOAc EtOH	9.5 9.7/31.0* 9.7/15.0*	*L. donovani*	AA[3]	m
Dumontiaceae	*Dumontia incrassata*	CF:MeOH	68.6	*L. donovani*	AA[3]	f
Furcellariaceae	*Furcellaria lumbricalis*	CF:MeOH	43.3	*L. donovani*	AA[3]	f
Galaxauraceae	*Scinaia furcellata*	EtOH	64.4	*L. donovani*	AA[3]	c
	Scinaia hatei	EtOH	14.1	*L. major*	P[1]	a, i
	Scinaia indica		na	*L. donovani*	-	b
		EtOH	59.6	*L. major*	P[1]	a
	Scinaia fascicularis	EtOH	59.6	*L. major*	P[1]	i
Gelidiaceae	*Gelidium crinale*	MeOH	19.9	*L. donovani*	AA[3]	l
	Gelidium latifolium	EtOAc	9.7/49.0*	*L. donovani*	AA[3]	m
	Gelidium pulchellum	CF:MeOH	32.5	*L. donovani*	AA[3]	f
Gigartinaceae	*Chondrus crispus*	EtOAc EtOH	9.7/12.0* 9.7/95.0*	*L. donovani*	AA[3]	m
Gracilariaceae	*Gracilaria caudata*	DCM:MeOH ME_2CO	50/9.3* 50/35.2*	*L. braziliensis*	P[1]	n
		DCM:H_2O	>100.0	*L. mexicana*	P[1]	d
	Gracilaria corticata	H_2O hot H_2O cold	38.0 65.0	*L. major*	P[2]	h
		EtOH	37.5	*L. major*	P[1]	a, i
	Gracilaria cervicornis	DCM:H_2O	>100.0	*L. mexicana*	P[1]	d
	Gracilaria damaecornis	DCM:H_2O	>100.0	*L. mexicana*	P[1]	d
	Gracilaria gracilis	CF:MeOH	53.3	*L. donovani*	AA[3]	f
		EtOAc EtOH	9.7/36.0* 9.7/29.0*	*L. donovani*	AA[3]	m
	Gracilaria salicornia	H_2O hot H_2O cold	46.0 74.0	*L. major*	P	h
	Gracilaria verrucosa	MeOH	36.0	*L. donovani*	AA[3]	l
	Hydropuntia cornea	DCM:H_2O	>100.0	*L. mexicana*	P[1]	d
Halymeniaceae	*Grateloupia cuneifolia*	DCM:MeOH	50/37.0*	*L. braziliensis*	P[1]	n
	Grateloupia turuturu	EtOAc	9.7/33.0*	*L. donovani*	AA[3]	m
	Halymenia floresii	DCM:H_2O	>100.0	*L. mexicana*	P[1]	d
Lomentariaceae	*Lomentaria articulata*	CF:MeOH	60.0	*L. donovani*	AA[3]	f
Palmariaceae	*Palmaria palmata*	EtOAc EtOH	9.7/10.0* 9.7/0.0*	*L. donovani*	AA[3]	m
Peyssonneliales	*Peyssonnelia decaisne*	HEX EtOH EtOAc MeOH	391.1 41.8 277.1 ≥500.0	*L. infantum*	P[2]	p
Phyllophoraceae	*Mastocarpus stellatus*	CF:MeOH	44.1	*L. donovani*	AA[3]	f
		EtOAc EtOH	9.7/39* 9.7/20*	*L. donovani*	AA[3]	m
Plocamiaceae	*Plocamium cartilagineum*	CF:MeOH	21.2	*L. donovani*	AA[3]	f
Polyidaceae	*Polyides rotundus*	CF:MeOH	57.3	*L. donovani*	AA[3]	f
Pterocladiaceae	*Pterocladiella capillacea*	HEX EtOH EtOAc MeOH	≥500.0 168.4 332.7 >500	*L. infantum*	P[2]	p
Rhizophyllidaceae	*Ochtodes secundiramea*	ME_2CO	50/99.7*	*L. braziliensis*	P[1]	n

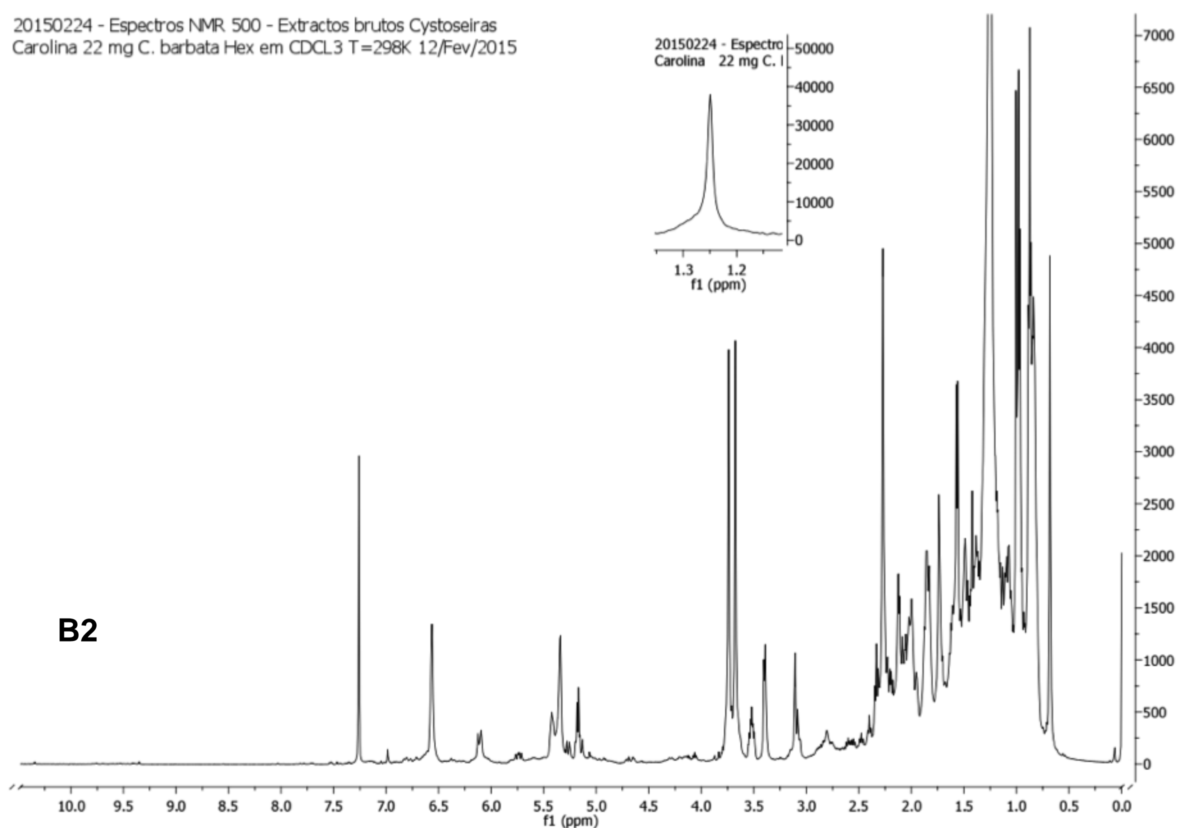
Phylum Family	Algal species	Crude extract	IC_{50} (µg/mL)	Tested organism	Parasite form	References (a-p)
Rhodomelaceae	*Boergeseniella fruticulosa*	CF:MeOH	26.6	*L. donovani*	AA[3]	f
	Bostrychia tenella	DCM:MeOH-HEX	1.5	*L. amazonensis*	P[2]	g
		DCM:MeOH-DCM	4.3			
	Botryocladia leptopoda	EtOH	60.8	*L. major*	P[1]	a, i
	Bryothamnion seaforthii	DCM:MeOH	50/33.5*	*L. braziliensis*	P[1]	n
	Bryothamnion triquetrum	DCM:H_2O	>100.0	*L. mexicana*	P[1]	d
	Digenea simplex	DCM:MeOH	50/26*	*L. braziliensis*	P[1]	n
	Halopitys incurvus	CF:MeOH	16.5	*L. donovani*	AA[3]	f
	Laurencia dendroidea	DCM:MeOH	50/14.6*	*L. braziliensis*	P[1]	n
	Laurencia microcladia	DCM:H_2O	16.3	*L. mexicana*	P[1]	d
	Melanothamnus afaqhusainii	EtOH	32.5	*L. major*	P[1]	a, i
	Osmundea hybrida	CF:MeOH	49.2	*L. donovani*	AA[3]	f
	Osmundea pinnatifida	CF:MeOH	32.7	*L. donovani*	AA[3]	f
		EtOH	6.3	*L. major*	P[1]	n
	Palisada flagellifera	DCM:MeOH	50/21.0*	*L. braziliensis*	P[1]	n
Solieriaceae	*Agardhiella* sp.	DCM:H_2O	>100.0	*L. mexicana*	P[1]	d
	Eucheuma isiforme	DCM:H_2O	>100.0	*L. mexicana*	P[1]	d
Wrangeliaceae	*Halurus equisetifolius*	CF:MeOH	69.2	*L. donovani*	AA[3]	f

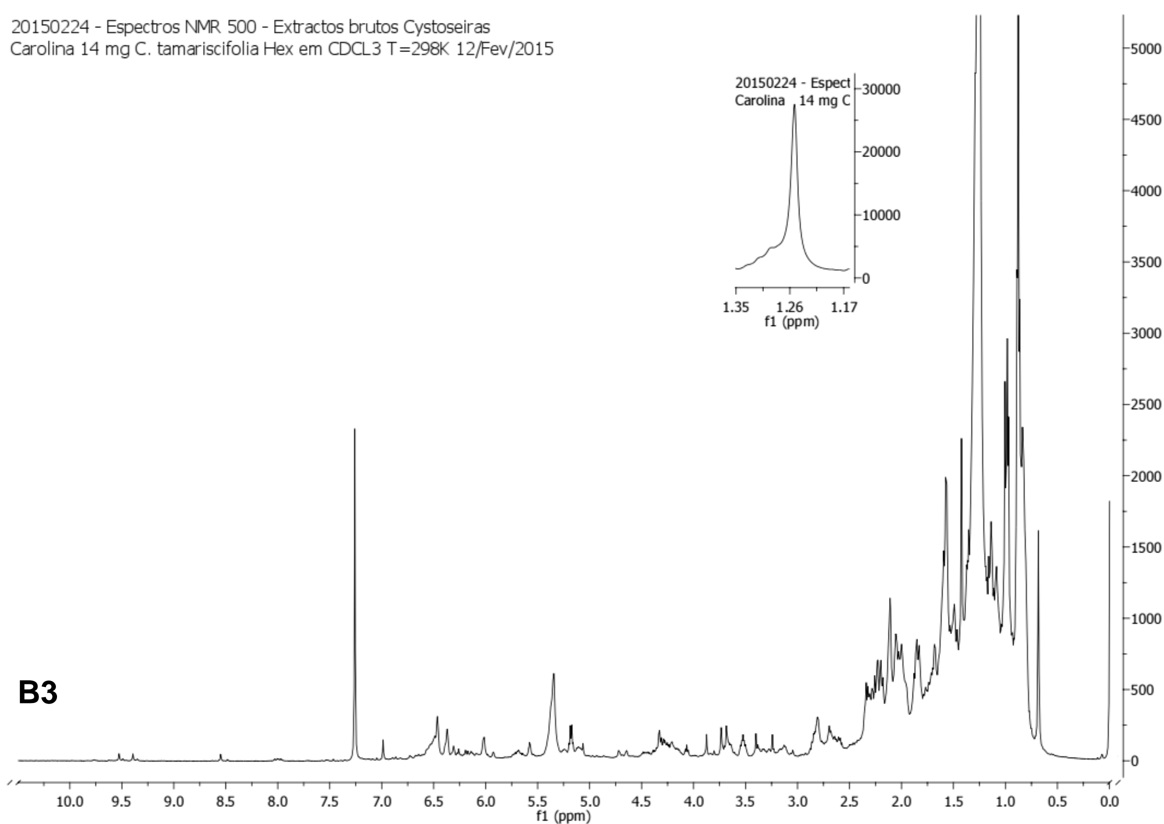
IC_{50} – half maximal inhibitory concentration µg/mL; *[Max conc.tested µg/mL / % cell inhibition]; HEX - hexane; CF - clorophorm; DCM - dicloromethane; EtOAc - ethyl acetate; Me_2CO - acetone; EtOH - ethanol; MeOH - methanol; H_2O - water; P - promastigotes; AA - axenic amastigotes; [1]Direct observation by optical microscopy; [2]MTT 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide assay; [3]Rezasurin, [a]Collected in Turkey - Sile, north Istanbul; [b]Collected in Turkey – Tekirdag; na - not active; n.d – not determined.

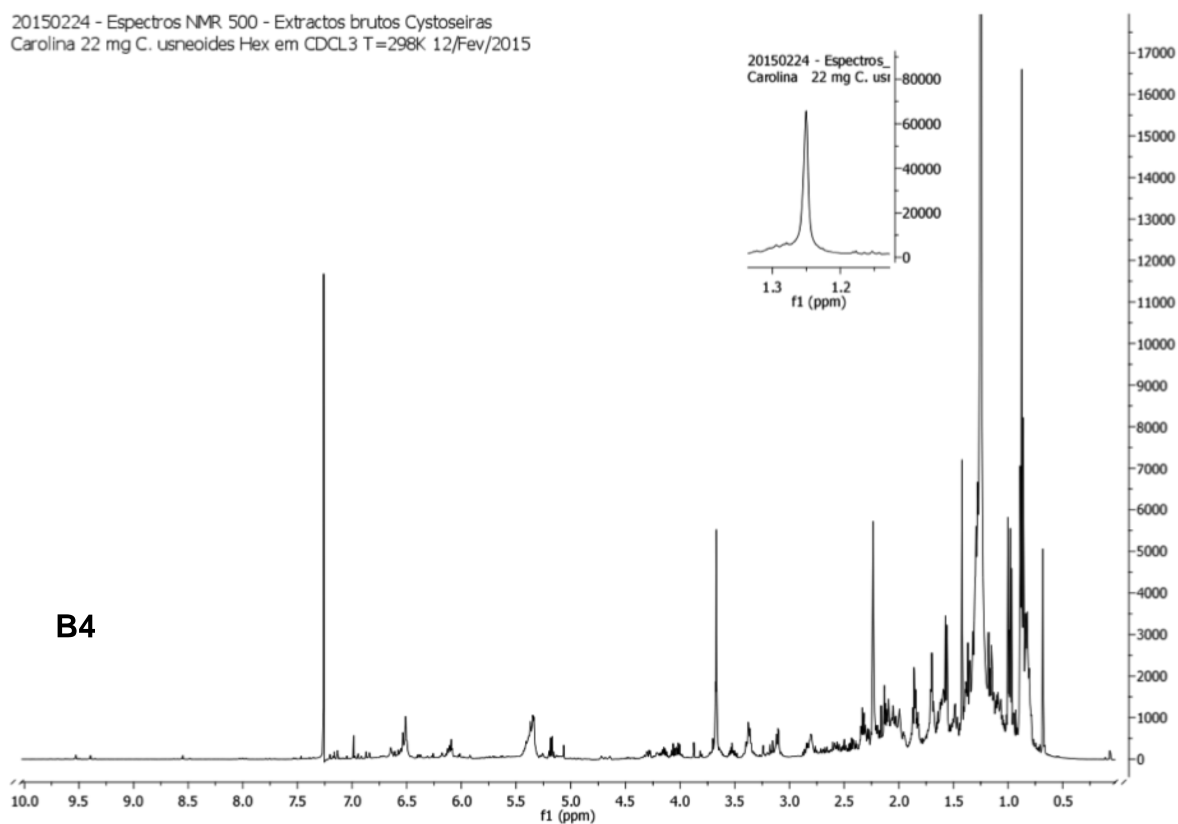
a - Sabina et al. 2005; b - Lakshmi et al.2006; c - Orhan et al. 2006; d - Freile-Pelegrin et al. 2008; e - Genovese et al. 2009; f - Allmendinger et al. 2010; g - Felício et al. 2010; h - Fouladvand et al. 2011; i - Sabina and Aliya, 2011; j - Spavieri et al. 2010a; k - Spavieri et al. 2010b; l - Süzgeç-Selçuk et al. 2010; m - Vonthron-Sénécheau et al. 2011; n - Bianco et al. 2013; o - Saher and Rahman 2013; p - Ainane et al. 2014.

ANNEX 2. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of the *C. baccata* (B1), *C. barbata* (B2), *C. tamariscifolia* (B3) and *C. usneoides* (B4) hexane extracts.

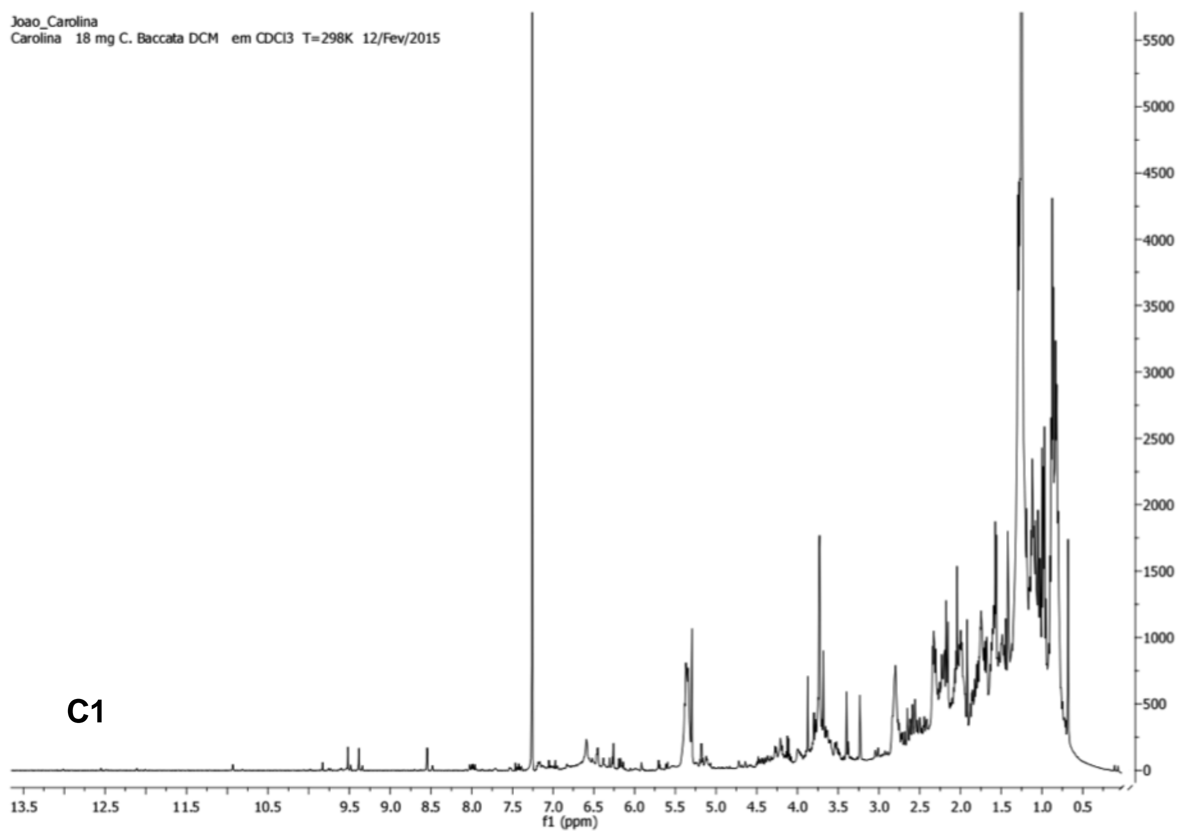


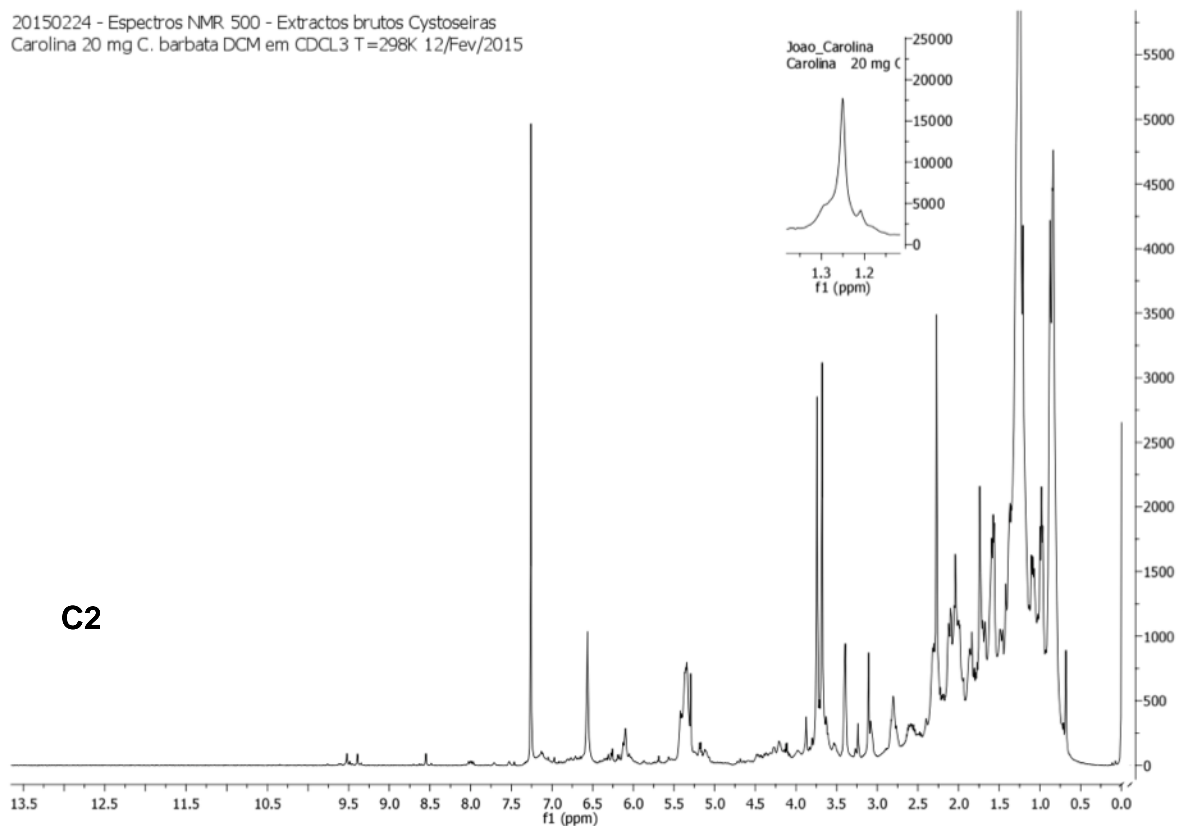


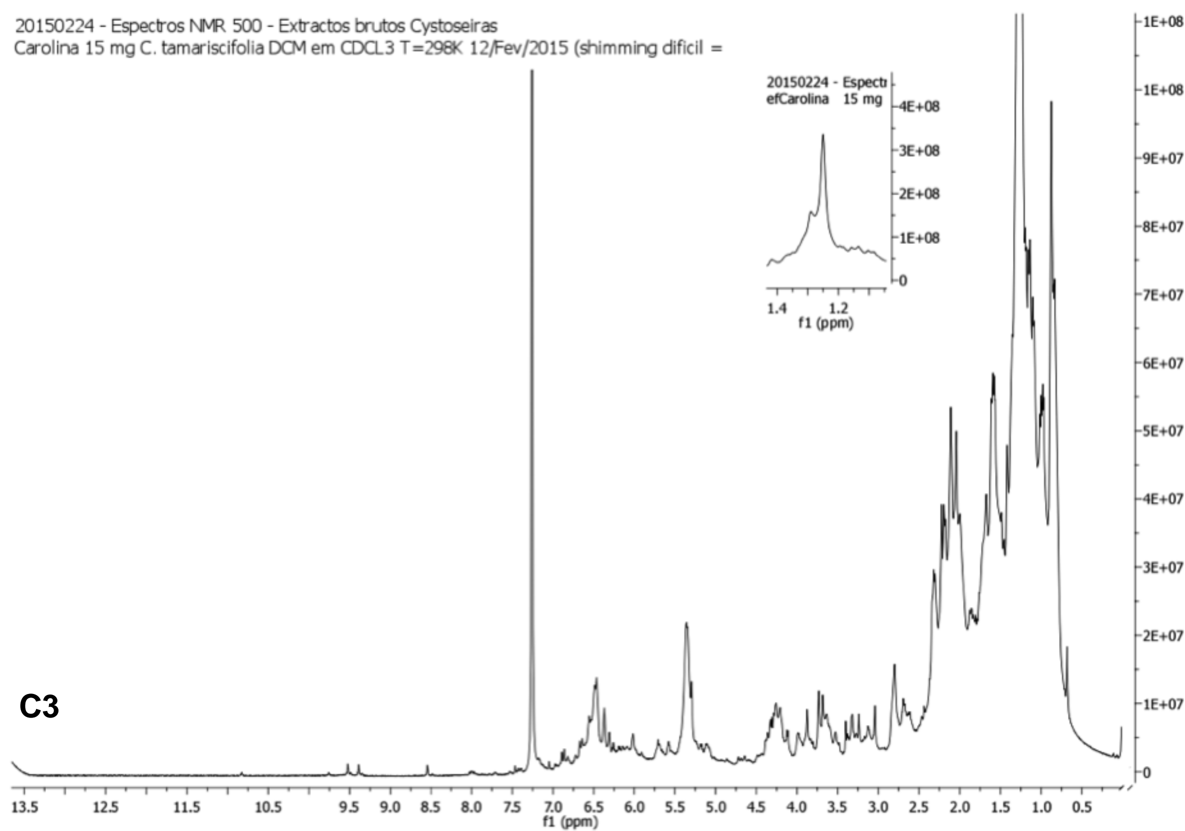

20150224 - Espectros NMR 500 - Extractos brutos Cystoseiras
Carolina 14 mg C. tamariscifolia Hex em CDCL3 T=298K 12/Fev/2015
B3
f1 (ppm)

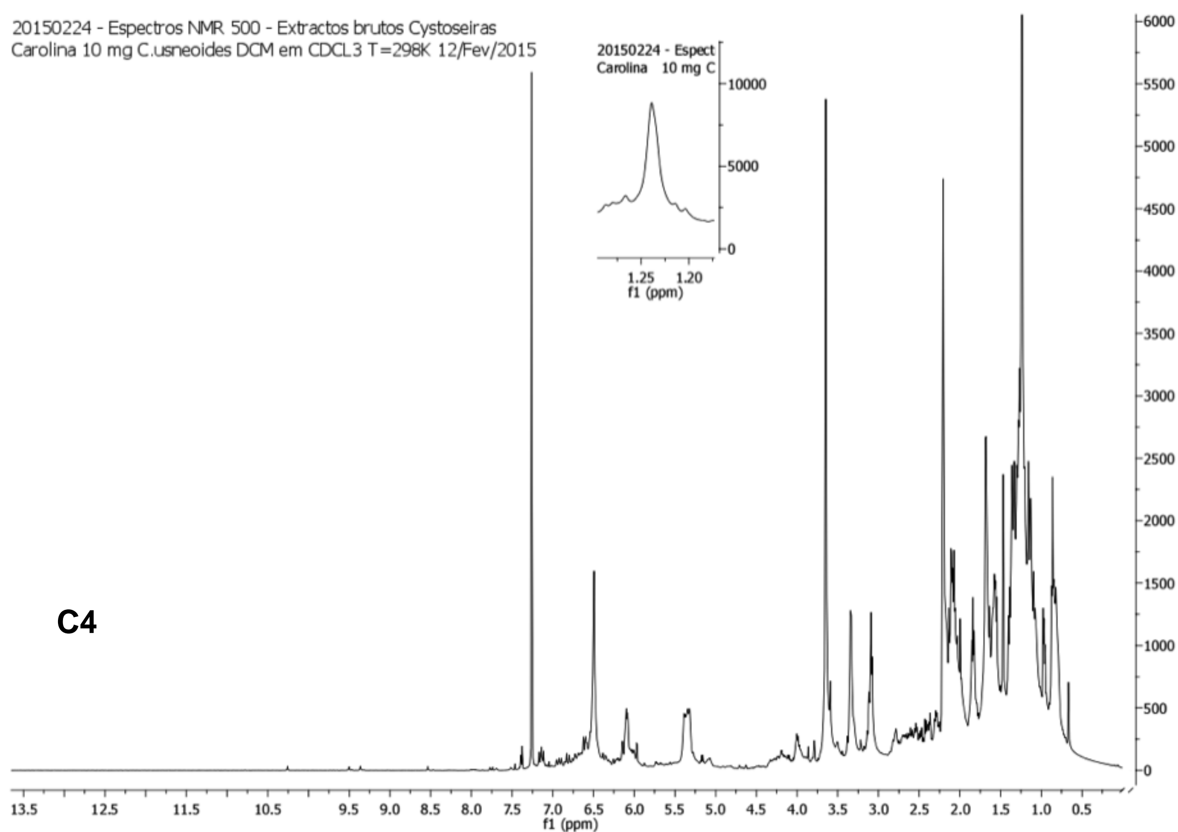

20150224 - Espectros NMR 500 - Extractos brutos Cystoseiras
Carolina 22 mg C. usneoides Hex em CDCL3 T=298K 12/Fev/2015
B4
f1 (ppm)

ANNEX 3. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of the *C. baccata* (C1), *C. barbata* (C2), *C. tamariscifolia* (C3) and *C. usneoides* (C4) CH_2Cl_2 extract.

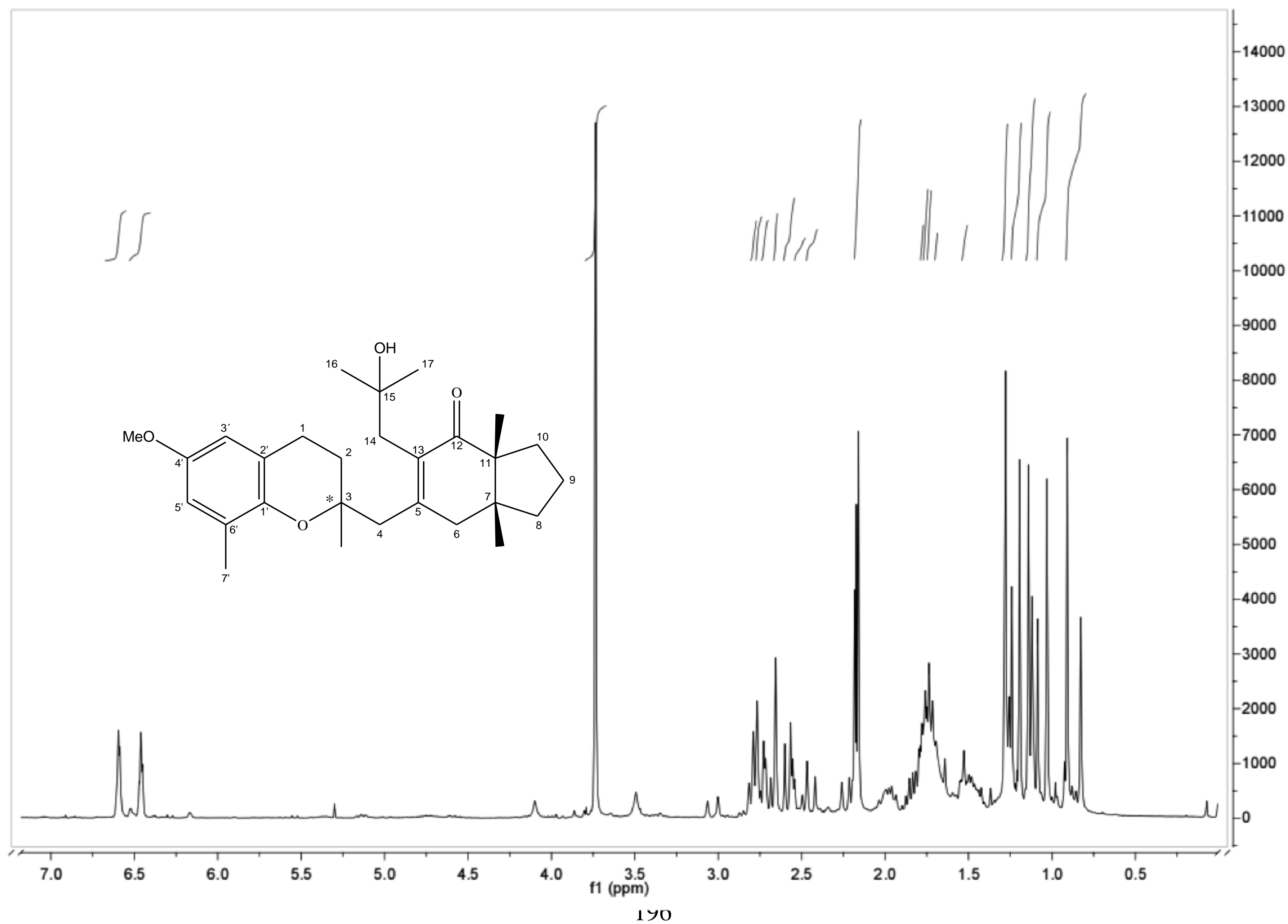




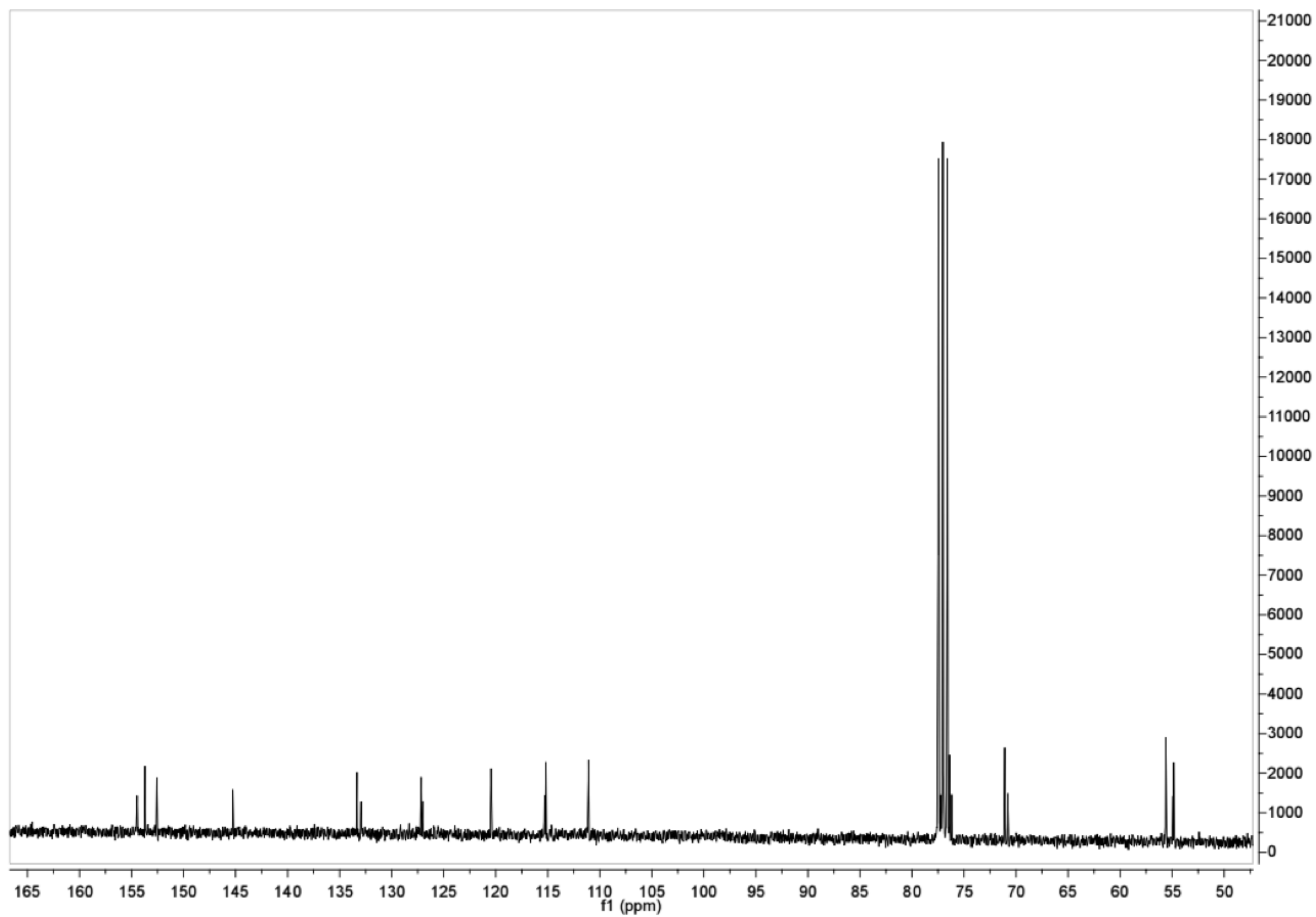

20150224 - Espectros NMR 500 - Extractos brutos Cystoseiras
Carolina 15 mg C. tamariscifolia DCM em CDCL3 T=298K 12/Fev/2015 (shimming dificil =
C3
f1 (ppm)


20150224 - Espectros NMR 500 - Extractos brutos Cystoseiras
Carolina 10 mg C.usneoides DCM em CDCL3 T=298K 12/Fev/2015
C4
f1 (ppm)

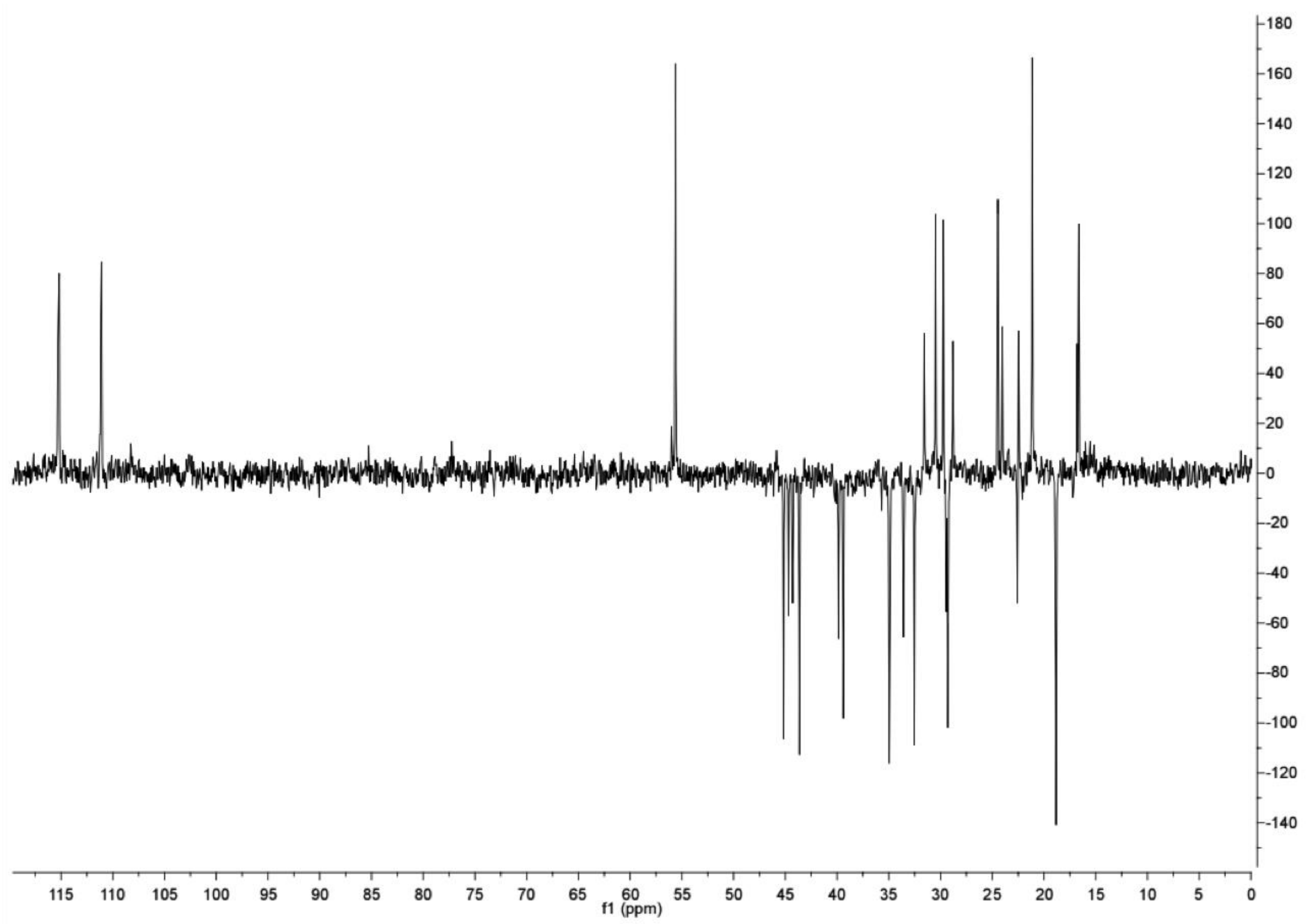
ANNEX 4. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of the tetraprenyltoluquinols **1a/1b**.



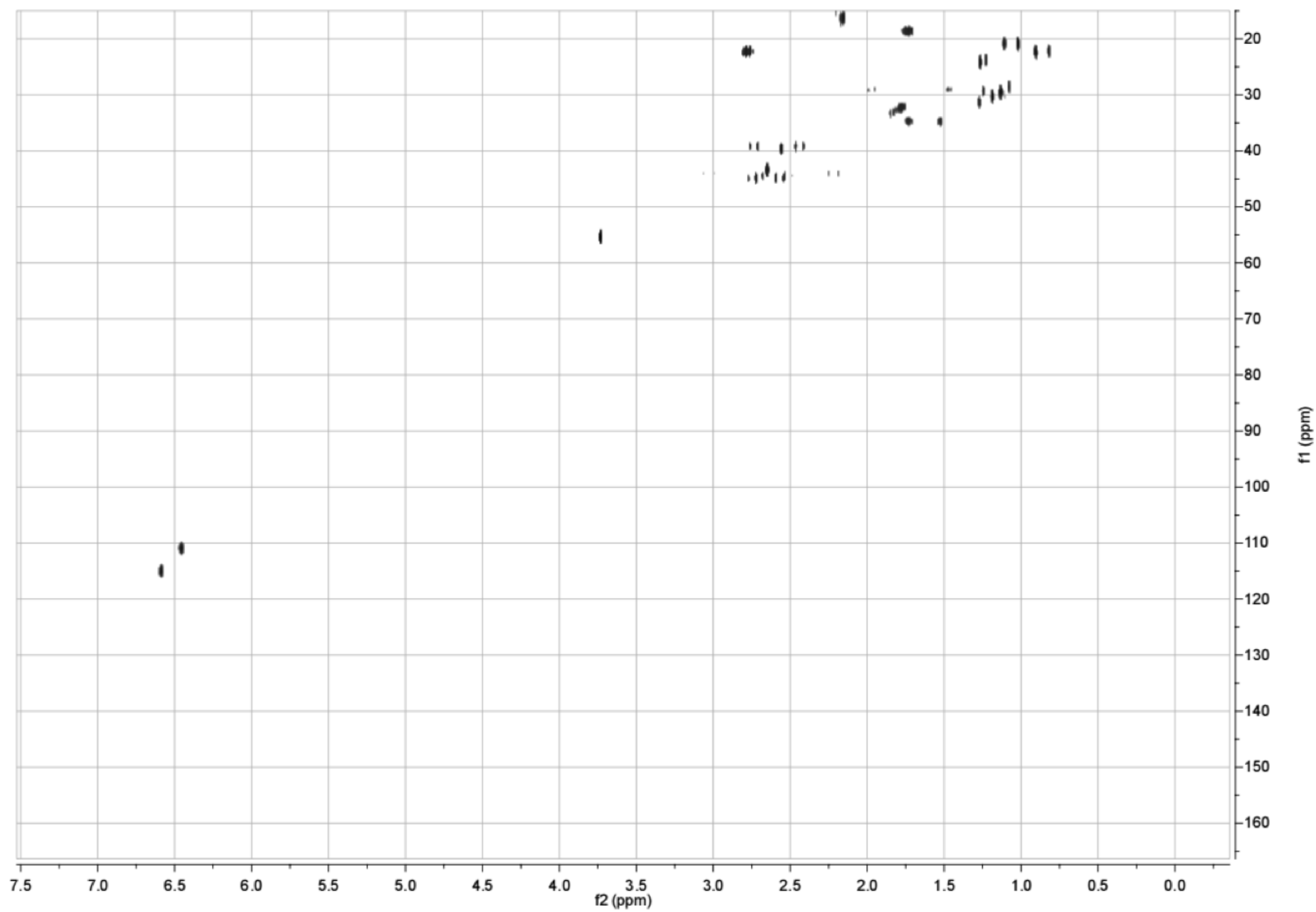
ANNEX 5. ^{13}C NMR spectrum (125 MHz, $CDCl_3$) of the tetraprenyltoluquinols **1a/1b**.



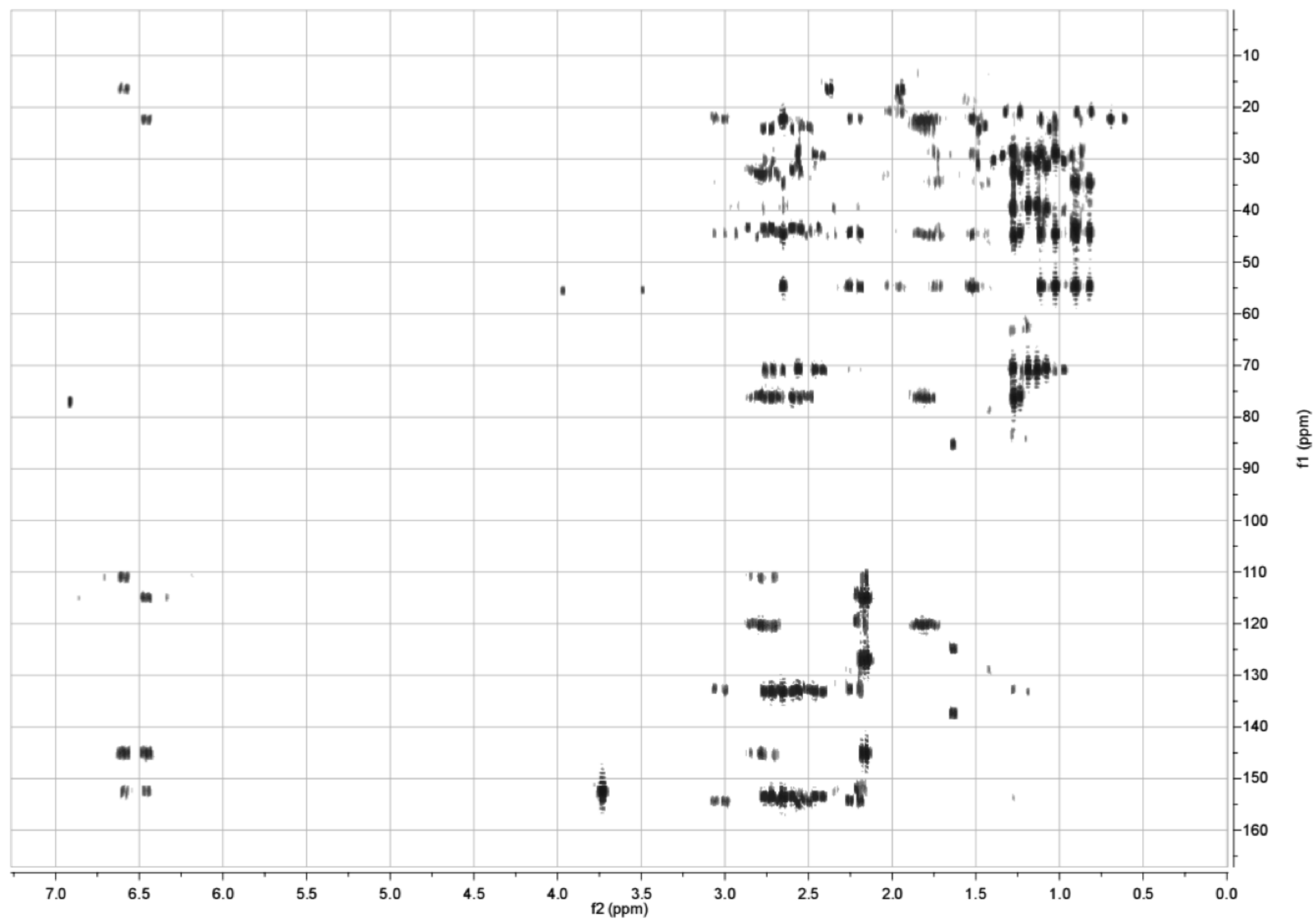
ANNEX 6. DEPT spectrum (125 MHz, $CDCl_3$) of the tetraprenyltoluquinols **1a/1b**.



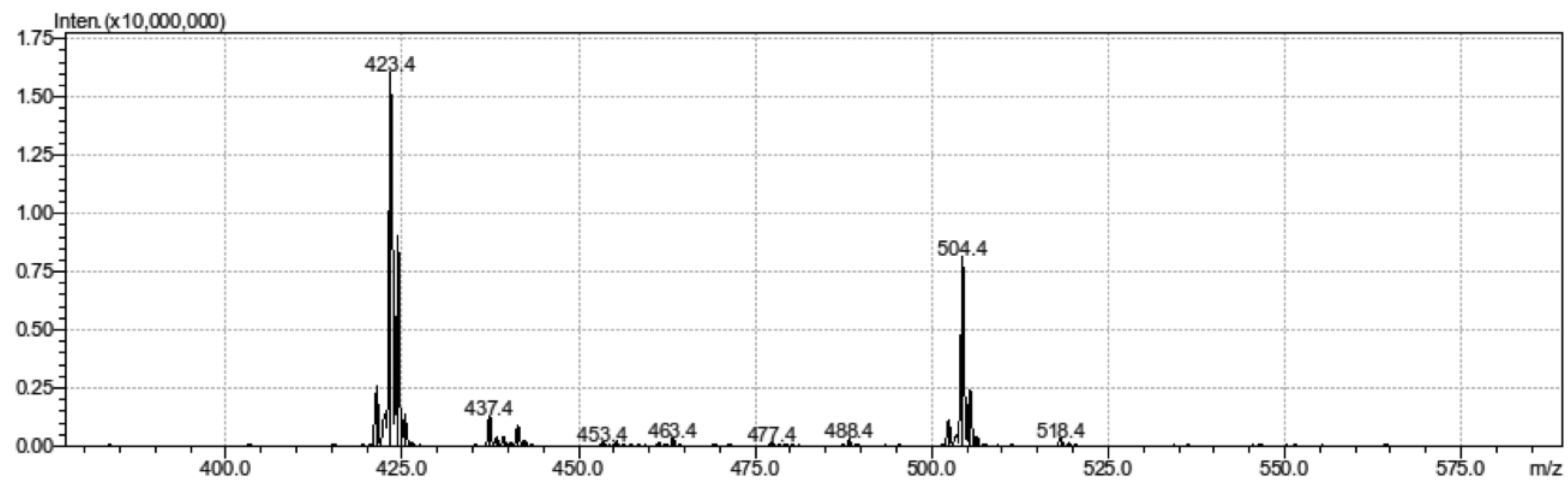
ANNEX 7. HSQC spectrum (500 and 125 MHz, $CDCl_3$) of the tetraprenyltoluquinols **1a/1b**.



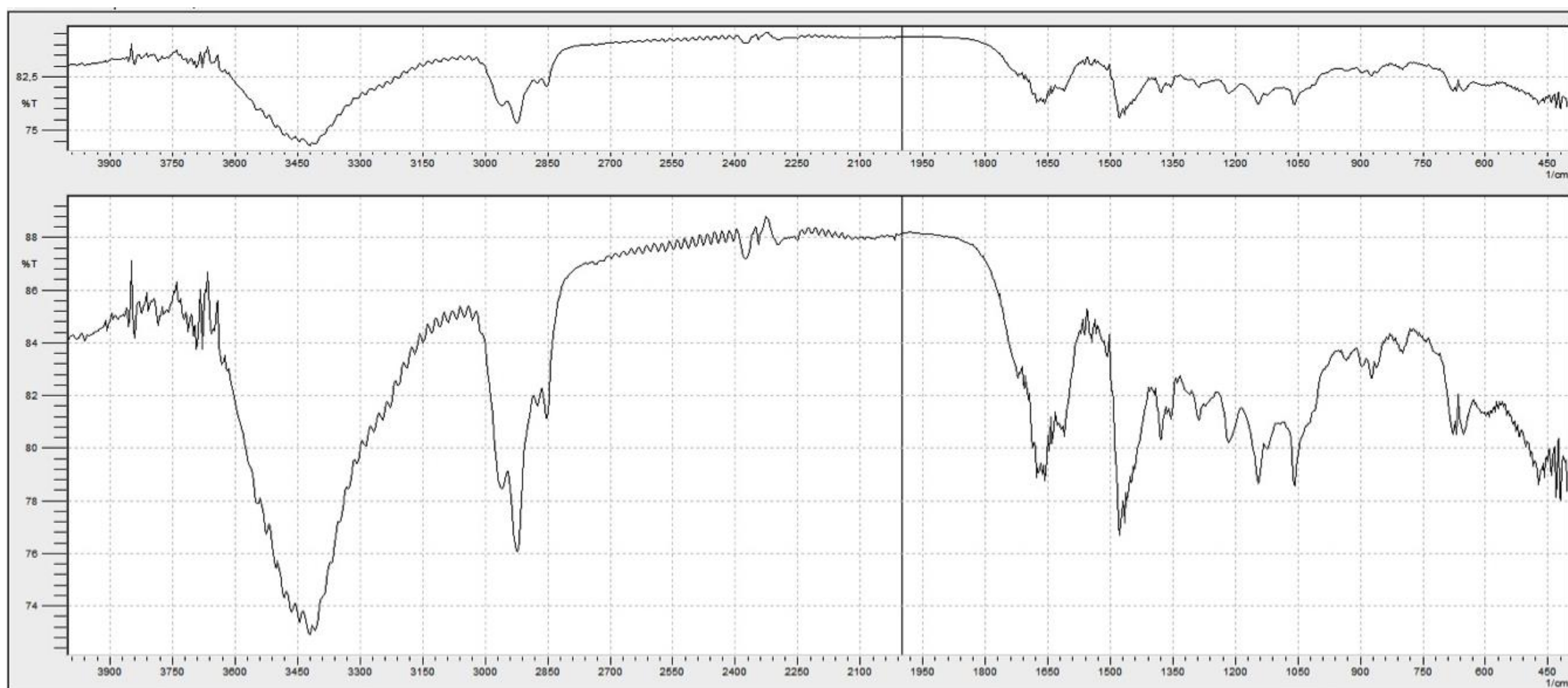
ANNEX 8. HMBC spectrum (500 and 125 MHz, $CDCl_3$) of the tetraprenyltoluquinols **1a/1b**.



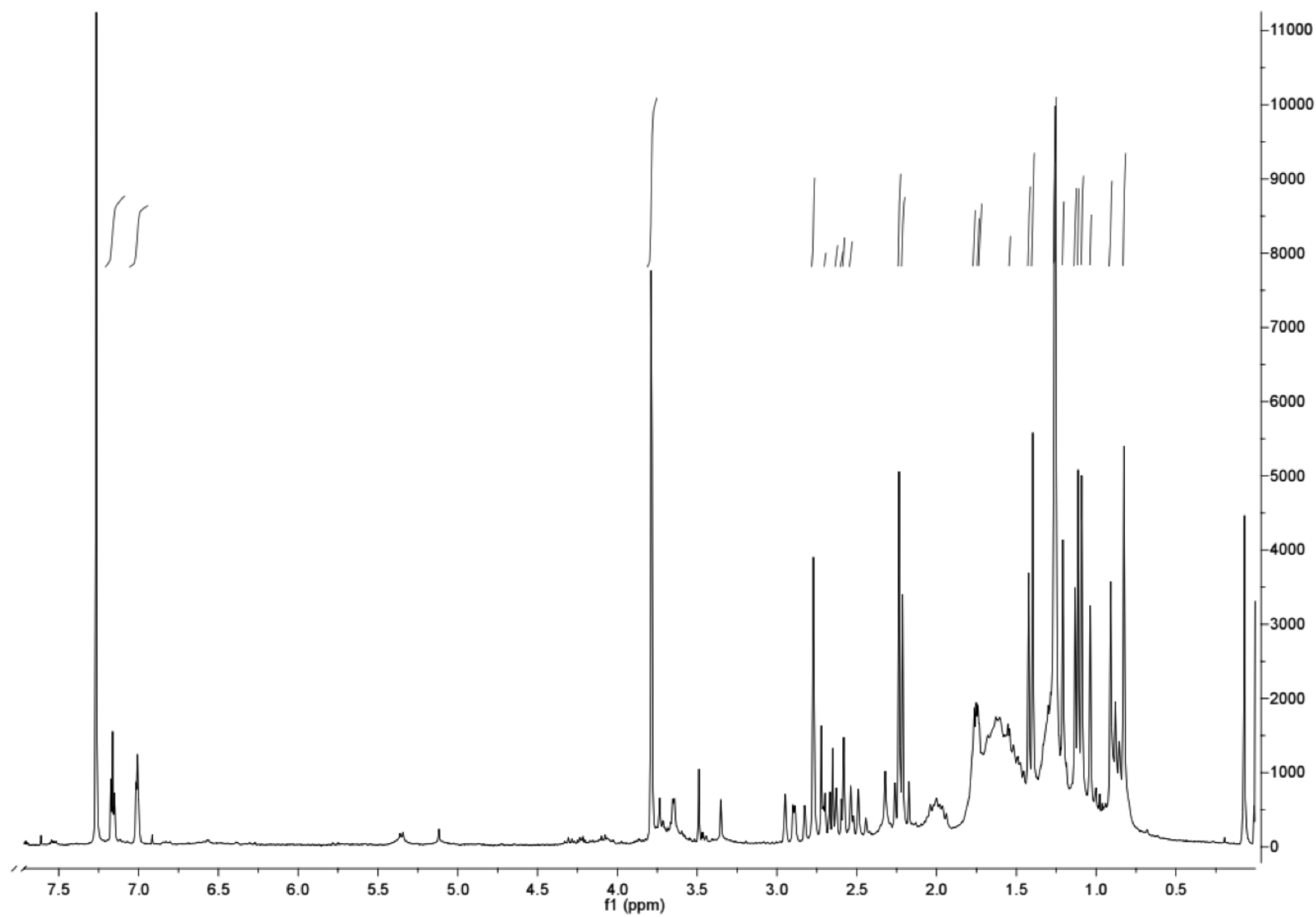
ANNEX 9. Positive HRESIMS spectrum of the tetraprenyltoluquinols **1a/1b**.



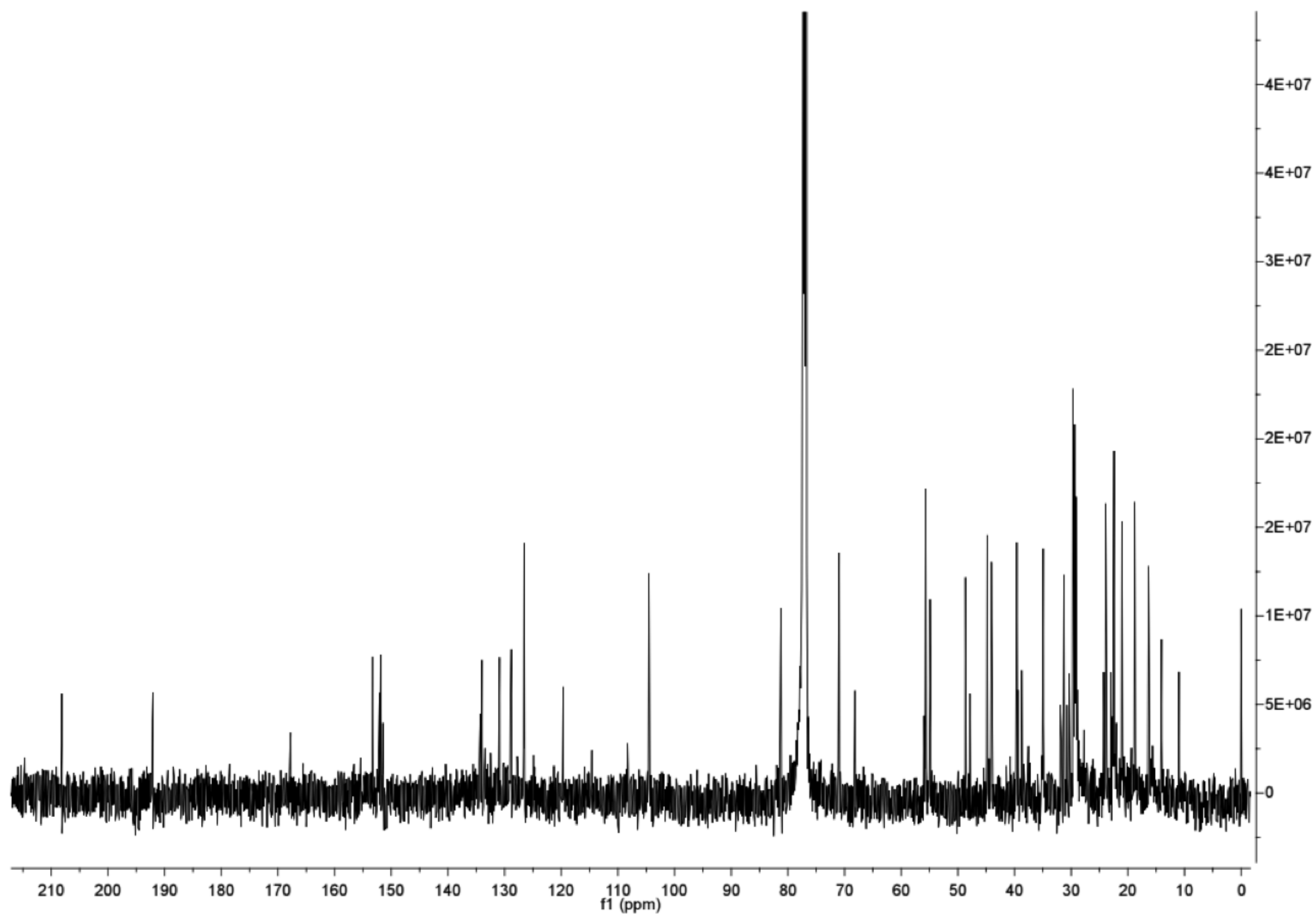
ANNEX 10. IR spectrum of the tetraprenyltoluquinols **1a/1b**.



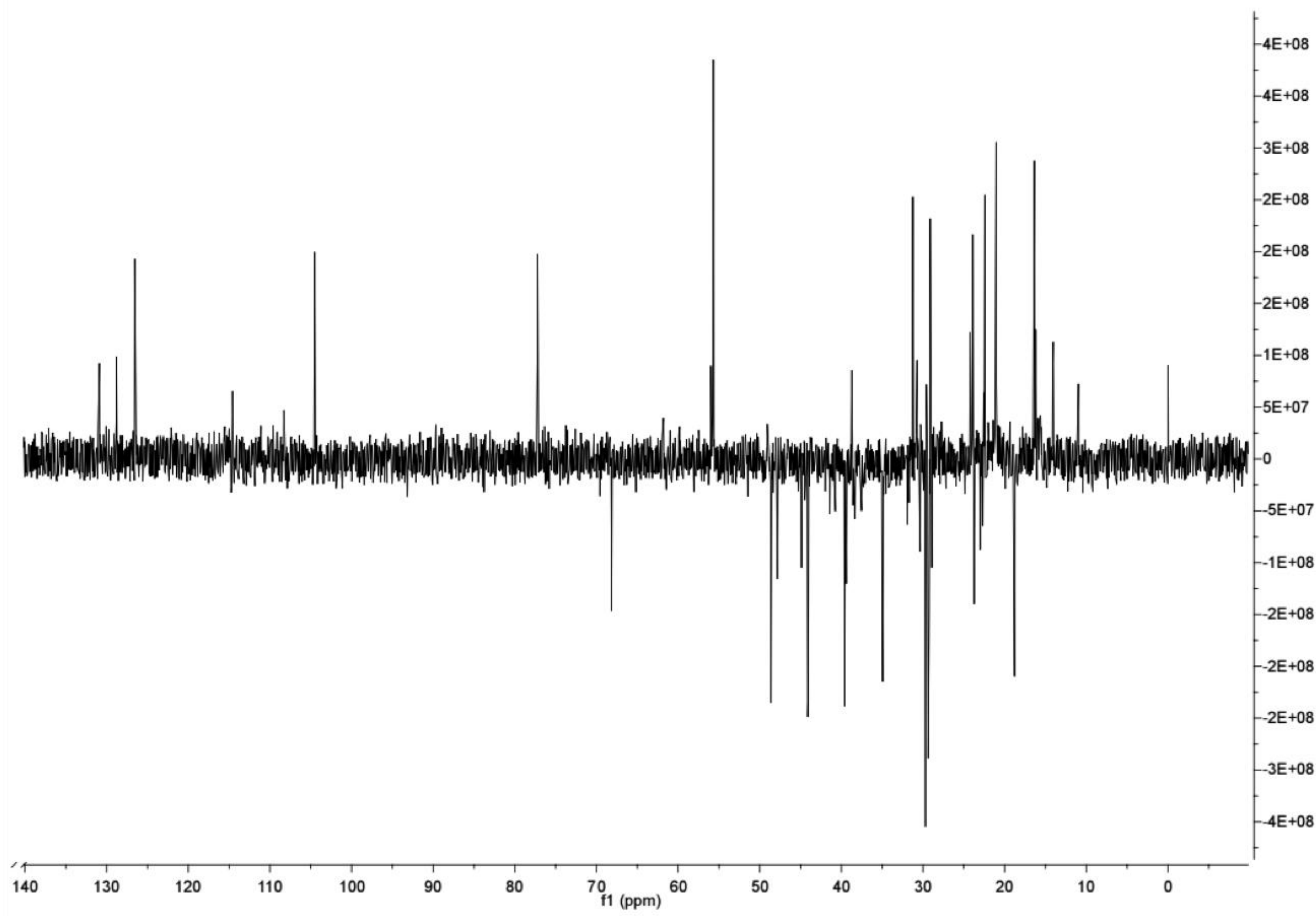
ANNEX 11. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of the tetraprenyltoluquinones **2a/2b**.



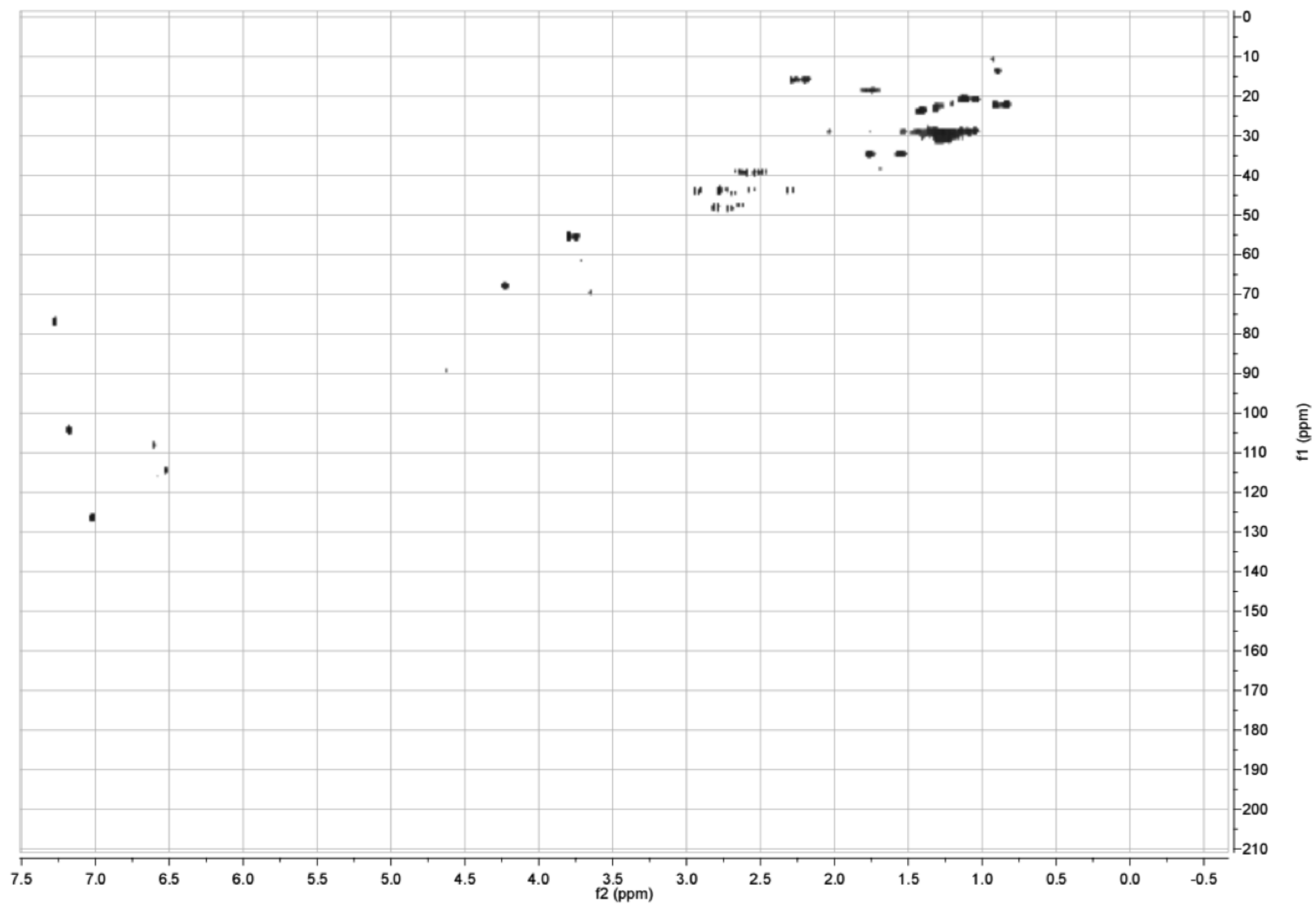
ANNEX 12. ^{13}C NMR spectrum (125 MHz, $CDCl_3$) of the tetraprenyltoluquinones **2a/2b**.



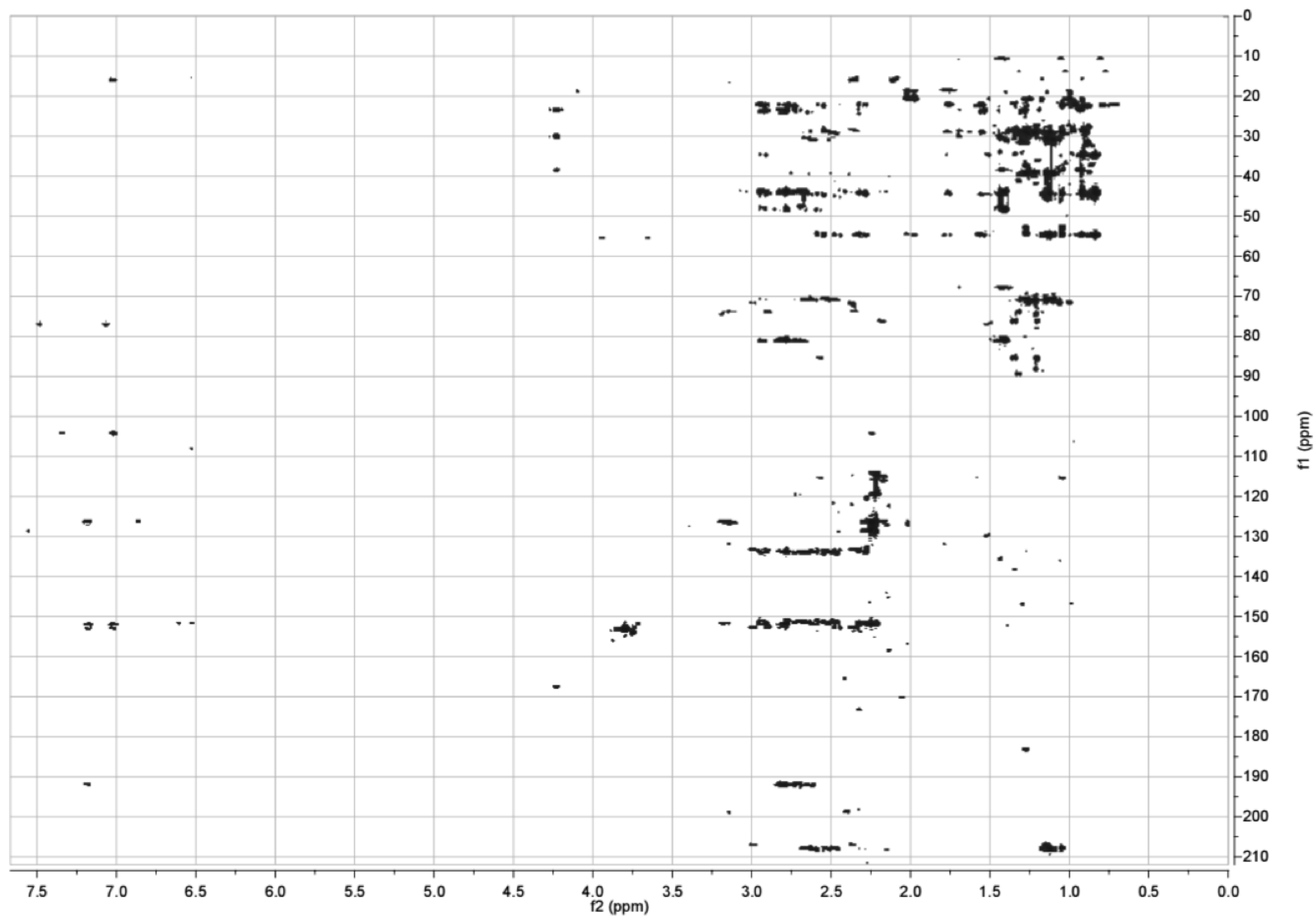
ANNEX 13. DEPT spectrum (125 MHz, $CDCl_3$) of the tetraprenyltoluquinones **2a/2b**.



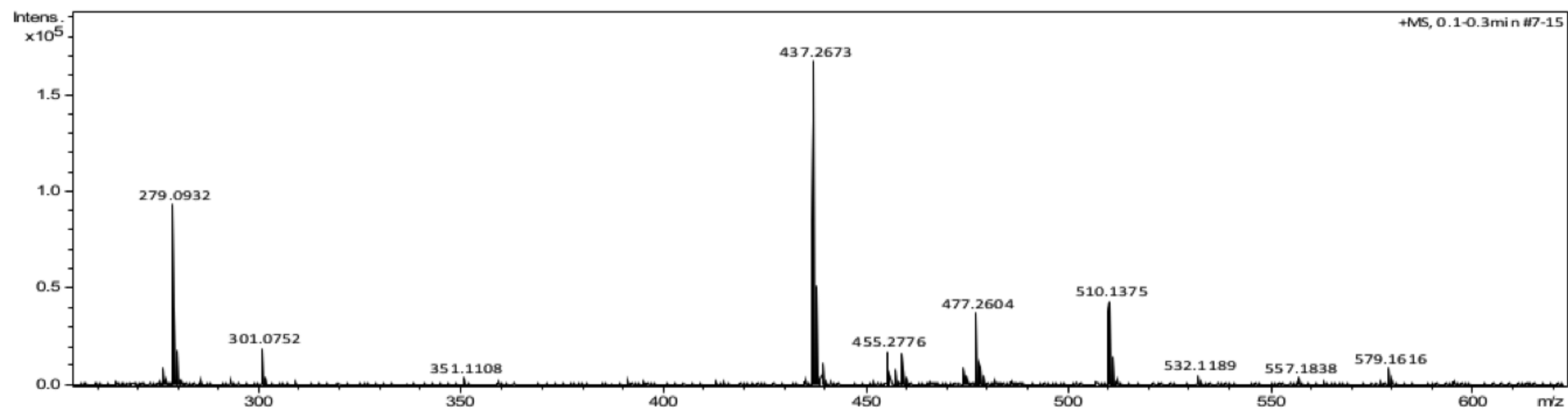
ANNEX 14. HSQC spectrum (500 and 125 MHz, $CDCl_3$) of the tetraprenyltoluquinones **2a/2b**.

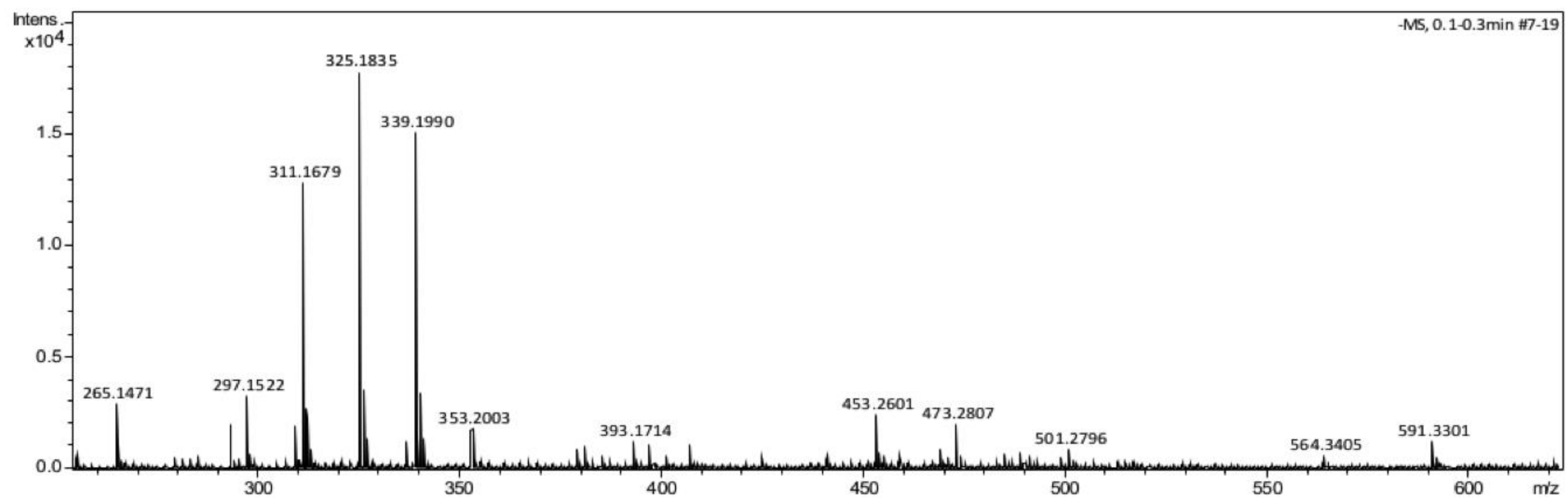


ANNEX 15. HMBC spectrum (500 ans 125 MHz, $CDCl_3$) of the tetraprenyltoluquinones **2a/2b**.

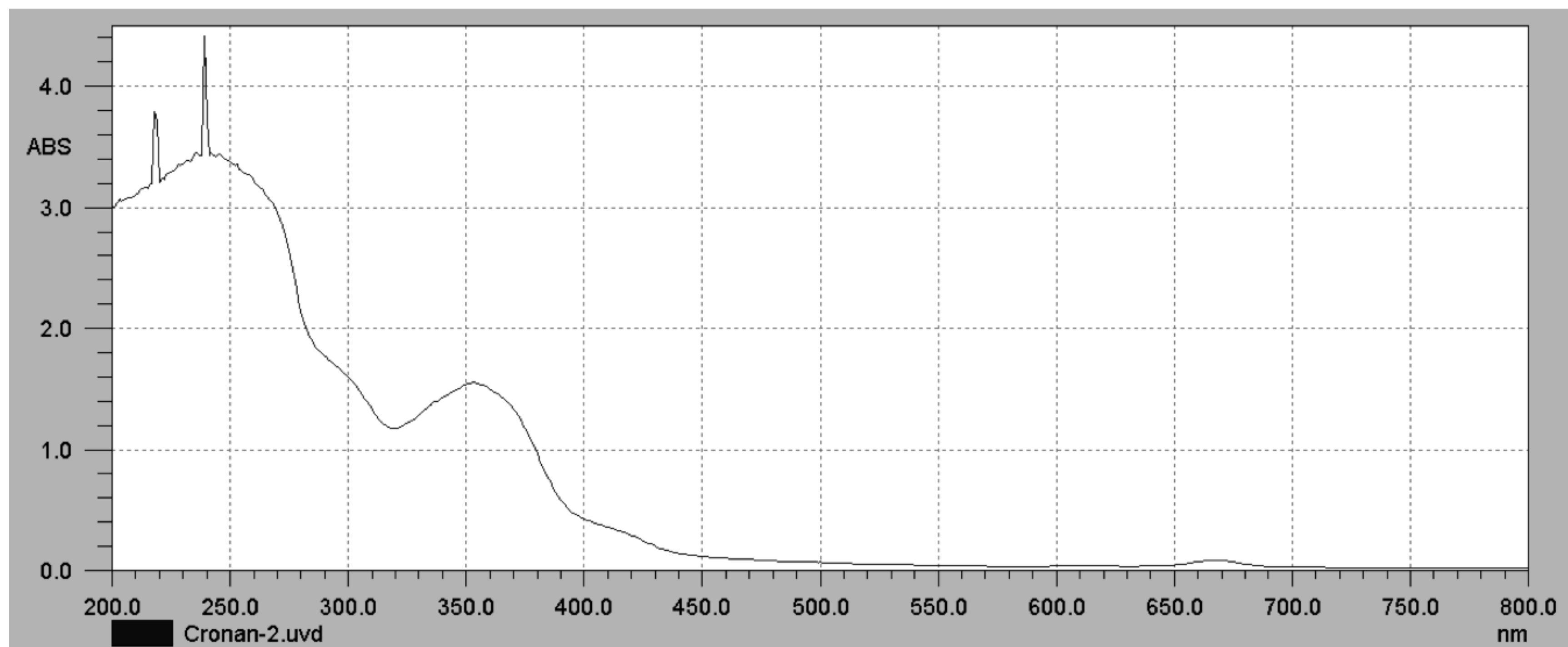


ANNEX 16. Positive (A) and negative (B) HRESIMS spectra of the tetraprenyltoluquinones **2a/2b**.

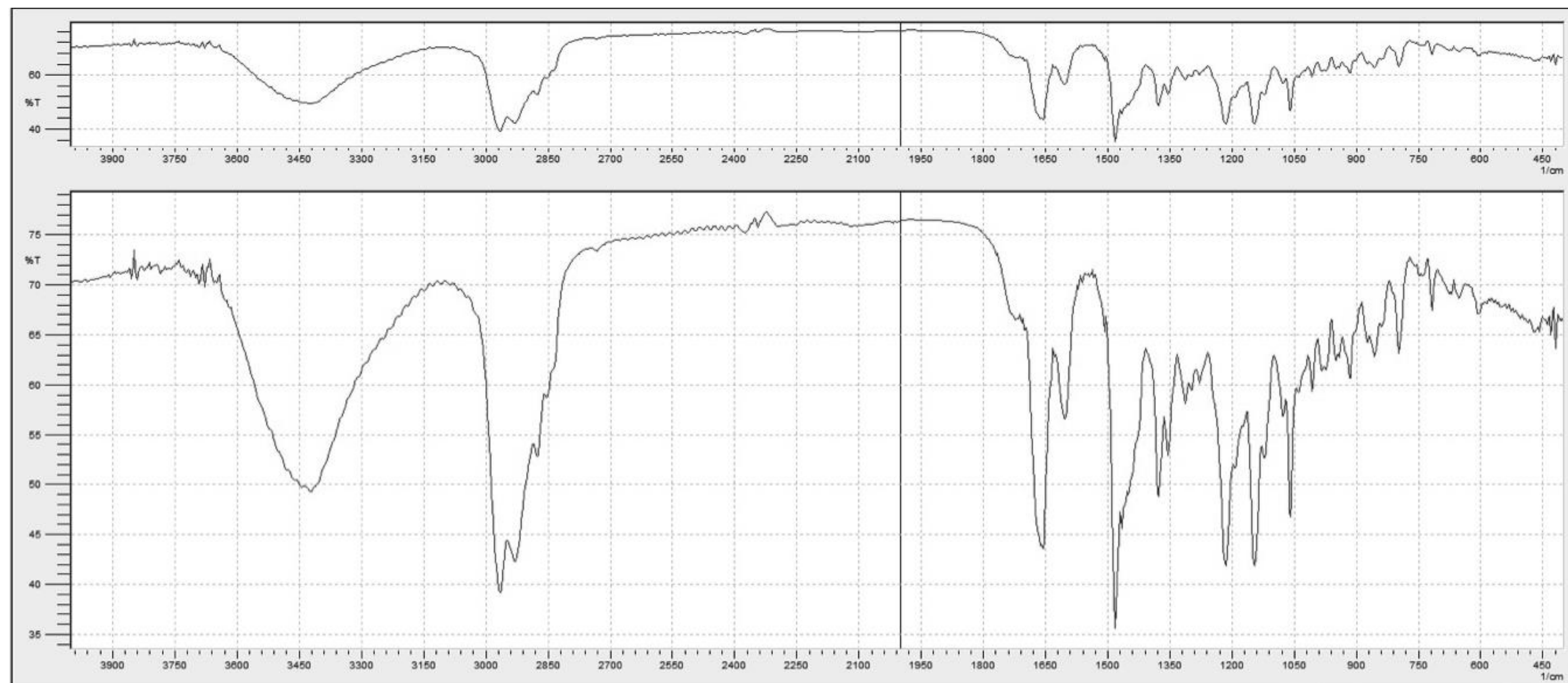




ANNEX 17. UV spectrum of the tetraprenyltoluquinones **2a/2b**.



ANNEX 18. IR spectrum of the tetraprenyltoluquinones **2a/2b**.



ANNEX 19. Information of the sequences included in this study - species, geographical origin, voucher, GenBank accession numbers and haplotypes.

Sample		**Geographic origin**		**Sample information and sequences GenBank acession n.º**				
Species	**Reference**	**Country**	**Location**	**Voucher**	**COI**	**23S**	**mt-spacer**	**Author**
Cystoseira abies-marina	C. abies marina 1	Spain	Tenerife, Mesa del Mar	L:0609335	MP3[#]	FM958377	FM993042	Draisma
Cystoseira abies-marina	C. abies marina 2	Spain	Tenerife, Bajamar	L:0609338	MP3[#]	FM958376	FM993042	Draisma
Cystoseira abies-marina	C. abies marina MP19	Spain	Tenerife, Punta del Hidalgo	MD0000400	x	x	-	This study
Cystoseira abies-marina	C. abies marina MP26	Spain	Tenerife, Punta del Hidalgo	MD0000522	x	x	-	This study
Cystoseira abies-marina	C. abies marina MP27	Spain	Tenerife, Punta del Hidalgo	MD0000557	x	x	x	This study
Cystoseira abies-marina	C. abies marina MP29	Portugal	S. Miguel ls, Ponta dos Mosteiros	MD0000746	x	x	x	This study
Cystoseira abies-marina	C. abies marina MP3	Spain	Tenerife, Punta del Hidalgo	PG072209	x	-	x	This study
Cystoseira abies-marina	C. abies marina MP30	Portugal	S. Miguel ls, Ponta dos Mosteiros	MD0000778	MP3[#]	x	x	This study
Cystoseira amentacea	C. amentacea	Italy	Sicily, Capo Passero	L:0609436	-	FM958359	FM993021	Draisma
Cystoseira amentacea	C. amentacea GV3	Spain	Almería, Guardias Viejas	GV3	x	x	x	This study
Cystoseira amentacea	C. amentacea MU1	Spain	Murcia, Cabo de Palos	MU1	x	x	-	This study
Cystoseira amentacea var. stricta	C. amentacea var. stricta 1	Italy	Sicily, Capo Gallo	L:0609384	-	FM958371	FM993017	Draisma
Cystoseira amentacea var. *stricta*	C. amentacea var. stricta 2	Italy	Sicily, S. Maria la Scala	L:0609446	-	FM958356	FM993016	Draisma
Cystoseira amentacea var. *stricta*	C. amentacea var. stricta RB95	Spain	Almería, Las Negras	MBR95	x	-	x	This study
Cystoseira baccata	C. baccata 1	Spain	A Coruña	SANT:16322	EU681399[”]	FM958368	FM993034	Draisma
Cystoseira baccata	C. baccata MB1	Portugal	Viana do Castelo, Areosa	MB1	x	x	x	This study
Cystoseira baccata	C. baccata MB2	Portugal	Caminha, Moledo	MB2	x	x	x	This study
Cystoseira barbata	C. barbata	Spain	Menorca, Moll d'es Miami	L:0609316	-	FM958378	FM993043	Draisma
Cystoseira barbata f. *repens*	C. barbata f repens RB87	Spain	Cádiz, Santibañez	MBR87	x	x	x	This study
Cystoseira barbata	C. barbata MB17	Spain	Cádiz, Santibañez	MB17	x	x	x	This study
Cystoseira barbata	C. barbata/susanensis	Italy	Sicily, Marzameni	L:SGAD1638	-	FM958379	FM993044	Draisma
Cystoseira barbatula	C. barbatula	Italy	Sicily, Marzameni	L:0609441	-	FM958365	-	Draisma
Cystoseira brachycarpa	C. sp RB105	Spain	Almería, El Playazo	MBR105	-	-	x	This study
Cystoseira brachycarpa	C. brachycarpa var. balearica 1	Spain	Menorca, Cala Viola de	L:0609251	-	FM958361	-	Draisma

Sample		Geographic origin		Sample information and sequences GenBank acession n.º				
Species	**Reference**	**Country**	**Location**	**Voucher**	**COI**	**23S**	**mt-spacer**	**Author**
var. balearica			Llevant					
Cystoseira brachycarpa var. *balearica*	C. brachycarpa var. balearica 2	Spain	Menorca, La Llosa d'en Patro Pere	L:0609308	-	FM958362*	FM993025	Draisma
Cystoseira brachycarpa var. *balearica*	C. brachycarpa var. balearica 3	Italy	Sicily, Capo Milazzo	L:0609404	-	FM958362*	FM993027	Draisma
Cystoseira brachycarpa var. *brachycarpa*	C. brachycarpa var. brachycarpa 1	Italy	Sicily, S. Maria la Scala	L:0609414	-	FM958362	FM993026	Draisma
Cystoseira brachycarpa var. *brachycarpa*	C. brachycarpa var. brachycarpa 2	Italy	Aeolian Is, Salina	L:SGAD1633	-	-	FM993028	Draisma
Cystoseira brachycarpa var. *brachycarpa*	C. brachycarpa var. brachycarpa 3	France	Cote Vermeille, Banyuls-sur-Mer	PC:FR194	-	-	FM993023	Draisma
Cystoseira compressa	C. compressa	Spain	Tenerife, Punta del Hidalgo	L:0609343	MP17#	FM958355	FM993015	Draisma
Cystoseira compressa	C. compressa MB4	Portugal	Albufeira, Arrifes	MB4	x	x	-	This study
Cystoseira compressa	C. compressa MB6	Portugal	Albufeira, Olhos de Água	MB6	x	x	x	This study
Cystoseira compressa	C. compressa MP17	Spain	Tenerife, Mesa del Mar	MD0000360	x	-	-	This study
Cystoseira compressa	C. compressa RB25	Spain	Girona, Blanes	MBR25	x	x	x	This study
Cystoseira compressa	C. compressa MP4	Spain	Tenerife, Mesa del Mar	PG072363	x	-	-	This study
Cystoseira compressa	C. compressa MP25	Spain	Tenerife, Mesa del Mar	MD0000506	x	x	-	This study
Cystoseira compressa var. *pustulata*	C. compressa var. pustulata	Italy	Sicily, Marzameni	L:0609427	-	FM958354	FM993014	Draisma
Cystoseira compressa var. *pustulata*	C. compressa var. pustulata RB67	Spain	Almería, La isleta del Moro	MBR67	x	-	-	This study
Cystoseira compressa var. *pustulata*	C. compressa var. pustulata RB103	Spain	Almería, La isleta del Moro	MBR103	x	x	x	This study
Cystoseira crinita	C. crinita 1	Spain	Menorca, Illots de Tirant	L:0609275	-	FM958363	FM993029	Draisma
Cystoseira crinita	C. crinita 2	Italy	Sicily, Marzameni	L:0609440	-	FM958360	FM993024	Draisma
Cystoseira crinita	C. crinita 3	Spain	Menorca, Cala Mica	L:0609314	-	-	FM993030	Draisma
Cystoseira crinita	C. crinita RB90	France	Antibes, Pointe I´lette	MBR90	-	x	x	This study
Cystoseira elegans	C. elegans	Italy	Sicily, Capo Passero	L:0609444	-	FM958375	FM993038	Draisma
Cystoseira elegans	C. elegans RB68	Spain	Almería, El Playazo	MBR68	-	x	x	This study
Cystoseira foeniculacea	C. foeniculacea	Spain	Tenerife, Punta del Hidalgo	L:0609350	-	FM958353	FM993013	Draisma
Cystoseira foeniculacea	C. foeniculacea MP20	Spain	Tenerife, Mesa del Mar	MD0000403	x	-	x	This study

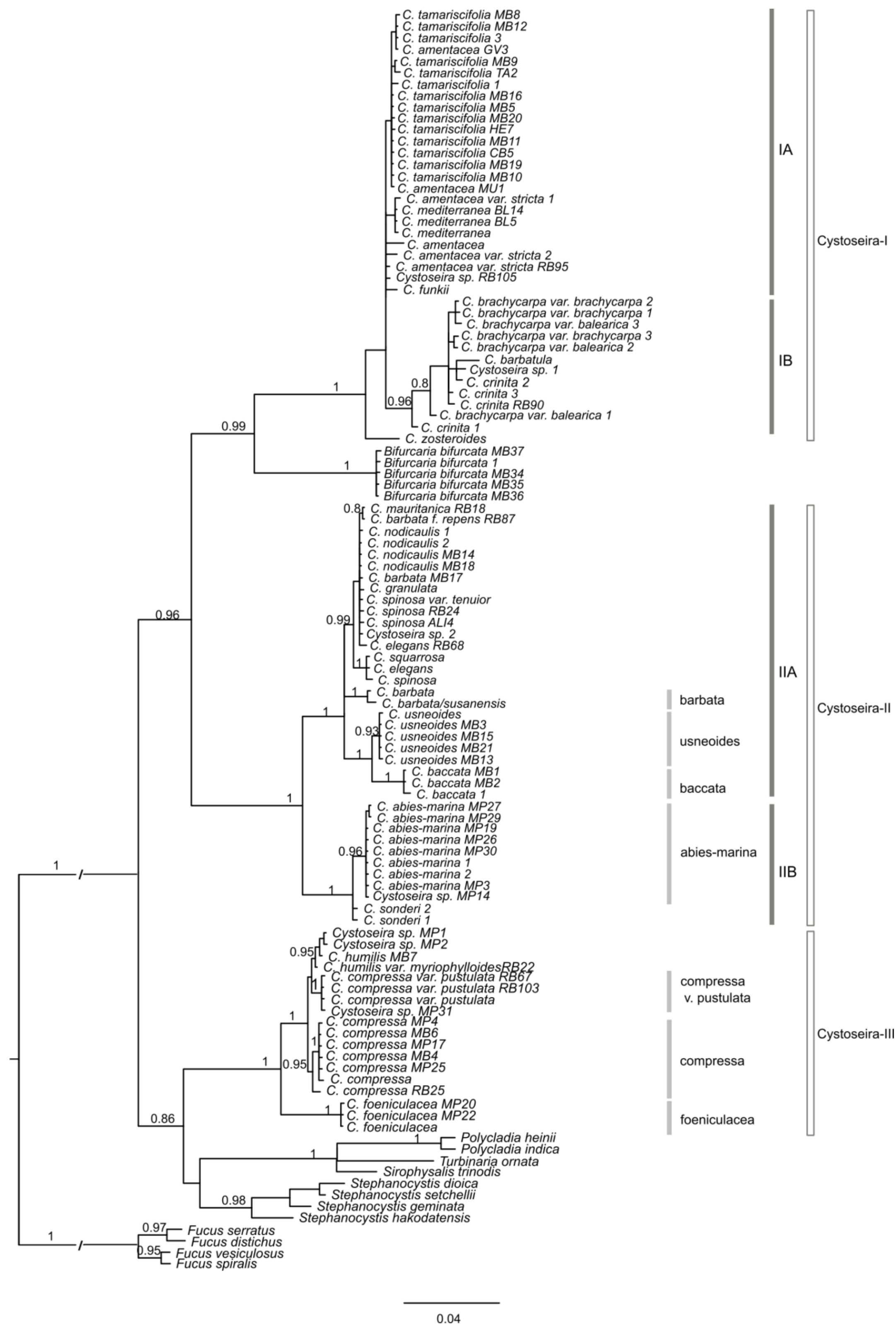
Sample		Geographic origin		Sample information and sequences GenBank acession n.º				
Species	**Reference**	**Country**	**Location**	**Voucher**	**COI**	**23S**	**mt-spacer**	**Author**
Cystoseira foeniculacea	C. foeniculacea MP22	Spain	Tenerife, Mesa del Mar	MD0000421	x	x	x	This study
Cystoseira funkii	C. funkii	Italy	Aeolian Is, Salina	L:0609449	-	FM958357	FM993018	Draisma
Cystoseira granulata	C. granulata	France	Brittany	PC:FR295	-	-	FM993039	Draisma
Cystoseira humilis	C. humilis MB7	Portugal	Albufeira, Manuel Lourenço	MB7	x	KF525359	-	This study
Cystoseira humilis var. *myriophyoides*	C. humilis var. myriophylloides RB22	Spain	Cádiz, El Mirlo	MBR22	x	x	x	This study
Cystoseira mauritanica	C. mauritanica RB18	Spain	Cádiz, El Mirlo	MBR18	x	x	x	This study
Cystoseira mediterranea	C. mediterranea	France	Cote Vermeille, Le Troc	L:0609379	-	FM958371[*]	FM993022	Draisma
Cystoseira mediterranea	C. mediterranea BL14	Spain	Girona, Blanes	BL14	x	KF525356	x	This study
Cystoseira mediterranea	C. mediterranea BL5	Spain	Girona, Blanes	BL5	x	KF525357	KF525365	This study
Cystoseira nodicaulis	C. nodicaulis 2	France	Brittany, Santec	PC FR289	EU681400	FM958369[*]	FM993036	Draisma
Cystoseira nodicaulis	C. nodicaulis MB14	Spain	A Coruña, Santa Mariña	MB14.2	x	x	x	This study
Cystoseira nodicaulis	C. nodicaulis MB18	Spain	A Coruña, Santa Mariña	MB18	x	x	x	This study
Cystoseira sonderi	C. sonderi 1	Cape Verde	Branco Is	L:CANCAP-VII 9718	-	-	FM993040	Draisma
Cystoseira sonderi	C. sonderi 2	Cape Verde	São Tiago ls, Tarrafal Bay	L:CANCAPVII 8621	-	-	FM993041	Draisma
Cystoseira sp.	Cystoseira sp. 1	Croatia	Prvic Island	GENT: KRK 005	-	FM958364	FM993031	Draisma
Cystoseira sp.	Cystoseira sp. 2	Spain	Menorca, Illa d'es Porros	L:0609306	-	FM958369	FM993035	Draisma
Cystoseira sp.	Cystoseira sp. MP1	Portugal	Madeira Is, Caniço	PG071164	-	x	x	This study
Cystoseira sp.	Cystoseira sp. MP14	Malta	Xghajra	PG081405	x	-	x	This study
Cystoseira sp.	Cystoseira sp. MP2	Portugal	Madeira Is, Caniço	PG071220	x	-	x	This study
Cystoseira sp.	Cystoseira <sp. MP31	Portugal	Graciosa Is, Carapacho	MD0003137	x	x	-	This study
Cystoseira spinosa	C. spinosa	Croatia	Brac Island	-	-	HQ438490	HQ438492	Puizina
Cystoseira spinosa	C. spinosa ALI4	Spain	Alicante, Santa Pola	ALI4	x	x	x	This study
Cystoseira spinosa	C. spinosa RB24	Spain	Almería, La Serena	MBR24	-	-	x	This study
Cystoseira spinosa var. *tenuior*	C. spinosa var. tenuior	Spain	Menorca, Cala Mica	L:0609312	-	FM958374	FM993037	Draisma
Cystoseira squarrosa	C. squarrosa	Croatia	Dubrovnik city area	-	-	HQ438491	HQ438494	Puizina
Cystoseira tamariscifolia	C. tamariscifolia 1	Spain	A Coruña	SANT16323	EU681401[**]	FM958358	FM993019	Draisma

Sample		**Geographic origin**		**Sample information and sequences GenBank acession n.º**				
Species	**Reference**	**Country**	**Location**	**Voucher**	**COI**	**23S**	**mt-spacer**	**Author**
Cystoseira tamariscifolia	C. tamariscifolia 3	Spain	A Coruña	SANT:16325	EU681401[”]	FM958370	FM993020	Draisma
Cystoseira tamariscifolia	C. tamariscifolia CB5	Spain	Málaga, Calaburras	CB5	x	x	x	This study
Cystoseira tamariscifolia	C. tamariscifolia HE7	Spain	Granada, Herradura	HE7	x	x	x	This study
Cystoseira tamariscifolia	C. tamariscifolia MB10	Portugal	Albufeira, Olhos de Água	MB10	-	x	x	This study
Cystoseira tamariscifolia	C. tamariscifolia MB11	Portugal	Albufeira, Olhos de Água	MB11	x	x	x	This study
Cystoseira tamariscifolia	C. tamariscifolia MB12	Portugal	Albufeira, Olhos de Água	MB12	x	x	x	This study
Cystoseira tamariscifolia	C. tamariscifolia MB16	Portugal	Albufeira, Olhos de Água	MB16	x	-	x	This study
Cystoseira tamariscifolia	C. tamariscifolia MB19	Portugal	Albufeira, Olhos de Água	MB19	x	x	x	This study
Cystoseira tamariscifolia	C. tamariscifolia MB20	Portugal	Albufeira, Olhos de Água	MB20	x	x	x	This study
Cystoseira tamariscifolia	C. tamariscifolia MB5	Portugal	Albufeira, Manuel Lourenço	MB5	x	-	KF525364	This study
Cystoseira tamariscifolia	C. tamariscifolia MB8	Portugal	Viana do Castelo, Areosa	MB8	x	x	x	This study
Cystoseira tamariscifolia	C. tamariscifolia MB9	Portugal	Albufeira, Manuel Lourenço	MB9	x	x	x	This study
Cystoseira tamariscifolia	C. tamariscifolia TA2	Spain	Cádiz, El Mirlo	TA2	x	x	x	This study
Cystoseira usneoides	C. usneoides	Spain	A Coruña	SANT:15803	MB13[#]	FM958367	FM993033	Draisma
Cystoseira usneoides	C. usneoides MB13	Portugal	Albufeira, Olhos de Água	MB13	x	-	-	This study
Cystoseira usneoides	C. usneoides MB15	Portugal	Albufeira, Manuel Lourenço	MB15	x	KF525360	KF525362	This study
Cystoseira usneoides	C. usneoides MB21	Portugal	Albufeira, Olhos de Água	MB21	x	x	x	This study
Cystoseira usneoides	C. usneoides MB3	Portugal	Aljezur, Odeceixe	MB3	x	x	x	This study
Cystoseira zosteroides	C. zosteroides	Italy	Sicily, S. Maria la Scala	L:0609421	-	FM958366	FM993032	Draisma
Bifurcaria bifurcata	Bifurcaria bifurcata 1	France	Brittany, Santec	PC:FR287/FRA0520	EU681394	FM958373	FM992996	Draisma
Bifurcaria bifurcata	Bifurcaria bifurcata MB34	Spain	A Coruña, Lires	MB34	MB37	x	x	This study
Bifurcaria bifurcata	Bifurcaria bifurcata MB35	Spain	A Coruña	MB35	MB37	-	x	This study
Bifurcaria bifurcata	Bifurcaria bifurcata MB36	Spain	Asturias, Porcia	MB36	MB37	x	MB35	This study
Bifurcaria bifurcata	Bifurcaria bifurcata MB37	Spain	Murcia, Lastra	MB37	MB37	-	x	This study
Polycladia heinii	Polycladia heinii	Oman	Al Ashkharah	GENT:ASH 030	-	FM958335	FM992993	Draisma
Polycladia indica	Polycladia indica	Oman	Dhofar, Mirbat	GENT:DHO2 0297	-	FN435994	FM992994	Draisma
Sirophysalis trinodis	Sirophysalis trinodis	Indonesia/ Australia	Thousand Is, Semak Daun	L:SGAD0509396/ AD-A95058A	KF285949	FM958348	FM993008	Draisma / Soisup
Stephanocystis geminata	Stephanocystis geminata	Canada	British Columbia	GWS004223	FJ409138	-	-	McDevit

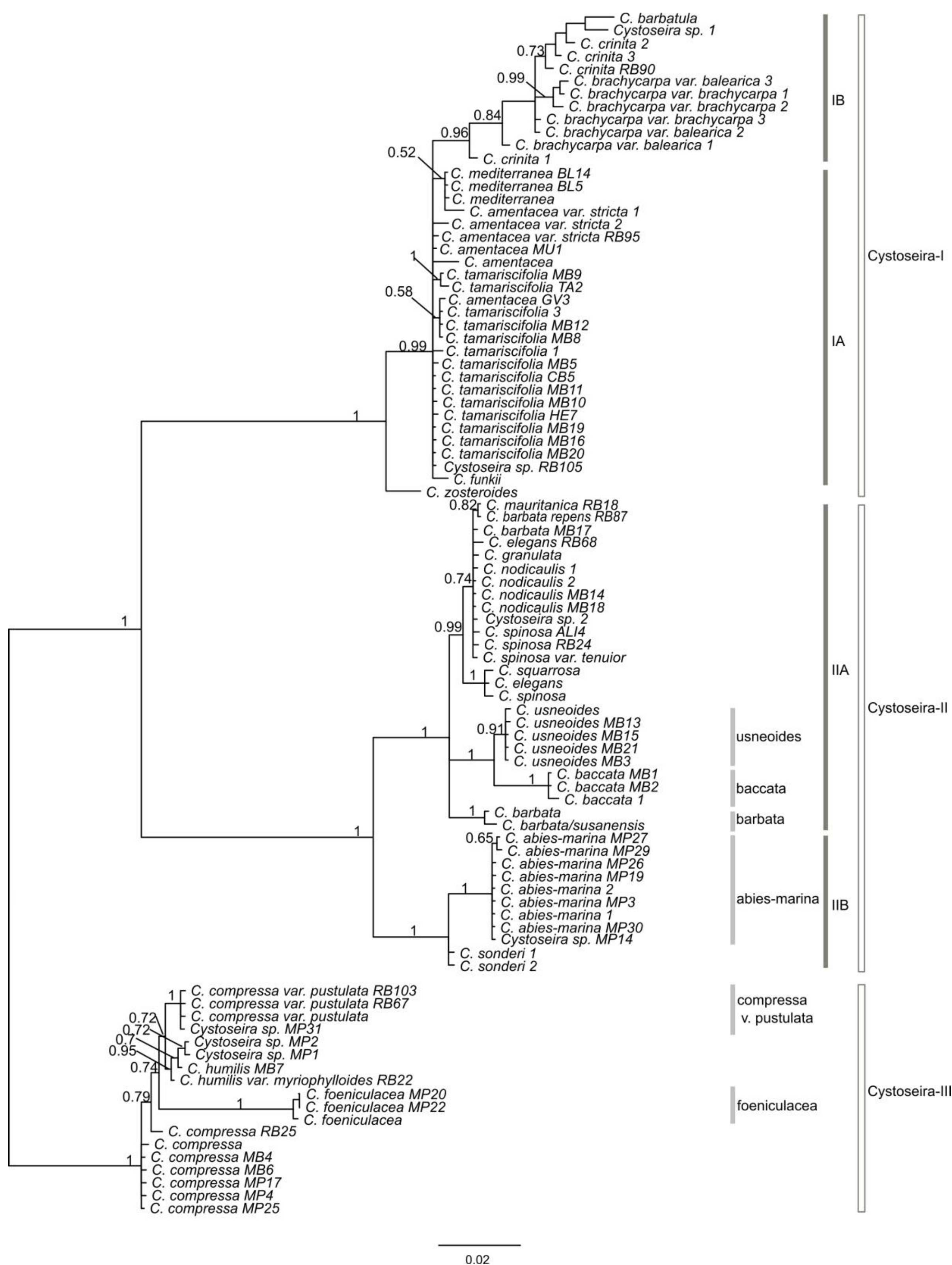
Sample		Geographic origin		Sample information and sequences GenBank acession n.º				
Species	**Reference**	**Country**	**Location**	**Voucher**	**COI**	**23S**	**mt-spacer**	**Author**
Stephanocystis hakodatensis	Stephanocystis hakodatensis	Japan	Hokkaido, Muroran	SAP:086290	-	FM958350	FM993010	Draisma
Stephanocystis setchelli	Stephanocystis setchelli	USA	California, Anacapa Island	AC2	-	FM958351	FM993011	Draisma
Stephanocystis dioica	Stephanocystis dioica	USA	California, Catalina Island	CT2	-	FM958352	FM993012	Draisma
Turbinaria ornata	Turbinaria ornata 3	Indonesia	Thousand Is, Pulau Sepa	L:SGAD0509269 / -	JF718405	FM958414	FM993083	Draisma/ Yu
Fucus distichus	Fucus distichus 1	Spain	Tenerife, Punta del Hidalgo	AY659916/ CSM007A	EU646709	AY659916	AY659884	Draisma/ Kucera
Fucus serratus	Fucus serratus 3	- / Canada	- / Nova Scotia	AY659920/ DM05-014	EU646717	AY659920	AY659875	Draisma/ Kucera
Fucus spiralis	Fucus spiralis 1	- / Canada	- / Nova Scotia	AY659921/ CSM009A	EU646738	AY659921	AY659907	Draisma/ Kucera
Fucus vesiculosus	Fucus vesiculosus 5	- / Canada	- / Nova Scotia	AY494079	AY494079	AY494079	AY494079	Draisma/ Kucera

X Sequences obtained in this study and under registration process in the GenBank database.[*] Sequences identical to, as indicated by Draisma; [#] Sequences assumed to be identical to the sequence given because available sequences from other individuals did not show any variation; [¨] Sequences assumed to be identical to other individual from the same specie available at GenBank database.

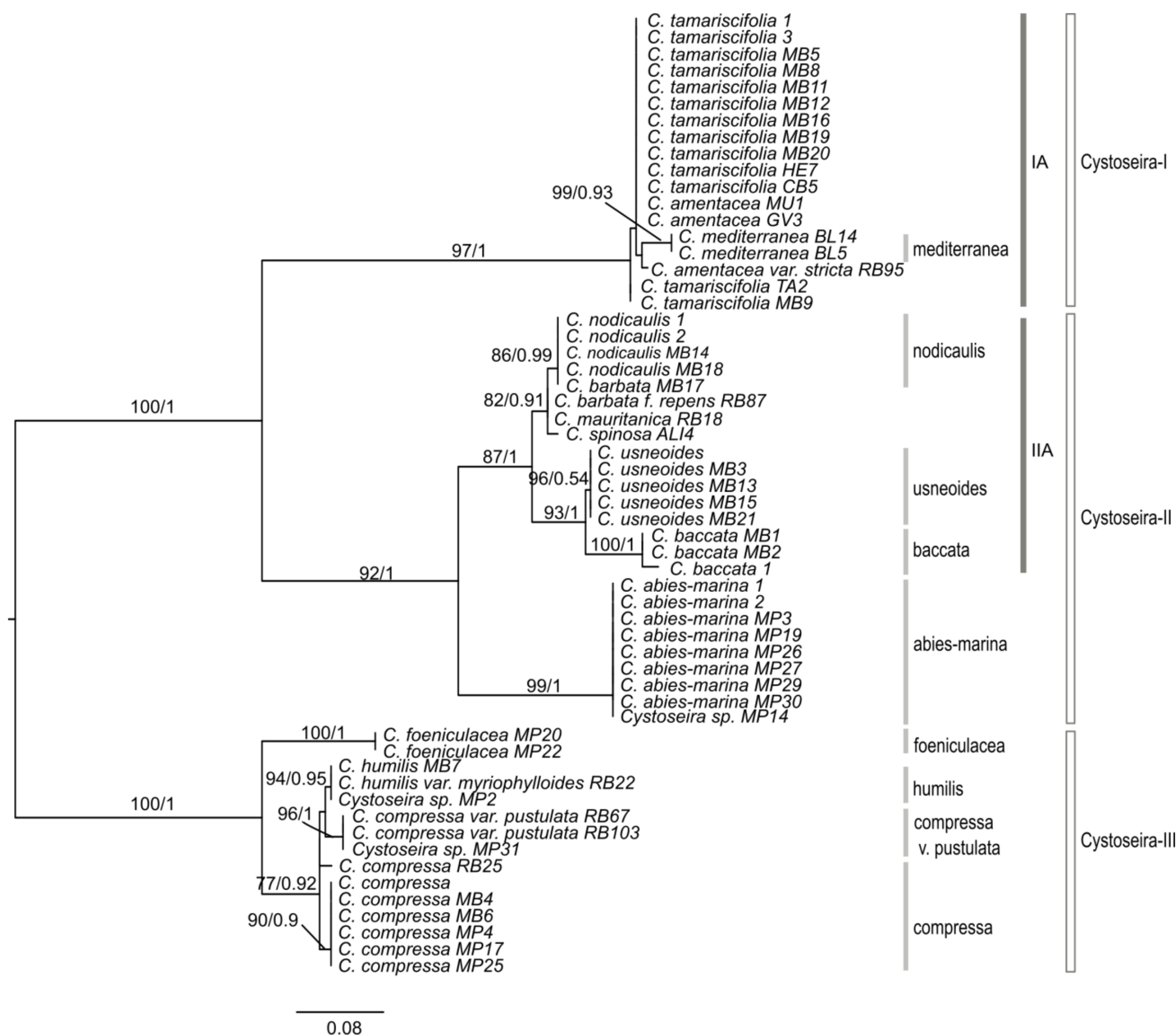
ANNEX 20. Bayesian phylogenetic tree obtained with MrBayes and based on concatenated COI-23S-mt-spacer sequences of the samples from the Sargassaceae family. Values on the branches represent Bayesian posterior probabilities ≥ 90%.



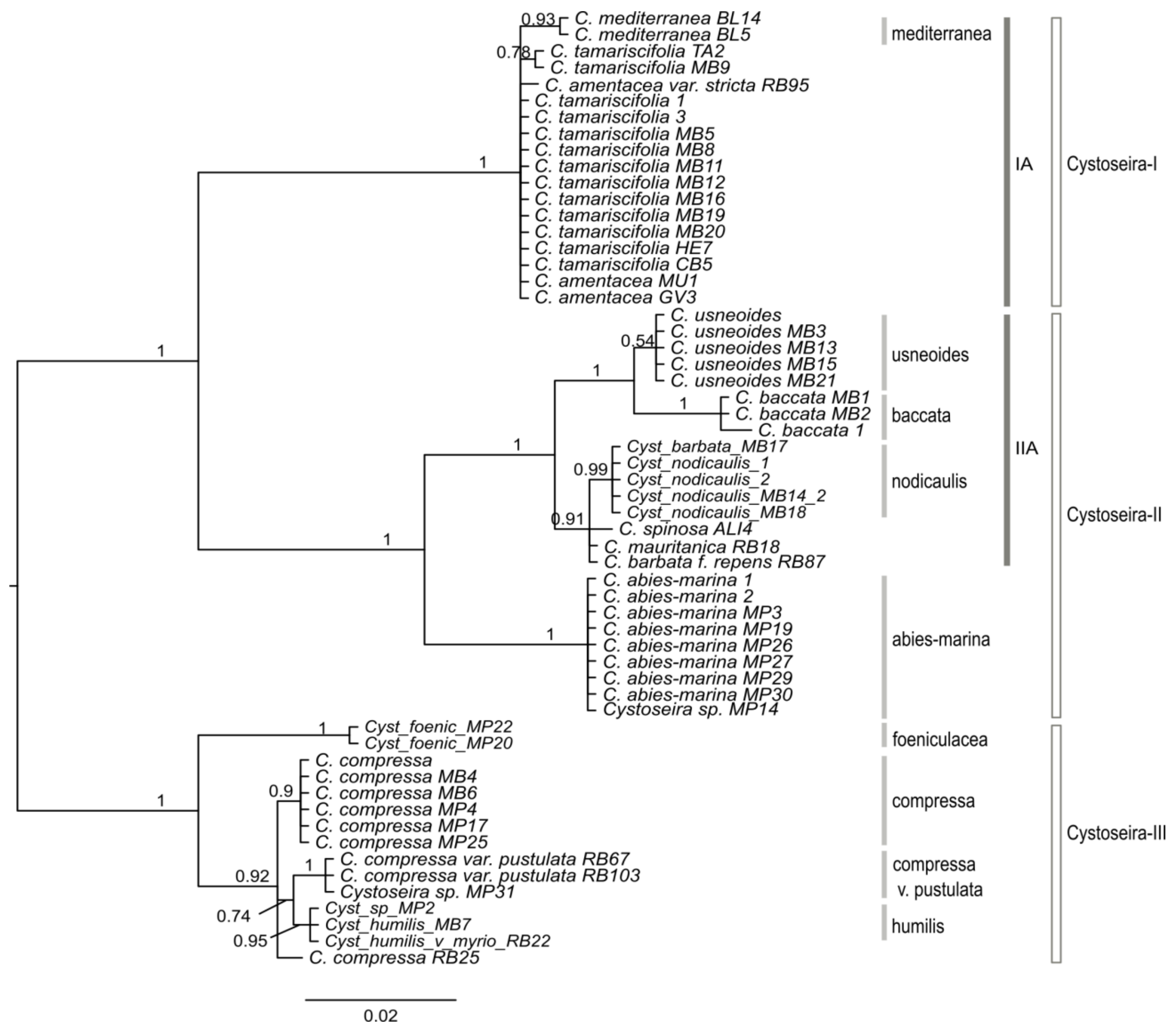
ANNEX 21. Bayesian phylogenetic tree obtained with MrBayes and based on concatenated COI-23S-mt-spacer sequences of the samples from *Cystoseira* genus. Values on the branches represent Bayesian posterior probabilities ≥ 90%.



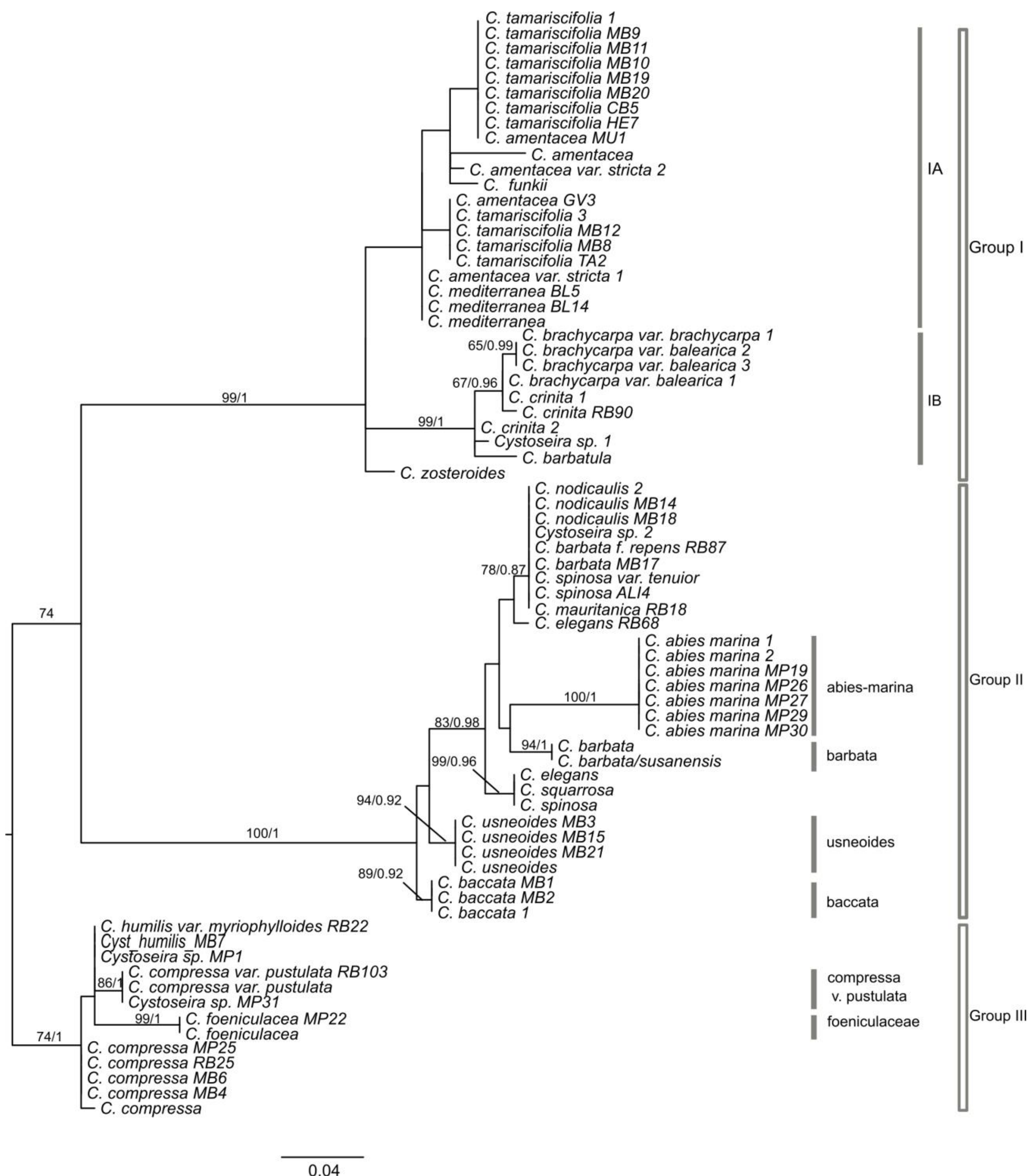
ANNEX 22. Maximum likelihood phylogenetic tree obtained with RAXML and based on the COI sequences of the samples from *Cystoseira* genus. Values on the branches represent maximum likelihood bootstrap support values ≥ 75 on the left, and Bayesian posterior probabilities ≥ 90% on the right.



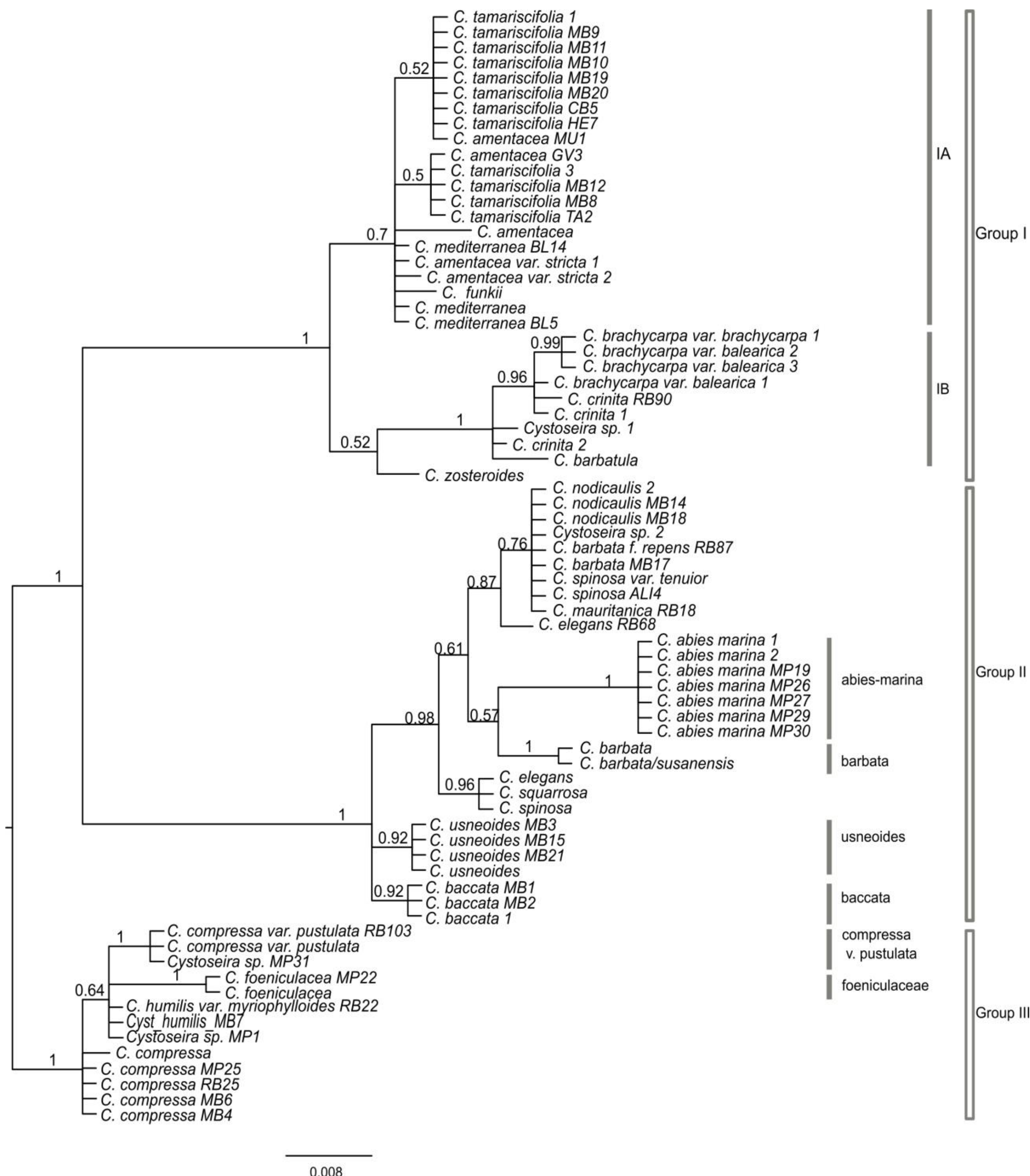
ANNEX 23. Bayesian phylogenetic tree obtained with MrBayes and based on the COI sequences of the samples from *Cystoseira* genus. Values on the branches represent Bayesian posterior probabilities ≥ 90%.



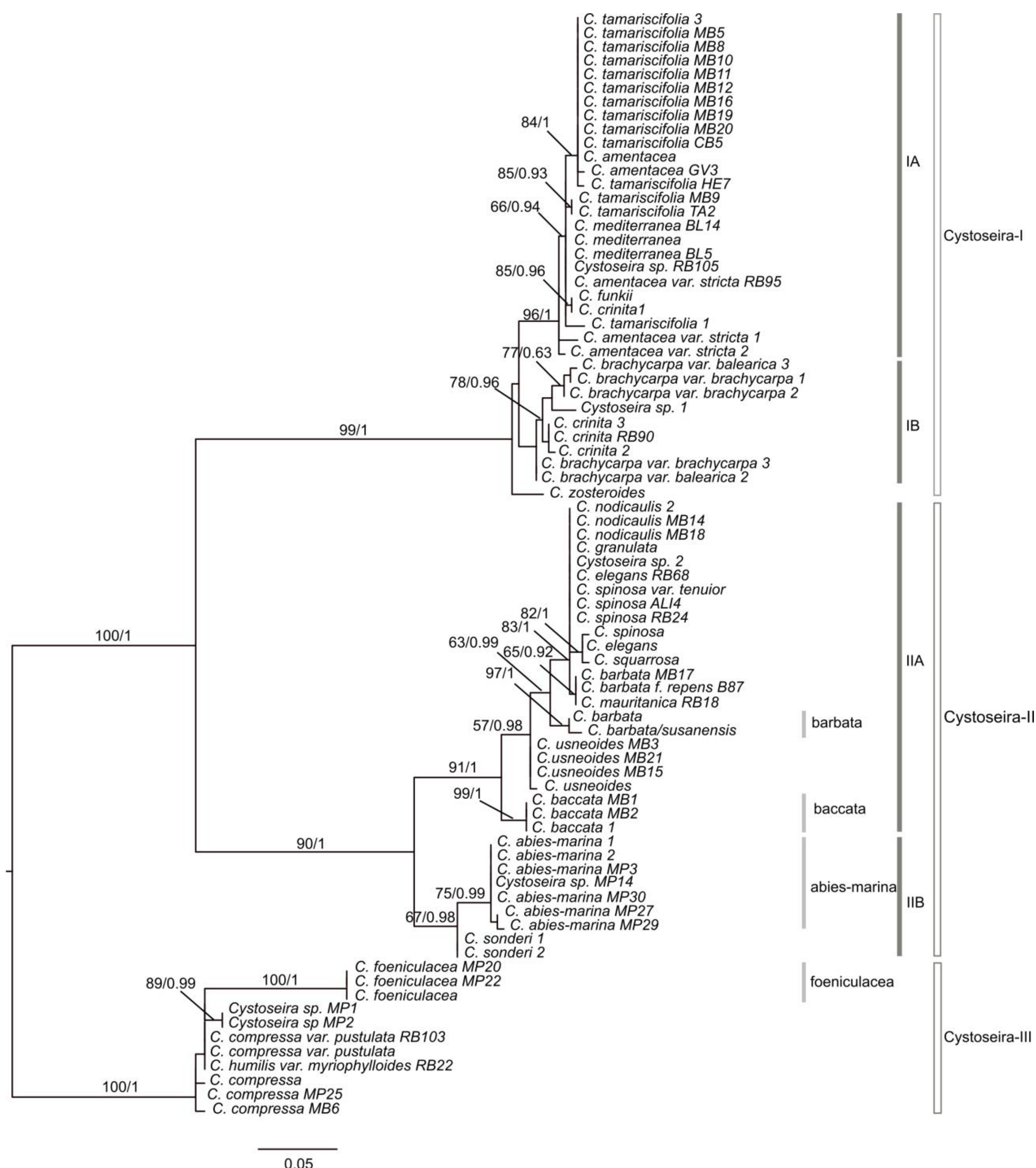
ANNEX 24. Maximum likelihood phylogenetic tree obtained with RAXML and based on the 23S sequences of the samples from the *Cystoseira* genus. Values on the branches represent maximum likelihood bootstrap support values ≥ 75 on the left, and Bayesian posterior probabilities ≥ 90% on the right.



ANNEX 25. Bayesian phylogenetic tree obtained with MrBayes and based on the 23S sequences of the samples from *Cystoseira* genus. Values on the branches represent Bayesian posterior probabilities ≥ 90%.



ANNEX 26. Maximum likelihood phylogenetic tree obtained with RAXML and based on the mt-spacer sequences of the samples from *Cystoseira* genus. Values on the branches represent maximum likelihood bootstrap support values ≥ 75 on the left, and Bayesian posterior probabilities ≥ 90% on the right.



ANNEX 27. Bayesian phylogenetic tree obtained with MrBayes and based on the mt-spacer sequences of the samples from *Cystoseira* genus. Values on the branches represent Bayesian posterior probabilities ≥ 90%.

ANNEX 28. Evolutionary divergence between COI *Cystoseira* sequences.

A.

Group I species	*C. amentacea* var. *stricta*[*]	*C. amentacea*	*C. tamariscifolia*	*C. mediterranea*
C. amentacea var. *stricta*[*]	*			
C. amentacea	0.3	0.3		
C. tamariscifolia	0.3 - 0.5	0.0 - 0.2	0.0 - 0.2	
C. mediterranea	0.9	0.9	0.9 – 1.1	0.0

B.

Group II species	*C. abies-marina*	*Cystoseira sp.* MP14[*]	*C. spinosa*[*]	*C. mauritanica*[*]	*C. barbata* f. *repens*[*]	*C. barbata*[*]	*C. nodicaulis*	*C. baccata*	*C. usneoides*
C. abies-marina	0.0								
Cystoseira sp. MP14[*]	0.0	*							
C. spinosa[*]	5.6	5.6	*						
C. mauritanica[*]	5.4	5.4	0.3	*					
C. barbata f. *repens*[*]	5.4	5.4	0.3	0.0	*				
C. barbata[*]	5.8	5.8	0.6	0.3	0.3	*			
C. nodicaulis	5.8	5.8	0.6	0.3	0.3	0.0	5.6		
C. baccata	6.3 – 6.8	6.3 – 6.8	3.0 – 3.4	3.0 – 3.4	3.0 – 3.4	3.3 – 3.8	3.3 – 3.8	0.0 – 0.5	
C. usneoides	5.9	5.9	2.3	2.0	2.0	2.3	2.3	1.7 – 2.2	0.0

C.

Group III species	*C. compressa*	*C. compressa var. pustulata*	*Cystoseira sp.* MP31*	*Cystoseira* sp. MP2*	*C. humilis**	*C. humilis* var. *myriophylloides**	*C. foeniculacea*
C. compressa	0.0 - 0.6						
C. compressa var. pustulata	0.9 – 1.0	0.0					
Cystoseira sp. MP31[*]	1.0	0.0	*				
Cystoseira sp. MP2[*]	0.6	0.6	0.6	*			
C. humilis[*]	0.6	0.6	0.6	0.0	*		
C. humilis var. *myriophylloides*[*]	0.6	0.6	0.6	0.0	0.0	*	
C. foeniculacea	4.2 – 4.4	4.2 – 4.3	4.2 – 4.4	4.2 – 4.4	4.2 – 4.4	4.2 – 4.4	0.0

*Species represented by only one specimen

ANNEX 29. Evolutionary divergence between 23S *Cystoseira* equences.

A.

Group I species	C. zosteroides*	C. amentacea	C. amentacea var. stricta	C. funkii*	C. mediterranea	Cystoseira sp. 1*	C. b. var. brachycarpa*	C. b. var. balearica	C. crinita	C. barbatula*	C. tamariscifolia
C. zosteroides*	*										
C. amentacea	1.9 – 2.4	0.5 - 2.2									
C. amentacea var. stricta	1.6 – 2.4	0.5 - 1.3	0.8								
C. funkii*	2.6	1.0 - 2.0	0.8 - 1.0	*							
C. mediterranea	1.6	0.5 - 1.9	0.0 - 0.8	1.0	0.0						
Cystoseira sp. 1*	2.6	3.7 - 4.3	3.2 - 3.5	4.3	3.2	*					
C. brachycarpa var. brachycarpa*	3.2	3.5 - 4.3	3.7	3.5	3.7	1.0	*				
C. brachycarpa var. balearica	2.9 – 3.2	3.4 - 4.3	3.2 - 3.7	3.5 - 3.7	3.5 - 3.5	0.8 - 1.0	0.0 - 0.3	0.0 - 0.3			
C. crinita	2.4 – 2.9	3.5 - 4.3	2.9 - 3.7	3.5 - 4.0	2.9 - 3.5	0.3 - 0.8	0.3 - 0.8	0.0 - 0.8	0.3 - 0.8		
C. barbatula*	3.2	4.3 - 4.9	3.7 - 4.3	4.9	3.7	0.8	1.6	1.3 - 1.6	0.8 - 1.6	*	
C. tamariscifolia	1.7 – 2.4	0.0 - 2.3	0.3 - 1.3	1.0 - 1.6	0.3 - 1.0	3.4 - 4.3	3.7 - 4.3	3.5 - 4.3	3.4 - 4.3	4.3 - 4.9	0.0 - 0.6

B.

Group II species	C. barbata	C. barbata f. repens*	C. elegans	C. nodicaulis	C. mauritanica*	C. spinosa var. tenuior*	C. spinosa	Cystoseira sp. 2*	C. squarrosa*	C. baccata	C. usneoides
C. barbata	0.0 - 1.6										
C. barbata f. repens*	0.0 - 1.6	*									
C. elegans	0.5 - 1.9	0.5 - 1.1	1.3								
C. nodicaulis	0.0 - 1.6	0.0	0.5 - 1.1	0.0							
C. mauritanica*	0.0 - 1.6	0.0	0.5 - 1.1	0.0	*						
C. spinosa var. tenuior*	0.0 - 1.6	0.0	0.5 - 1.1	0.0	0.0	*					
C. spinosa	0.0 - 1.9	0.0 - 1.1	0.0 - 1.3	0.0 - 1.1	0.0 - 1.1	0.0 - 1.1	1.1				
Cystoseira sp. 2*	0.0 - 1.6	0.0	0.5 - 1.1	0.0	0.0	0.0	0.0 - 1.1	*			
C. squarrosa*	1.1 - 1.9	1.1	0.0 - 1.3	1.1	1.1	1.1	0.0 - 1.1	1.1	*		
C. baccata	2.1 - 3.1	2.1 - 2.3	2.1 - 2.6	2.1 - 2.3	2.1 - 2.3	2.1 - 2.3	2.1 - 2.3	2.1 - 2.3	2.1 - 2.3	0.0	
C. usneoides	1.9 - 2.9	1.9	1.9 - 2.1	1.9	1.9	1.9	1.9	1.9	1.9	1.0 - 1.1	0.0

C.

Group III species	C. compressa var. pustulata	Cystoseira sp. MP31*	C. compressa	C. humilis var. myriophylloides*	C. humilis*	Cystoseira sp. MP1*	C. foeniculacea	C. abies-marina
C. compressa var. pustulata	0.0							
Cystoseira sp. MP31*	0.0	*						
C. compressa	0.8 - 1.0	0.8 - 1.0	0.0 - 0.3					
C. humilis var. myriophylloides*	0.5	0.5	0.3 - 0.5	*				
C. humilis*	0.5	0.5	0.3 - 0.5	0.0	*			
Cystoseira sp. MP1*	0.6	0.6	0.3 - 0.3	0.0	0.0	*		
C. foeniculacea	2.1	2.1	1.8 - 2.1	1.6	1.6	1.7	0.0	
C. abies-marina	10.4	10.4	10.4 - 10.7	10.1	10.1	10.1	9.7	0.0

* Species represented by only one specimen

ANNEX 30. Evolutionary divergence between mt-spacer *Cystoseira* sequences.

A.

Group I species	*C. amentacea* var. *stricta*	*C. amentacea*	*C. mediterranea*	*C. tamariscifolia*	*C. funkii**	*C. brachycarpa**	*C. b.* var. *balearica*	*C. b.* var. *brachycarpa*	*C. crinita*	*Cystoseira sp. 1**	*C. zosteroides**
C. amentacea var. *stricta*	1.1 - 2.6										
C. amentacea	0.7 - 4.4	0.4									
C. mediterranea	0.4 - 3.0	1.1 - 1.7	0.0								
C. tamariscifolia	0.4 - 3.9	0.0 - 3.0	0.7 - 1.4	0.0 - 2.6							
*C. funkii**	1.1 - 3.4	1.8 - 2.6	0.7	1.5 - 2.2	*						
*C. brachycarpa**	0.4 - 3.0	1.1 - 1.7	0.0	0.7 - 1.5	0.7	*					
C. brachycarpa var. *balearica*	5.6 - 7.0	6.0 - 7.6	5.2 - 5.7	6.0 - 6.8	4.9 - 5.4	5.3 - 5.7	2.9				
C. brachycarpa var. *brachycarpa*	5.7 - 7.7	7.6 - 8.5	5.2 - 6.0	6.0 - 7.4	4.9 - 6.1	5.3 - 6.0	0.0 - 2.9	0.4 - 2.5			
C. crinita	1.1 - 9.0	1.8 - 9.5	0.7 - 6.8	1.4 - 8.3	0.0 - 6.5	0.7 - 6.8	2.1 - 3.3	2.1 - 6.8	0.3 - 7.6		
*Cystoseira sp. 1**	6.4 - 7.3	6.8 - 8.5	6.0	6.8 - 7.4	6.1	6.0	1.8 - 3.2	1.4 - 3.2	3.5 - 6.8	*	
*C. zosteroides**	7.3 - 8.7	7.7 - 9.6	7.3	7.3 - 8.1	6.6	7.3	5.1 - 6.8	5.1 - 7.5	6.2 - 7.7	7.7	*

B.

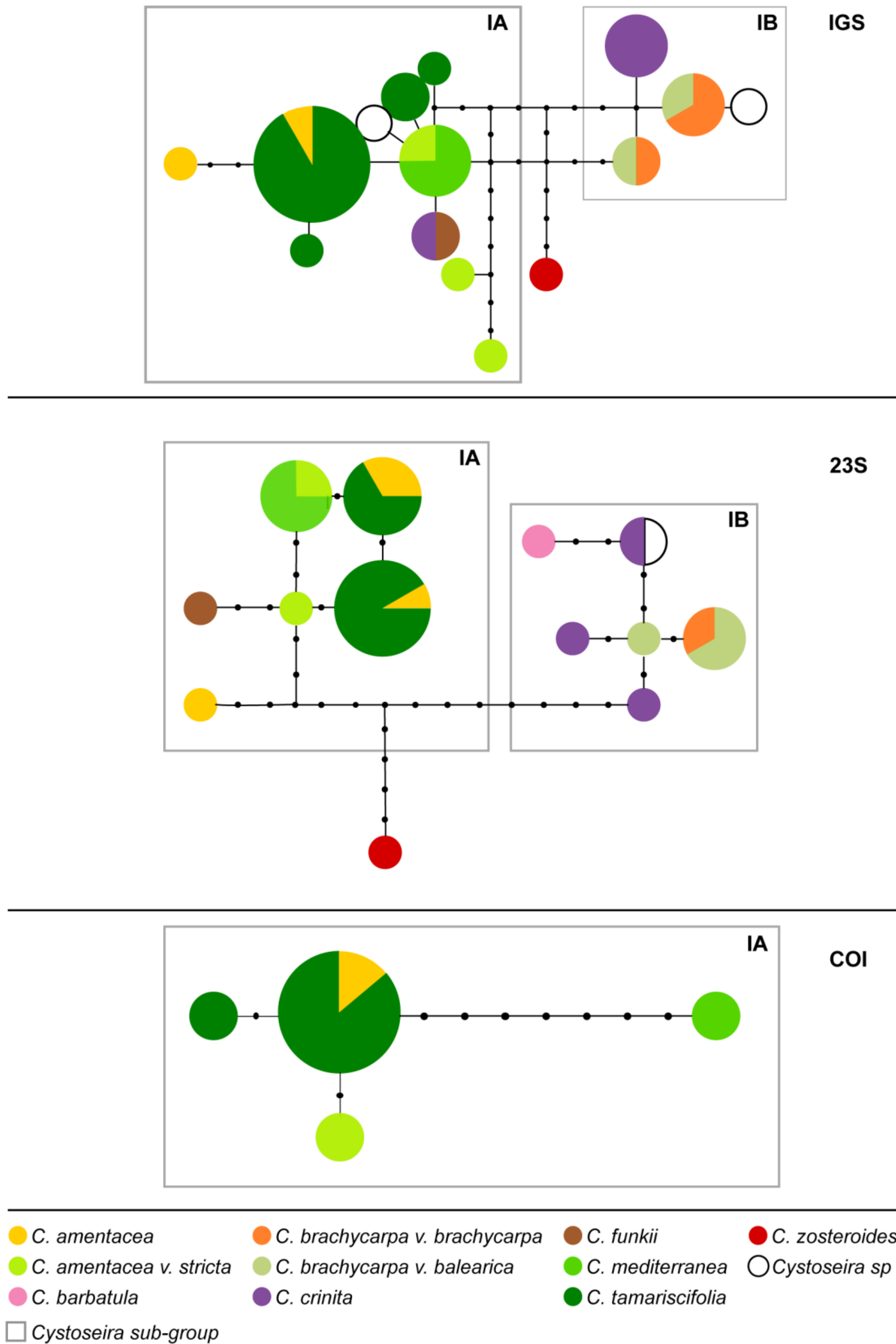
Group II species	*C. abies-marina*	*Cystoseira sp. MP14**	*C. sonderi*	*C. squarrosa**	*C. elegans**	*C. baccata*	*C. barbata*	*C. mauritanica**	*C. barbata* f. *repens**	*C. elegans**	*C. granulata**	*C. nodicaulis*	*Cystoseira sp. 2**	*C. spinosa*	*C. spinosa* var. *tenuior**	*C. usneoides*
C. abies-marina	0.0 - 0.4															
*Cystoseira sp. P14**	0.0 - 0.7	*														
C. sonderi	2.4 - 3.5	2.7 - 2.4	0.0													
*C. squarrosa**	11.1 - 12.0	11.1	9.3	*												
*C. elegans**	11.5 - 12.4	11.5	9.7	0.3	*											
C. baccata	11.7 – 13.1	11.2 - 12.1	9.8 - 10.4	7.3 - 8.1	6.9 - 7.6	0.0										
C. barbata	12.0 – 13.8	11.5 - 12.9	9.7 - 11.5	3.9 - 4.7	1.7 - 4.3	5.4 - 7.2	0.7 - 3.9									
*C. mauritanica**	11.1 - 12.0	11.1	9.7	2.1	1.7	6.5 - 7.2	0.0 - 3.9	*								
C. barbata f. *repens**	11.5 - 12.4	11.5	9.7	2.1	1.7	6.5 - 7.2	0.0 - 3.9	0.0	*							
*C. elegans**	10.9 - 11.8	10.9	9.5	1.8	1.4	6.7 - 7.4	1.1 - 3.3	1.1	1.1	*						
*C. granulata**	11.6 - 12.5	11.6	10.2	2.1	1.7	6.9 - 7.7	0.7 - 3.9	0.7	0.7	1.1	*					
C. nodicaulis	11.5 - 14.0	11.5 – 13.5	10.1 - 11.8	2.1 - 2.4	1.7 - 2.0	7.6 - 8.9	0.7 - 4.5	0.7 - 0.8	0.7 - 0.8	1.1 - 1.2	0.0	0.0				
*Cystoseira sp. 2**	10.6 - 11.5	10.6	9.3	1.4	1.0	6.2 - 6.8	0.7 - 3.2	0.7	0.7	0.4	0.7	0.7 - 0.8	*			
C. spinosa	10.6 - 12.9	10.6 - 12.0	9.3 - 10.1	0.7-1.4	0.3 - 1.0	6.2 - 8.1	0.7 - 4.7	0.7 - 2.1	0.7 - 2.1	0.4 - 1.8	0.7 - 2.1	0.7 - 2.4	0.0 - 1.4	0.0 - 1.4		
C. spinosa var.*tenuior**	10.6 - 11.5	10.6	9.3	1.4	1.0	6.2 - 6.8	0.7 - 3.2	0.7	0.7	0.4	0.7	0.7 - 0.8	0.0	0.0-1.4	*	
C. usneoides	12.0 - 12.9	11.5 - 12.0	9.9 - 11.0	4.5 - 4.9	4.1 - 4.5	4.0 - 4.5	2.2 - 4.1	3.7 - 4.1	3.7 - 4.1	3.4 - 3.8	3.3 - 3.7	3.3 - 4.2	2.9 - 3.3	2.9 – 4.4	2.9 - 3.3	0.0 - 0.4

C.

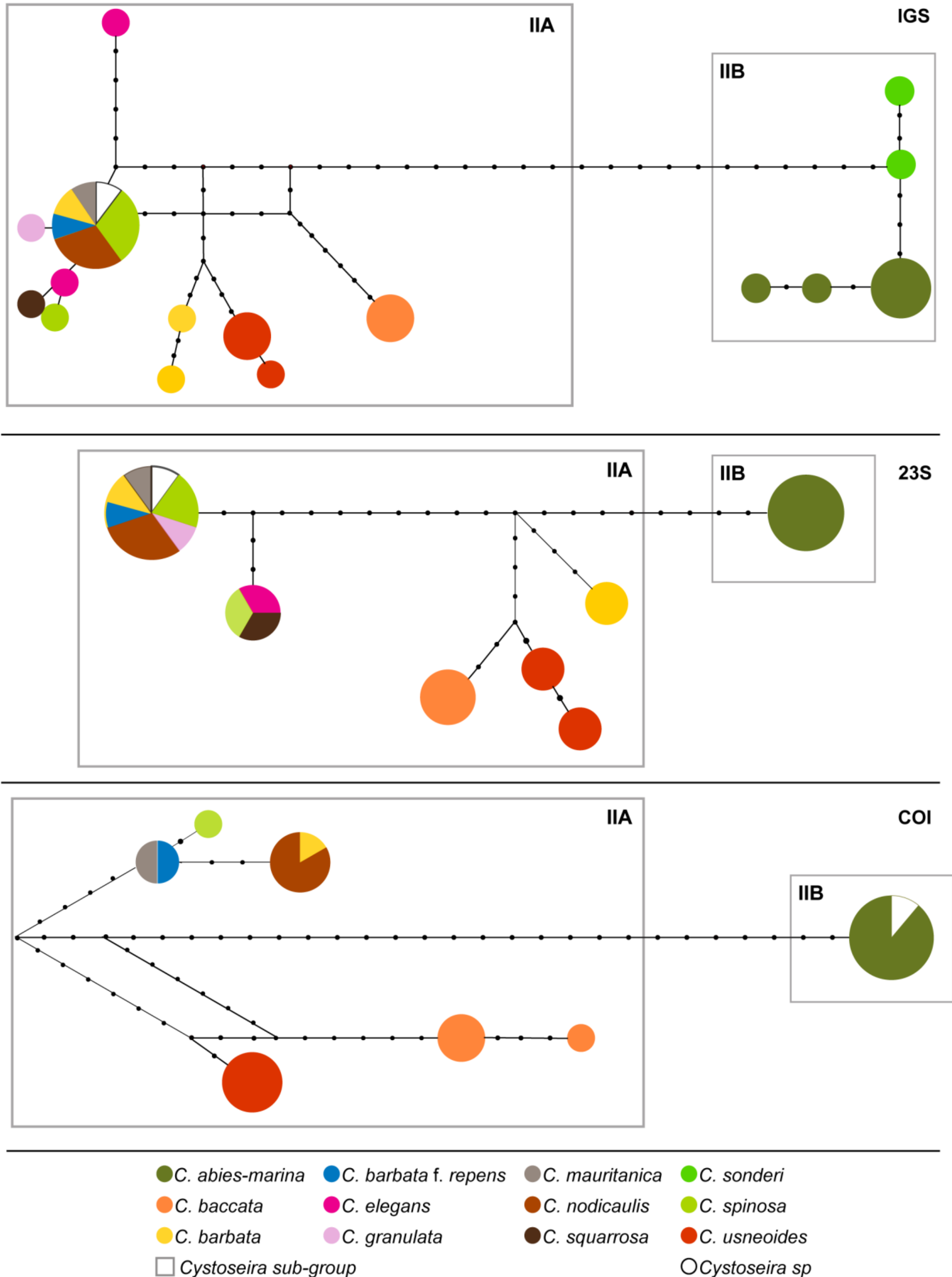
Group III species	*C. compressa*	*C. c.* var. *pustulata**	*C. humilis* var. *myriophyoides**	*Cystoseira sp. MP2**	*Cystoseira sp. MP1**	*C. foeniculacea*
C. compressa	0.4 - 0.8					
C. compressa var. *pustulata**	0.7 - 1.5	*				
C. humilis var. *myriophyoides**	0.4 - 0.8	0.4 - 0.8	*			
*Cystoseira sp. MP2**	1.5 - 1.9	1.5 - 1.9	1.1	*		
*Cystoseira sp. MP1**	1.5 - 1.9	1.5 - 1.9	1.1	0.0	*	
C. foeniculacea	9.7 - 10.9	8.8 - 9.3	9.3 - 9.9	10.6 - 11.4	10.6 - 11.4	0.0

* Species represented by only one specimen

ANNEX 31. Median-Joining networks of Cystoseira-I mt-spacer, 23S and COI haplotypes. Pie charts are proportional to haplotype frequencies. Theoretical median vectors are represented by black dots. Colors represent the different *Cystoseira* species as described in the legend.



ANNEX 32. Median-Joining networks of Cystoseira-II mt-spacer, 23S and COI haplotypes. Pie charts are proportional to haplotype frequencies. Theoretical median vectors are represented by black dots. Colors represent the different *Cystoseira* species as described in the legend.



ANNEX 33. Median-Joining networks of Cystoseira-III mt-spacer, 23S and COI haplotypes. Pie charts are proportional to haplotype frequencies. Theoretical median vectors are represented by black dots. Colors represent the different *Cystoseira* species as described in the legend.