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Antifungal, antibacterial and antiviral activity of *Chondracanthus teedei* var. *lusitanicus* (Gigartinaceae, Rhodophyta)

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biodiversidade e Biotecnologia Vegetal, área de especialização em Biotecnologia, realizada sob a orientação científica do Professor Doutor Leonel Pereira (Universidade de Coimbra) e da Professora Doutora Teresa Gonçalves (Universidade de Coimbra)

Fabiana Soares

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"Há um tempo em que é preciso abandonar as roupas usadas...
Que já têm a forma do nosso corpo...
E esquecer os nossos caminhos que nos levam sempre
aos mesmos lugares...

É o tempo da travessia...
E se não ousarmos fazê-la...
Teremos ficado... para sempre...
À margem de nós mesmos..."

Fernando Pessoa
(1888-1935)

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Abbreviations

[#] Online reference
AIDS – Acquired Immune Deficiency Syndrome
API – Analytical Profile Index
ATCC – American Type Culture Collection
ATR – Attenuated Total Reflectance
Caco – Human Caucasian Colon Adenocarcinoma
CBS – Centraalbureau voor Schimmelcultures
cDNA – complementary DNA
CFU – Colony-forming Unit
CLSI – Clinical & Laboratory Standards Institute
CMV – Cytomegalovirus
CO_2 – Carbon Dioxide
DAPI – 4',6-diamidino-2-phenylindole
DENV – Dengue Virus
DMEM – Dulbecco's Modified Eagle's Medium
DNA – Deoxyribonucleic acid
DW – Dry Weight
em – Emission
exc – Excitation
FBS – Fetal Bovine Serum
FG – Female Gametophyte
FT – Fourier Transform
FTIR – Fourier Transform Infrared
FW – Final Weight
HCl – Hydrogen Chloride
HEK – Human Embryonic Kidney
HIV – Human Immunodeficiency virus
HSV – Herpes Simplex Virus
IMTA – Integrated Multitrophic Aquaculture
IW – Initial Weight
kDa – Kilodalton
Ln – Natural logarithm

LPS – Lipopolysaccharide

MEC – Minimum Effective Concentration

MIC – Minimum Inhibitory Concentration

n – Number of replicates

n.s. – non significant

Na_2CO_3 – Sodium Carbonate

NaOH – Sodium Hydroxide

nm – nanometer

NMR – Nuclear Magnetic Resonance

OD – Optical Density

PDA – Potato Dextrose Agar

PES – Provasoli's Enriched Seawater

qPCR – quantitative polymerase chain reaction

RGR – Relative Growth Rate

RNA – Ribonucleic acid

rpm – Rotations per minute

RPMI – Roswell Park Memorial Institute

RT-PCR – Reverse transcription polymerase chain reaction

SE – Standard Error

Tetra – Tetrasporophyte

UV – Ultra Violet

VSE – von Stosch enriched

VSV – Vesicular Stomatitis Virus

wk – Week

YME – Yeast Malt Extract

YPD – Yeast Extract Peptone Dextrose

Resumo

Neste trabalho, a espécie *Chondracanthus teedei* var. *lusitanicus* foi estudada a fim de se avaliar a sua propagação vegetativa através do cultivo laboratorial e do cultivo em aquacultura multitrófica integrada. A actividade antifúngica, antibacteriana e antiviral dos seus extractos de carragenana foi também avaliada.

Apesar do cultivo laboratorial desta espécie não ter sido bem-sucedido, os resultados demonstraram que as maiores taxas de crescimento (0.56 ± 1.9 % dia) e productividade (1.02 ± 2.55 (g (dw) m^{-2} dia)) foram alcançadas nas densidades de cultivo 1 g L^{-1} e 2 g L^{-1}, respectivamente. Relativamente ao seu cultivo num sistema de aquacultura, este revelou melhores resultados comparativamente àqueles registados no cultivo laboratorial. Assim, as maiores taxas de crescimento (2.04 ± 1.9 % dia) e productividade (53.1 ± 1.2 g (dw) m^{-2} dia)) foram alcançadas na densidade de cultivo 8 g L^{-1}.

As extracções alcalinas desta espécie revelaram que a fase tetrasporófita produziu o maior rendimento de carragenanas (38 ± 1.1 %), seguida da fase do gametófito feminino (33 ± 0.01 %).

A análise de espectroscopia vibracional permitiu confirmar a presença da carragenana híbrida kappa/iota pertencente à fase gametófita e a presença da carragenana lambda pertencente à fase tetrasporófita. Estes extractos foram testados em *Alternaria infectoria* e *Aspergillus fumigatus* e os resultados demonstraram a indução de alterações morfológicas nas hifas destes fungos. Os extractos pertencentes ao gametófito feminino e à fase tetrasporófita induziram alterações morfológicas em *A. infectoria* após a exposição deste fungo a uma concentração mínima de 125 µg/mL e 60 µg/mL, respectivamente. Relativamente ao fungo *Asp. fumigatus*, apenas o extracto pertencente à fase do gametófito feminino revelou induzir alterações morfológicas após uma exposição deste fungo a uma concentração mínima de 87.5 µg/mL de extracto. Contudo, nenhum dos extractos revelou inibir o crescimento ou causar alterações morfológicas na levedura *Candida albicans*.

Os extractos de carragenana foram também avaliados na modulação dos componentes de parede, quitina e β-glucano, de *A. infectoria* e *Asp. fumigatus*. Os resultados obtidos demonstraram uma diminuição significativa na concentração de β-glucano em *A. infectoria* após exposição deste fungo a 150 µg/mL do extracto pertencente à fase do gametófito feminino e a 100 µg/mL do extracto pertencente à fase

tetrasporófita. A concentração da quitina, no entanto, permaneceu praticamente sem alterações aquando da exposição aos dois extractos. Pelo contrário, o conteúdo em quitina do fungo *Asp. fumigatus* decresceu significativamente aquando da exposição a 150 µg/mL de ambos os extractos. No que concerne ao conteúdo em β-glucano, apenas o extracto pertencente ao tetrasporófito revelou aumentar significativamente este componente de parede.

Em relação à actividade antibacteriana, os resultados obtidos revelaram que ambos os extractos foram ineficazes contra *Escherichia coli* e *Staphylococcus aureus*.

No que respeita à actividade antiviral, os resultados obtidos no pré-tratamento revelaram uma tendência de ambos os extractos para uma inibição da infecção viral por Lentivirus após exposição do mesmo a uma concentração de 200 µg/mL de extracto. No ensaio virucida, apenas o extracto do gametófito feminino revelou possuir tendência inibitória. Por outro lado, no ensaio virucida com Coxsackie virus A-12, apenas o extracto da fase do tetrasporófito revelou inibição da infecção viral.

Pode-se, então, concluir que ambos os extractos de carragenanas revelaram um potencial efeito antifúngico contra *A. infectoria* e *Asp. fumigatus* e uma tendência inibitória em relação ao Lentivirus e ao Coxsackie virus A-12.

Contudo, nenhum dos extractos se revelou eficaz contra *C. albicans*, *E. coli* and *S. aureus*.

Palavras-chave: *Chondracanthus teedei* var. *lusitanicus*; cultivo laboratorial; cultivo em aquacultura; carragenanas; antimicrobiano; antiviral

Abstract

Chondracanthus teedei var. *lusitanicus* (Gigartinales, Rhodophyta) was studied in order to evaluate its vegetative propagation both at laboratory and IMTA system, and to determine the antifungal, antibacterial and antiviral activity of its carrageenan extracts.

Although the laboratory cultivation revealed to be mostly unsuccessful, the highest relative growth rate was achieved at cultivation density 1 g L^{-1} (0.56 ± 1.9 % day^{-1}) and the highest productivity was achieved at cultivation density 2 g L^{-1} (1.02 ± 2.55 (g (dw) m^{-2} day^{-1})). The cultivation of *C. teedei* var. *lusitanicus* in an IMTA system showed better results than the laboratory cultivation. The highest relative growth rate (2.04 ± 1.9 % day^{-1}) and productivity (53.1 ± 1.2 g (dw) m^{-2} day^{-1})) were achieved at cultivation density 8 g L^{-1}.

The alkali extractions of this species revealed that the tetrasporophyte lifecycle phase produced a carrageenan content of 38 ± 1.1 %, followed by the female gametophyte, which produced a carrageenan content of 33 ± 0.01 %.

The FTIR-ATR spectroscopic analysis allowed confirming the presence of a hybrid kappa/iota carrageenan belonging to the gametophyte phase and the presence of a lambda carrageenan in the tetrasporophyte phase. These carrageenan extracts were tested against *Alternaria infectoria* and *Aspergillus fumigatus* and revealed to induce morphological changes in the hyphae of these fungi. The extracts belonging to the female gametophyte (FG) and the tetrasporophyte (Tetra) induced these morphological alterations in *A. infectoria* after exposure to a Minimum Effective Concentration (MEC) of 125 µg/mL and 60 µg/mL, respectively. Regarding *Asp. fumigatus*, only FG extract revealed to induce hyphal morphological alterations after exposure to a Minimum Effective Concentration of 87.5 µg/mL. However, none of the tested extracts revealed to inhibit or cause a morphological change in yeast *Candida albicans*.

The same carrageenan extracts were evaluated on the modulation of chitin and β-glucan cell wall components of *A. infectoria* and *Asp. fumigatus*. Results showed a significantly decreased in the concentration of β-glucan content in *A. infectoria* after exposure to 150 µg/mL of FG extract and 100 µg/mL of Tetra extract. Chitin cell wall content remained almost unchanged upon exposure to both extracts. On the other hand, the chitin cell wall content of *Asp. fumigatus* decreased significantly upon exposure to

150 µg/mL of both FG and Tetra extracts. As regards to β-glucan, only Tetra extract revealed a significantly increase in this cell wall component.

In relation to the antibacterial activity, both FG and Tetra extracts revealed to be ineffective against *E. coli* and *S. aureus.*

In what concerns to the antiviral activity, results obtained in the pre-treatment showed a tendency of both extracts to inhibit the Lentivirus viral infection upon exposure to 200 µg/mL. In the virucidal assay, only FG extract revealed a tendency in inhibit the viral infection. On the other hand, only Tetra extract revealed to have a tendency to inhibit the viral infection of Coxsackie virus A-12 in the virucidal assay.

It can be concluded that both carrageenan extracts revealed a potential antifungal activity against *A. infectoria* and *Asp. fumigatus*, and a tendency to inhibit the viral infection of Lentivirus and Coxsackie virus A-12. However, none of the extracts revealed to be effective against *C. albicans*, *E. coli* and *S. aureus*.

Key words: *Chondracanthus teedei* var. *lusitanicus*; laboratory cultivation; IMTA cultivation; carrageenan; antimicrobial; antiviral

Chapter I - Introduction

1.1 What are macroalgae - Seaweeds?

Algae are photosynthetic aquatic organisms that can be small and single-celled or large and have a multiplicity of cells. Due to its high morphological diversity and structure algae can be divided in to microalgae and macroalgae (marine seaweeds) (Fig. 1). Microalgae are unicellular or colonial organisms that can be found in oceans, lakes and rivers, as well as in the bark of trees or on the side of buildings. They can also be found in desert and ice areas. On the other hand, seaweeds are larger algae, visible to the human eye, and that can grow more than 60 m in length (kelp forests). Some seaweed grow attached to surfaces that are bathed by water – underside of boats, ropes or rock faces – while others attached themselves to the shell of crabs, or grow on the surfaces of other seaweeds. Some species such as *Sargassum* (Ochrophyta, Phaeophyceae) can be free-floating (Thomas, 2002).

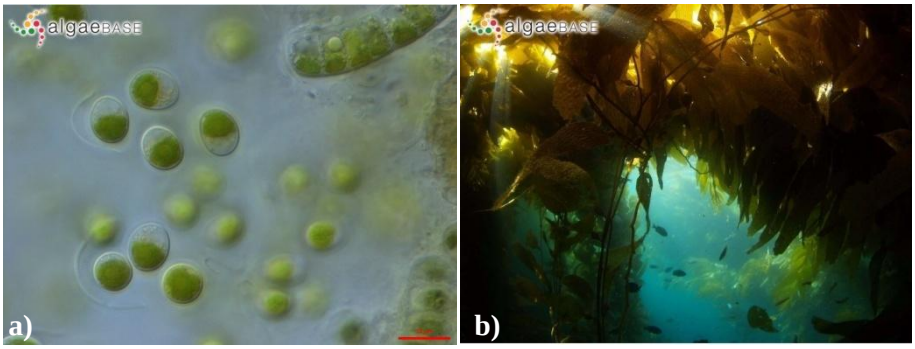

Figure 1 – Example of micro and macroalgae: **a)** *Chlorella vulgaris* (Chlorophyta); **b)** *Macrocystis pyrifera* (Ochrophyta – Phaeophyceae) [1], [2].

Marine seaweeds play an important role in the ecosystem. They are the primary producers in the oceans and so they are crucial to life not only in the aquatic food webs but to the rest of the planet, since they are the original source of fossil carbon found in crude oil and natural gas, and have a contribution of 40% to 50% on the total amount of oxygen in the atmosphere (Preisig & Andersen, 2005; Kilinç *et al*., 2013).

As well as land plants, marine seaweeds belong to Domain Eukarya and Kingdom Plantae (red and green algae) and although their appearance may be similar between each other, marine seaweeds are much smaller and less structurally complex than plants (Graham & Wilcox, 2000; Pereira, 2009); the brown algae belong to Kingdom Chromista (Pereira, 2009).

Seaweeds are divided into three main groups, according to its pigment composition and the way that its photosynthetic membranes are organized (Kilinç *et al*., 2013). Green algae - Phylum Chlorophyta – possess chlorophyll a, b and carotenoids. Chlorophyll a is the pigment responsible for their green color and appearance similar to land plants (Graham & Wilcox, 2000). This pigment is essential for photosynthesis, which requires great amounts of light and therefore, green seaweeds cannot be found at deep and shadowed places.

Some green seaweed such as *Codium* and *Ulva* (formerly *Enteromorpha*) are commonly used as food source. Brown algae – Phylum Heterokontophyta or Ochrophyta – are often found on rocky intertidal shores. This group of algae includes species such as the giant kelp *Macrocystis* or the invasive alga *Sargassum* (Fig. 2).

Figure 2 – Example of green and brown seaweeds: **a)** *Ulva lactuca* (Chlorophyta); **b)** *Sargassum muticum* (Ochrophyta – Phaeophyceae) [3], [4].

As well as the greens and the reds, the brown algae can be used as food source and can be consumed by humans as edible raw, dried or cooked. Brown algae are also harvested for industrial and pharmaceutical uses (Chapman, 2013; Kilinç *et al*., 2013).

Red algae – Phylum Rhodophyta – dominate intertidal rocky shores and exhibit a broad range of morphologies, simple anatomy and display a wide array of life cycles. About 98% of the species are marine, 2% freshwater and a few rare terrestrial/sub-aerial representatives (Gurgel & Lopez 2007; Pereira, 2012).

This Phylum is easily distinguished from other groups of eukaryotic algae due to some characteristics: total absence of centrioles and any flagellate phase; presence of chlorophylls a and d, and accessory pigments (light-harvest) called phycobilins (phycoerythrin and phycocyanin); plastids with unstacked thylakoids, and no external endoplasmic reticulum; absence of parenchyma and presence of pit-connections between cells (i.e. incomplete cytokinesis); floridean starch as storage product.

Some species such as *Gelidium* and *Gracilaria* (Rhodophyta) are used in the manufacture of agar and others, like *Eucheuma* and *Kapaphycus alvarezzi* (Rhodophyta), are used in the production of carrageenan. The red alga *Porphyra* (Rhodophyta) also known as Nori, is commonly used in sushi all over the world (Fig. 3).

Figure 3 – Red alga: a) *Porphyra umbilicalis* (Rhodophyta); b) Nori sushi rolls [5], [6].

Besides food and commercial purposes, red algae have also gain importance in the pharmaceutical field proving to have effects against bacteria, viruses and cancers (Chapman, 2013).

1.2 Red Algae (Rhodophyta)

1.2.1 Gigartinales

Traditionally, red algae can be morphologically separated in three major groups: (1) a unicellular group with reproduction by binary cell division only, (2) a multicellular group where a carpogonial branch is absent or incipient (Bangiophyceae *sensu lato*) and (3) a multicellular group with well developed carpogonial branches (Florideophyceae).

Gigartinales is one of the most extended Order of the red algae and includes a lot of Families (Freshwater *et al.*, 1994; Ragan *et al.*, 1994). This order presents development of a specialized female filament called carpogonial branch. The female gamete (carpogonium) is easily recognizable by the presence of the trichogyne, an elongated extension responsible for receiving the male gametes (spermatium). Germination *in situ* of the zygote leads to the formation of a group of spores (carpospores) which produce carpospores (carposporophyte) inside the cystocarp. The cystocarp is composed of the carposporophyte plus all protective sterile haploid tissue

of the female gametophyte encircling and interacting with it (pericarp). Carpospores develop into a second free-living phase called tetrasporophyte, which can be morphologically similar (isomorphic alternation of generations) or different (heteromorphic alternation of phases) from the gametophytes. Tetrasporophyte plants produce tetrasporangia by meiosis, which release tetraspores. When released, each tetraspore will give rise to either a male or a female haploid gametophyte (Gurgel & Lopez 2007; Pereira, 2012). In general, Gigartinales present triphasic isomorphic or heteromorphic, diplo-haplotic (haploid gametophyte, diploid carposporophyte and diploid tetrasporophyte) or diphasic diplo-haplotic lifecycles (Maggs 1990; Brown *et al*. 2004; Thornber 2006).

Hommersand *et al*. (1993) proposed a revised classification of red algae belonging to the Gigartinaceae family, in which 69 species were reclassified into four previously existing genera (*Chondrus*, *Gigartina*, *Iridae* and *Rhodoglossum*) and 3 reintegrated genera (*Chondracanthus*, *Sarcothalia* and *Mazzaella*), based on morphological characteristics and development. Thus, the specimens of the genus *Gigartina* were transfered to genus *Chondracanthus*, including *Gigartina teedei* and *G. teedei* var. *lusitanicus*, which began to be called *Chondracanthus teedei* and *C. teedei* var. *lusitanicus*, respectively.

A large number of species of high economic interest (carrageenophytes) are members of this order and can be found along the coastline of Portugal (e.g. *Ahnfeltiopsis devoniensis*, *Calliblepharis jubata*, *Chondracanthus acicularis*, *C. teedei* var. *lusitanicus*, *Chondrus crispus*, *Gigartina pistillata*, *Gymnogongrus crenulatus* and *Mastocarpus stellatus* (Barbara & Cremades, 1996; Gaspar, 2002; Pereira & Mesquita, 2004; Pereira & van de Velde, 2011). In this work only *Chondracanthus teedei* var. *lusitanicus* was studied.

1.2.1.1 *Chondracanthus teedei* var. *lusitanicus* (J.E. de Mesquita Rodrigues) Bárbara & Cremades

Chondracanthus teedei (Roth) Kützing (formerly *Gigartina teedei*) presents flattened main axes, regularly pinnately branched (Guiry, 1984; Zinoun *et al*. 1993). The fronds, cartilaginous-membranous, have a purplish-violet colour that darkens by desiccation, becoming greenish-yellow through decay (Rodrigues 1957; Gayral 1982).

This species is abundant throughout the year in the lower horizon of the intertidal zone (Zinoun *et al*. 1993). *Chondracanthus teedei* is widespread in the northeastern Atlantic, Mediterranean and the Black Sea (Ardré, 1970; Orfandis, 1993). It has also been reported from Japan (Mikami, 1965), the Indian Ocean (Silva *et al*., 1996) and Brazil (Ugadim, 1975)**.** According to Rodrigues (1958) and Pereira (2004), the specimens collected in Buarcos bay (Portugal) have very obvious differences compared to specimens taken from Brittany (France), Barcelona (Spain) and the Mediterranean Sea. The specimens collected in Portugal look more robust due to the principal axes of the fronds and their ramifications being wider (reaching 1 cm in the older portions), often reaching 20 cm in length; its branches are more dense and lush and the pinnules not only develop on the margins of the branches, but also on their surfaces; the cystocarps are spherical and sessile, being present in large numbers on the pinnules, margins of the branches and thalli surface (Fig. 4).

Due to these characteristics, the specimens collected in central and northern Portugal belong to the taxon *Chondracanthus teedei* var. *lusitanicus* (Rodrigues, 1958; Bárbara & Cremades, 1996; Pereira, 2004).

Figure 4 – *Chondracanthus teedei* var. *lusitanicus* [7].

This alga has an isomorphic triphasic life cycle (see Fig. 5) (Guiry 1984; Braga 1985, 1990; Pereira, 2012) and appears on the rocks and intertidal pools, generally in shallow water (Gayral, 1982).

This species was object of many studies such as ecology (Braga, 1985, 1990; Orfanidis, 1993; Pereira, 1996); ultrastructure (Tsekos, 1981, 1982, 1983; Tsekos *et al.*, 1985; Tsekos & Diannelidis, 1990; Tsekos & Schnepf, 1991; Tsekos e Reiss, 1993; Tsekos *et al.*, 1993; Pereira & Mesquita, 1994; Tsekos e Reiss, 1994; Tsekos, 1996; Delivopoulos, 2003; Ouriques & Bouzon, 2003); life cycle and reproductive morphology (Rodrigues, 1958; Guiry, 1984; Braga, 1985; Guiry *et al.*, 1987; Braga,

1990; Barbara & Cremades, 1996); and analysis of polysaccharides production (Zinoun, 1993; Zinoun *et al.*, 1993a, b).

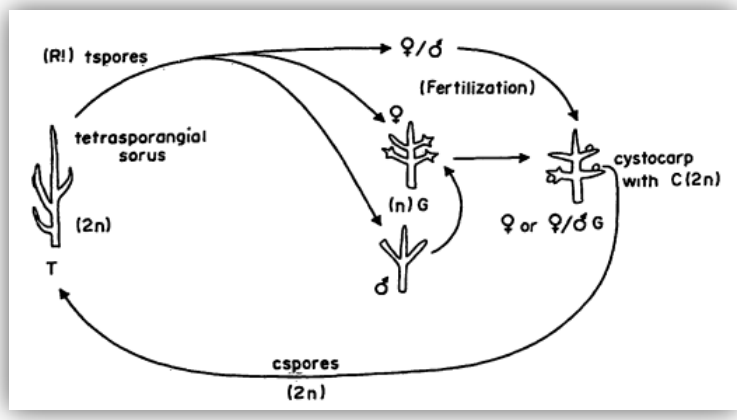

Figure 5 – Life cycle of *Chondracanthus teedei* (From Braga, 1990).

1.3 Cultivation methods of *C. teedei* var. *lusitanicus*

According to Braga (1990), the population of *Chondracanthus teedei* (formerly *Gigartina teedei*) is capable of maintaining itself by vegetative propagation and completion of life-history. This maintenance can also be verified in laboratory culture under proper conditions of light, temperature, pH, photoperiod, etc. Braga concluded that vegetative production of small ramets is an alternative method used by this population to occupy and maintain space. The importance of vegetative propagation as a reproductive strategy is related to the fact that this population is part of a turf-like community.

However, the cultivation of *Chondracanthus teedei* var. *lusitanicus* in laboratory revealed to be mostly unsuccessful. The same was verified in an integrated multitrophic aquaculture system.

1.4 Integrated Multitrophic Aquaculture (IMTA)

IMTA is the practice which combines, in the appropriate proportions, the cultivation of fed aquaculture species (e.g. finfish/shrimp) with organic extractive aquaculture species (e.g. shellfish/herbivorous fish) and inorganic extractive aquaculture species (e.g. seaweed) to create balanced systems for environmental sustainability (biomitigation), economic stability (product diversification and risk reduction) and social acceptability (better management practices). Thus, species such as fish and shrimp produce organic matter and excrete ammonia and CO_2. In turn these sub-products are recovered by seaweeds and other filtrating animals that absorb and incorporate them (Fig. 6) (Barrington *et al.*, 2009; Abreu *et al.*, 2011).

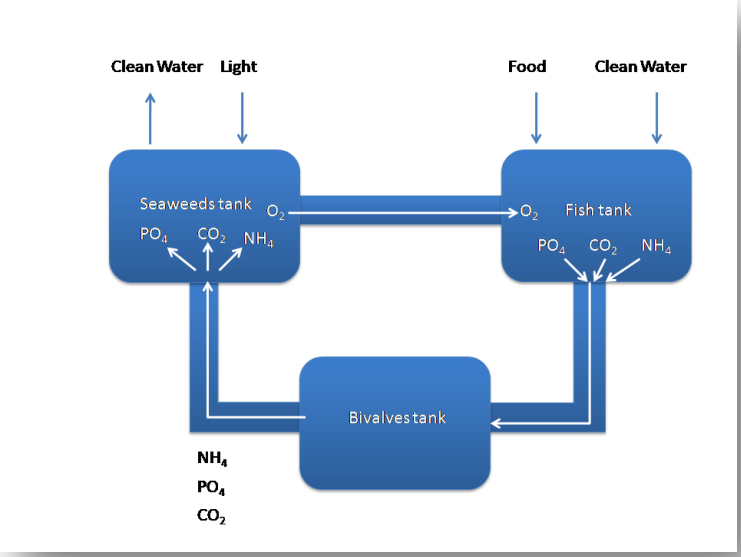

Figure 6 – Closed IMTA system.

Canada, Chile, China, Ireland, South Africa, the United Kingdom (mostly Scotland), Ireland and the United States of America are the only countries to have IMTA systems near commercial scale, or at commercial scale, at present. France, Portugal and Spain have ongoing research projects related to the development of IMTA (Barrington *et al.*, 2009). In Portugal, the implementation of IMTA concept emerged from the interest of investigators who work with seaweeds. And even though

the seaweed's industry in Portugal is practically inexistent, the country has a long tradition in the transformation and exploitation of this resource (Abreu *et al*., 2011).

The use of seaweeds in Portugal goes back to the 14th Century when harvesting of Kelp (called "Sargaço"), still done in the north region, was regulated by King D. Dinis. Due to the abundance and quality of local red seaweeds (mainly *Gelidium corneum* and *Pterocladiella capillacea*), and the lack of Japanese Agar during World War II, the Portuguese agar industry emerged and remained constant until the 20th Century. By that time, due to unfavorable international economic conditions this industry began to significantly drop leading to only one company left today – Iberagar. This company is responsible for the manufacture and distribution of hydrocolloids derived from seaweeds. These seaweed-derived hydrocolloids, or phycocolloids, are very important in terms of their industrial commercialization. In 2009 these phycocolloids had an estimated global value of approximately $US 1 billion and represented more than half of the non-food macroalgal market products (Cardoso *et al*., 2014).

1.5 Phycocolloids

Phycocolloids are structural polysaccharides with high molecular weight found in the cell wall and intercellular spaces of freshwater and marine algae. It usually forms colloidal solutions – an intermediate state between a solution and a suspension – which gives these polysaccharides the ability to be used as thickeners, gelling agents and stabilizers for suspensions and emulsions in diverse industries. Sulfated galactans (e.g., agars and carrageenans) can be obtained from red algae, and alginates and other sulfated polysaccharides (e.g., laminaran and fucoidan) are obtained from brown algae. Phycocolloids are used in food industries as natural additives and have different European codes: E400 (alginic acid), E401 (sodium alginate), E402 (potassium alginate), E403 (ammonium alginate), E404 (calcium alginate), E405 (propylene glycol alginate), E406 (agar), E407 (carrageenan) and E407A (semi-refined carrageenan or "processed *Eucheuma* seaweed"). Agar, alginates and carrageenans are the ones with the highest significant commercial value (Pereira *et al*., 2013; Cardoso *et al*., 2014).

1.5.1 Agar

Agar was the first colloid to be developed and it has applications as a gelling agent for food and also as an inert support medium for microbial culture. This polysaccharide is the dried hydrophilic, colloidal substance extracted commercially from certain marine algae of the phylum Rhodophyta. The most important commercial agarophyte genera are *Gelidium*, *Pterocladiella*, *Gelidiella*, and *Gracilaria*. Agar has also been found in species of *Ceramium*, *Phyllophora*, *Ahnfeltia*, *Campylaephora*, *Acanthopholis, and Gracilariopsis*. It is a polysaccharide, consisting primarily of D- and L-galactose units. About every tenth D-galactopyranose unit contains a sulphate ester group (Pereira *et al*., 2013). Agar may be separated into two fractions. One is a neutral polymer, agarose, composed of repeating units, referred to as agarobiose, and of alternating 1,3-linked β-D-galactopyranose and 1,4-linked 3,6-anhydro-α-L-galactopyranose (Fig. 7). The second fraction has agaropectin, a more complicated structure. It contains residues of sulphuric, pyruvic, and uronic acids, in addition to D-galactose and 3,6-anhydro-L-galactose (Pereira *et al*., 2013; Pereira *et al*., 2015).

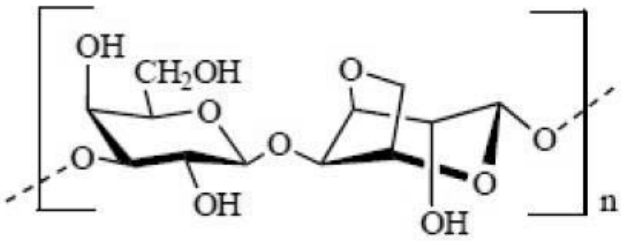

Figure 7 – Idealized structure of the chemical units of agarose (After Cardoso *et al*., 2014).

Agar was, and still is, prepared and sold as an extract in solution (hot) or in gel form (cold), to be used promptly in areas near the factories (FAO, 2010). The product was known as Tokoroten. Its industrialization as a dry and stable product began in the early 18th century and since then, has been called Kanten. Presently, Agar-Agar and Agar are the most accepted worldwide terms. However, it is also called Gelosa in French and Portuguese-speaking countries (Armisen & Galatas, 1987; Minghou, 1990; FAO, 2010).

1.5.2 Alginates

Alginic acid was discovered in 1883 by E. C. C. Stanford, a British pharmacist, who called it algin. Alginic acid is present in the cell walls of brown seaweeds, where it is partially responsible for their flexibility. In this context, brown seaweeds that grow in more turbulent conditions usually have higher alginate content than those in calmer waters (McHugh, 2003). Chemically, alginates are linear copolymers of β-D-mannuronic acid (M) and α-L-guluronic acid (G) (1-4)-linked residues, arranged either in heteropolymeric (MG) and/or homopolymeric (M or G) blocks (Fig. 8) (Chopin, 1999; Larsen *et al*., 2003; Pereira & Mesquita, 2003). Alginates extracted from different sources differ in their M and G ratios, as well as on the length of each block. This polysaccharide is derived from several genera of brown algae (e.g., mixed Fucales and Laminariales) that are utilized as raw materials by commercial alginate producers (Pereira *et al*., 2015).

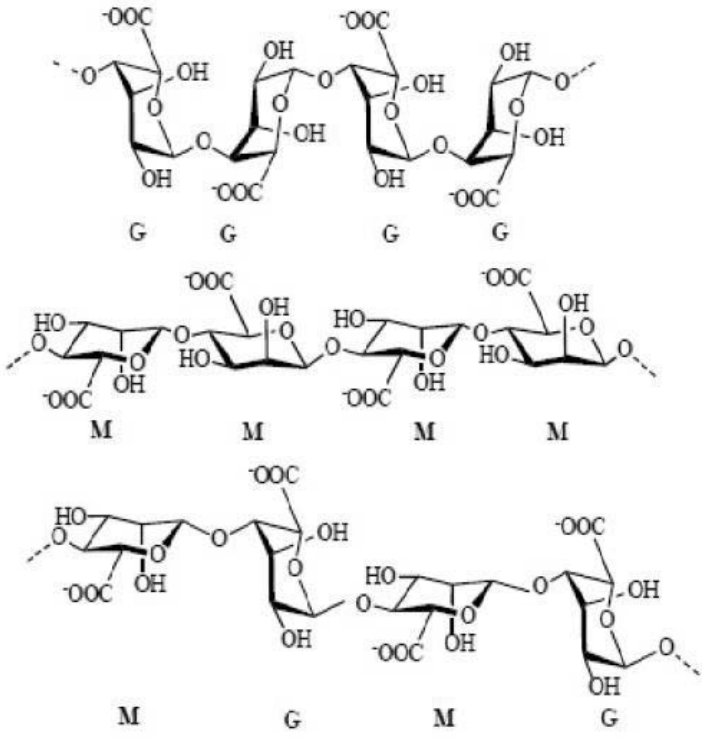

Figure 8 – Idealized structure of the chemical units of poly L-guluronic acid (G blocks), poly-mannuronic acid (M blocks) and alternate L-guluronic and D-mannuronic acid (GM blocks) in alginates (After Cardoso *et al*., 2014).

Alginates have several commercial applications based on their thickening, gelling, emulsifier and stabilizing abilities. They are used in the food industry for improving the textural quality of numerous products such as salad dressing, ice-cream, beer, jelly and lactic drinks, but also in cosmetics, pharmaceuticals, textiles and painting industries (Murata *et al*., 2001; Kim *et al*., 2008).

Carrageenans represent one of the major texturizing ingredients used by the food industry. They are natural ingredients, which have been used for decades in food applications and are generally regarded as safe (GRAS) (Pereira *et al*., 2009; Pereira & van de Velde, 2011). As the seaweed *Chondracanthus teedei* var. *lusitanicus* is a carrageenophyte, this study will only focus on the phycocolloid carrageenan, its properties, types and applications.

1.6 Carrageenan

Carrageenans are sulfated polysaccharides present in the cell walls of members of the Gigartinales (Pereira *et al*., 2007). Polysaccharides are carbohydrates that can be composed of only one kind of repeating monosaccharide, or formed by two or more different monomeric units (heteropolysaccharides; e.g. agar, alginate, carrageenan). The conformation of the polysaccharide chains depends not only on the pH and ionic strength of the medium, but also on the temperature and the concentration of certain molecules. Polysaccharides are divided into two sub-types: anionic and cationic polysaccharides. Carrageenans are naturally occurring anionic sulfated linear polysaccharides (Vipul *et al*., 2014).

The phycocolloid "carrageenin", as first called, was discovered by the British pharmacist Stanford in 1862, who extracted it from Irish moss (*Chondrus crispus*) (Fig. 9). The name was later changed to carrageenan so as to comply with the "-an" suffix for the names of polysaccharides.

Figure 9 – *Chondrus crispus* [8].

The industry of carrageenans dates from the 1940's where it was found to be the ideal stabilizer for the suspension of cocoa in chocolate milk (Pereira & van de Velde, 2011). In the last decades, due to its physical functional properties, such as gelling, thickening, emulsifying and stabilizing abilities, carrageenans have been employed in food industry to improve the texture of cottage cheese, puddings and dairy desserts, and in the manufacture of sausages, patties and low-fat hamburgers (Li *et al*., 2014).

1.6.1 Chemical structure of carrageenans

The molecular chains of carrageenans have two fundamental characteristics: they are composed with a monomer - galactose - and contain a high proportion of sulfate esters (O-SO3), to which the negative charge is provided by the compound. Galactose, in aqueous solution, is capable of fixating a water molecule to the carbon 1 (C1), which leads to an unstable structure that is self-cyclized in a pyranose form to form an oxygen bridge between C_1 and C_5. The cyclization can occur in three different ways: in the form of β-D-galactose (G-units), α-D-galactose (D-units) or 3,6 anidro-α-D-galactose (DA-units), through an intermediate oxygen bridge between C_3 and C_6 of α-D-galactose (Perez *et al.*, 1992). Carrageenans are mainly composed of D-galactose residues linked alternately in 3-linked-ß-D-galactopyranose and 4-linked-α-D-galactopyranose units, and are classified according to the degree of substitution that occurs on their free hydroxyl groups. Substitutions are generally either the addition of ester sulfate or the presence of the 3,6-anhydride on the 4-linked residue (Campo *et al*., 2009). In addition to D-galactose and 3,6-anhydro-D-galactose as the main sugar residues and sulfate as the main substituent, other carbohydrate residues commonly exist in carrageenan, such as xylose, glucose, and uronic acids (Vipul *et al*., 2014).

Thus, the structure of the various types of carrageenans is defined by the number and position of sulfate groups, by the presence of 3,6-anhydro-D-galactose, and conformation of the pyranose ring. According to Chopin *et al*., (1999) there are about fifteen idealized carrageenan structures traditionally identified by Greek letters. Commercial carrageenans are usually classified into κ (kappa), ι (iota) and λ (lambda) carrageenans (Pereira *et al*., 2012). The idealized disaccharide repeating units of these carrageenans are given in Figure 10. Kappa-carrageenans have alternating (1-3)-β-D-galactose-4-sulfate and (1-4)-3,6-anhydro-α-D-galactose units, while the iota-carrageenans have an additional sulfate group on C-2(O) of the (1-4)-3,6-anhydro-α-D-galactose sugars, resulting in two sulfates per disaccharide repeating unit (Jiao *et al*., 2011). Moreover, the lambda-carrageenans have an additional sulfate group linked to the C-6 position of the 4-linked residue, but in turn this is a (1-4)-α-D-galactopyranose. However, generally seaweeds do not produce these idealized and pure carrageenans, but rather a range of hybrid structures: ν (nu), μ (mu), θ (theta), β (beta) and ξ (xi) (see Fig.10). The precursors (mu and nu), when exposed to alkali conditions, are modified into kappa and iota, respectively, through formation of the 3,6-anhydro-galactose bridge (Rudolph, 2000; Pereira & van de Velde, 2011).

The different types of carrageenan are obtained from different species of the Gigartinales (Rhodophyta) (Pereira *et al*., 2012). Kappa-carrageenan is predominantly obtained by extraction from the cultivated, tropical seaweed *Kappaphycus alvarezii*. *Eucheuma denticulatum* is the main species for the production of iota-carrageenan (Pereira *et al*., 2009). Lambda-carrageenan is obtained from different species from the genera *Gigartina* and *Chondrus* (Van de Velde & de Ruiter, 2002). Kappa and iota carrageenan have gelling properties, while lambda-carrageenan acts as thickening agent (Vipul *et al*., 2014). The kappa-type usually forms gels that are hard, strong and brittle, whereas iota-carrageenan forms soft and weak gels. This is the result of the anhydro-galactose bridge of the 4-linked galactose residue, respectively DA and DA2S, which adopts the $^{1}C_{4}$-chair conformation. This conformation is responsible for the formation of the helical structure and, therefore, the ability to form a gel. On the other hand, lambda-carrageenan and the precursors mu- and nu-carrageenan lack the 3,6-anhydro bridge and, therefore, the 4-linked residue adopts the $^{4}C_{1}$-chair conformation, which disturbs the helical conformation. Thus, lambda-carrageenan acts simply as a thickening agent (Pereira *et al*., 2013).

The industrial applications of phycocolloids (alginates, agar and carrageenans) are based on their particular properties to form gels in aqueous solution. However, the need for a more accurate identification of the natural composition of the polysaccharides produced by these seaweeds lead to the use of new spectroscopic techniques. With the combination of two spectroscopic techniques (FTIR-ATR and FT-Raman) it is possible to identify the principal seaweed colloids in ground seaweed samples as in extracted material (Pereira *et al*., 2009). Another technique that has been used since the 80's is the NMR (Nuclear Magnetic Resonance) spectroscopy which detects the nature and position of the substituent in carrageenans and agars (Zinoun, 1993).

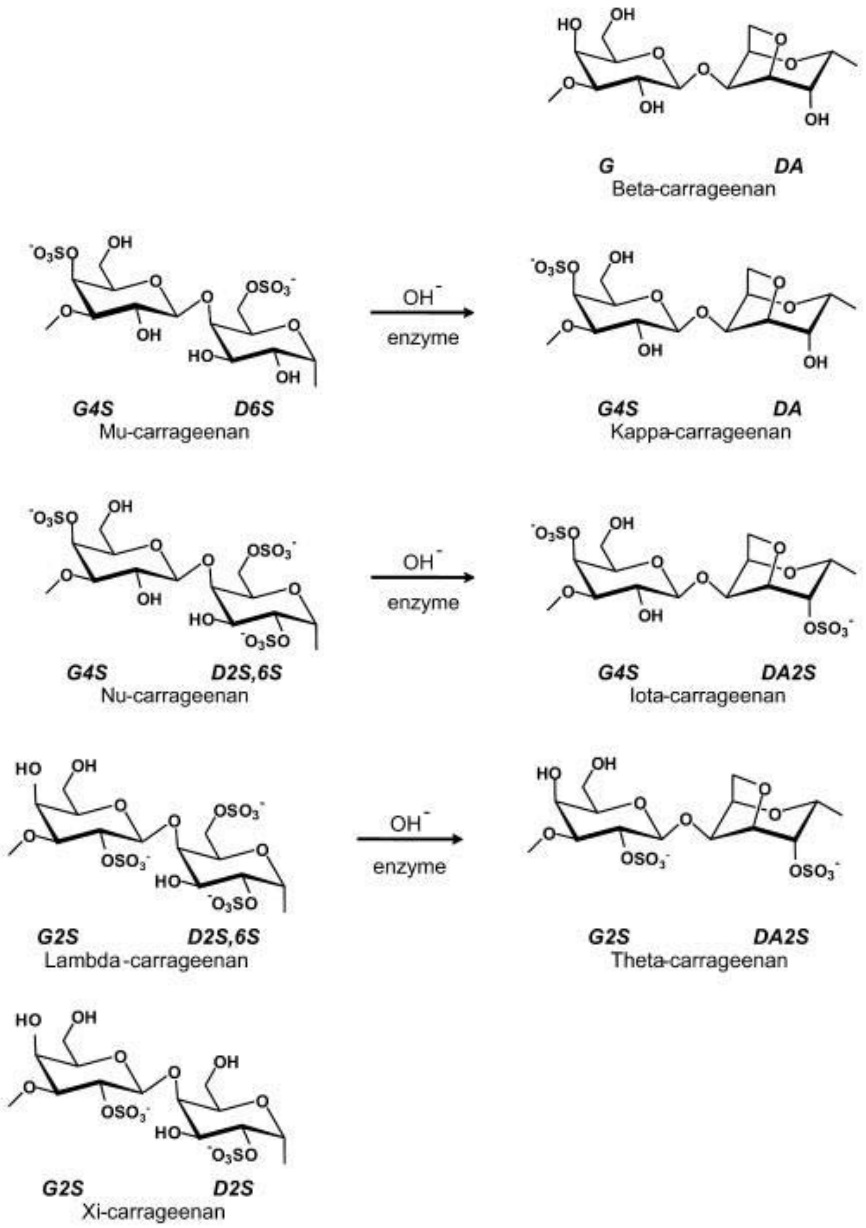

Figure 10 - Idealized units of the main carrageenan types (According to van de Velde *et al.*, 2004)

1.6.2 Spectroscopic analysis (Vibrational Spectroscopy)

1.6.2.1 FTIR-ATR FT-Raman

Infrared (IR) spectroscopy has been applied for many years in the characterization of sulfated polysaccharides from seaweeds and it was, until recently, the most frequently used vibrational technique for the studying natural products (Matsuhiro, 1996; Pereira *et al*, 2003). This technique presents three main advantages: it's fast, non-aggressive, and it requires small sample quantities (milligrams) (Pereira *et al*., 2003). However, conventional IR spectroscopy requires laborious procedures to obtain spectra with a good signal/noise ratio (Chopin & Whalen, 1993). Thus, to overcome this limitation, it was developed an interferometric IR technique (associated with the Fourier transform algorithm), known as FTIR spectroscopy (Fourier transform IR) (Pereira *et al*., 2009). FTIR spectroscopy is useful in distinguishing agar-producing from carrageenan-producing algal material (Matsuhiro, 1996). More recently, Pereira and collaborators had used a technique of analysis on the basis of FTIR-ATR (from attenuated total reflectance) spectroscopy, allowing for the determination of the composition of the different phycocolloids from dried ground seaweed (Pereira, 2006; Pereira & Mesquita, 2004).

In contrast, the application of traditional Raman spectroscopy was limited until recently, due to the need for an incident visible laser in dispersive spectrometers: the visible laser light often excites electronic transitions in biochemical samples, which can lead to either sample degradation or strong background signal from unwanted laser-induced fluorescence (Pereira *et al*., 2009). Moreover, recording the spectra with good signal-to-noise ratio was often time-consuming (Pereira *et al*., 2003). These limitations were greatly overcome through the development of near-IR Fourier transform Raman spectroscopy (FT-Raman), with which fluorescence, risk of sample destruction and spectra recording time are greatly reduced (Matsuhiro, 1996) while the spectral resolution and signal-to-noise ratio are enhanced (Pereira *et al*., 2003). Raman spectra are similar to infrared, but certain low intensity bands in FTIR appear clear in the Raman spectra, a fact which facilitates the identification of the different fractions present in the samples (Givernaud-Mouradi, 1992). According to Pereira *et al*. (2003) FT-Raman spectra present, in general, better resolution than the infrared spectra, allowing identifying a number of characteristic bands useful in the identification of

different types of carrageenan. In some cases, phycocolloids can be identified only with the use of Raman spectroscopy. For example, some variants of the family of lambda carrageenan (xi and theta) can be easily identified by FT-Raman.

Table 1 and Table 2 summarize the absorption peaks of the 8 main carrageenan types by FTIR and FT-Raman spectroscopy, respectively.

Table 1 - Identification of the different types of carrageenan by FTIR-ATR (According to Pereira, 2004a; Pereira *et al.*, 2009; Pereira & Ribeiro-Claro, 2014).

Wave number (cm^{-1})	Bridge (s) /Group (s)	Letter code	Carrageenan type							
			Kappa (κ)	**Mu (μ)**	**Iota (ι)**	**Nu (ν)**	**Beta (β)**	**Teta (θ)**	**Lambda (λ)**	**Xi (ξ)**
1240-1260	S=O of sulphate esters		++	++	++	+++	-	++	+++	++
1070	C-O of the 3,6-anhydrogalactose	DA	+	-	+	-	+	S	-	-
970-975	Galactose	G/D	+	S	+	S	+	+	-	-
930	C-O of the 3,6-anhydrogalactose	DA	+	-	+	-	+	+	-	-
905	C-O-SO_3 on C_2 of the 3,6 anhydrogalactose	DA2S	-	-	+	-	-	+	-	-
890-900	β-D-non sulphated galactose	G/D	-	-	-	-	+	-	-	-
867	C-O-SO_3 on C_6 of the galactose	G/D6S	-	+	-	+	-	-	+	-
845	C-O-SO_3 on C_4 of the galactose	G4S	+	+	+	+	-	-	-	-
825-830	C-O-SO_3 on C_2 o the galactose	G/D2S	-	-	-	+	-	+	+	Sh
815-820	C-O-SO_3 on C_6 of the galactose	G/D6S	-	+	-	+	-	-	+	-
805	C-O-SO_3 on C_2 of the 3,6 anhydrogalactose	DA2S	-	-	+	-	-	+	-	-

(-), non-existent; (+), medium; (++), prominent; (+++) high prominence; (s), shoulder form peak; (sh), sharp peak.

Table 2 - Identification of the different types of carrageenan by FT-Raman (According to Pereira, 2004a; Pereira et al., 2009; Pereira & Ribeiro-Claro, 2014).

Wave number (cm^1)	Bridge (s) /Group (s)	Letter code	Carrageenan type							
			Kappa (κ)	Mu (μ)	Iota (ι)	Nu (ν)	Beta (β)	Teta (θ)	Lambda (λ)	Xi (ξ)
1240-1260	S=O of sulphate esters		++	++	++	+++	-	++	++	++
1075-1805	C-O of the3,6-anhydrogalactose	DA	+++	-	+++	-	+	+	-	-
970-975	Galactose	G/D		+	S	S	+	+	-	-
925-935	C-O of the 3,6-anhydrogalactose	DA	+	-	+	-	+	+	-	-
905-907	C-O-SO_3 on C_2 of the 3,6 anhydrogalactose	DA2S	-	-	+	-	-	+	+	+
890-900	β-D-non sulphated galactose	G/D	-	-	-	-	+	-	-	-
867-871	C-O-SO_3 on C_6 of the galactose	G/D6S	-	S		+	-	-	+	-
845-850	C-O-SO_3 on C_4 of the galactose	G4S	++	+	++	+	-	-	-	+
825-830	C-O-SO_3 on C_2 of the galactose	G/D2S	-	-	-	+	-	+	+	+
815-825	C-O-SO_3 on C_6 of the galactose	G/D6S	-	S	-	S	-	-	+	-
804-808	C-O-SO_3 on C_2 of the 3,6 anhydrogalactose	DA2S	-	-	++	-	-	+	-	-

(-), non-existent; (+), medium; (++), prominent ; (+++) high prominence; (s), shoulder form peak;

1.7 Sources and extraction methods of carrageenans

Most carrageenan is extracted from *Kappaphycus alvarezii* and *Eucheuma denticulatum* (Fig. 11). The original source of carrageenan was *Chondrus crispus*, and this is still used to a limited extent. *Betaphycus gelatinum* is used for a particular type of carrageenan (beta-carrageenan). Some South American species, previously used to a limited extent, are now gaining favor with carrageenan producers since they look for more diversification in the species available and the types of carrageenan that can be extracted (McHugh, 2003).

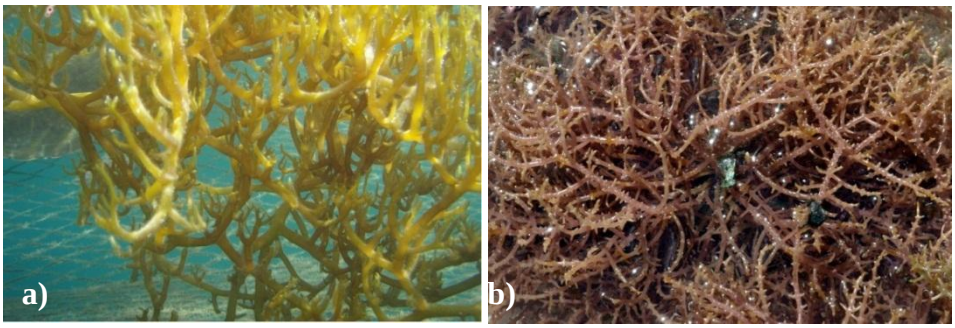

Figure 11 – Example of seaweeds used as a source of carrageenan: a) *Kappaphycus alvarezii*; b) *Eucheuma denticulatum* [9], [10].

1.7.1 Extraction methods

The industrial extraction of carrageenan had its start in 1930 in New-England, from *Chondrus crispus* and *Mastocarpus stellatus* stalks, for the preparation of chocolate milk. The interruption of agar imports during World War II, led to its replacement by carrageenan. This situation was the starting point of a booming industry (Ribier & Godineau, 1984).

1.7.1.1 Industrial extraction

After being harvested, carrageenophytes must be washed, in order to remove sand and salts, and dried to preserve its quality. In tropical regions, carrageenophytes are dried in the sun, but in cold regions carrageenophytes are dried using rotatory air driers. After drying, seaweeds are transported to factories. Factories located close to the harvesting sites use fresh seaweeds allowing a substantial reduction of the costs (Bixler, 1996).

The industrial extraction is performed using the alkali extraction method, described in section 2.7 of "Materials and Methods" chapter.

1.8 Applications of carrageenan

In recent years, much attention has been focused on polysaccharides isolated from natural sources. During the last decade, numerous bioactive polysaccharides with interesting functional properties have been discovered from seaweeds. The biological features of the sulfated polysaccharides reported till now are antioxidant, antitumor, immunomodulatory, inflammation, anticoagulant, antiviral, antibacterial and antilipemic (Patel, 2012).

1.8.1 Antimicrobial activity

1.8.1.1 Fungi

Fungal kingdom is constituted by a wide variety of eukaryotic organisms with a diverse range of forms and functions. Among other reasons, these organisms are grouped in a different kingdom due to the presence of chitin in their cell walls (Rai & Bridge, 2009). Based on their morphologic, physiologic and reproductive structures characteristics, fungi are classified into seven phyla: Microsporidia, Chytridiomycota, Blastocladiomycota, Neocallimastigomycota, Glomeromycota, Basidiomycota and Ascomycota (Hibbett *et al*., 2007). Of these, Ascomycota constitutes the largest, most diverse and ubiquitous phylum, occurring in a wide variety of ecosystems. Species belonging to this phylum possess a specialized sac-like structure (ascus) in which meiotic spores (ascospores) are produced. However, a great number of Ascomycota occur as a single celled yeast that reproduce by budding or binary fission (Schoch *et al*., 2009).

Among the wide variety of species belonging to this phylum, some filamentous fungi and yeasts have a special importance from the health care point of view, since these can cause opportunistic infections and skin and mucosa diseases, respectively (Richardson & Warnock, 2012).

The frequency of invasive mycoses due to opportunistic fungal pathogens has increased significantly over the past two decades (Rees *et al*., 1998; Trick *et al*., 2002; Ostrosky-Zeichner *et al*., 2003; Hajjeh *et al*., 2004; Pfaller & Diekema, 2004; Walsh *et al*., 2004). Serious life-threatening infections are being reported with an ever increasing array of pathogens, including the well-known opportunists *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* (Mirza *et al*., 2003; Hajjeh *et al*., 2004; Pfaller & Diekema, 2004).

Although *A. fumigatus* heads the list of these opportunistic molds (Denning *et al*., 1998; Diekema *et al*., 2003; Lin *et al*., 2005), infections due to less common but antifungal-resistant species such as *A. terreus* and dematiaceous filamentous fungi (e.g., *Bipolaris*, *Cladophialophora*, and *Alternaria*) are being reported with greater frequency (Iwen *et al*., 1998; Flemming *et al*., 2002; Baddley *et al*., 2003; Walsh *et al*., 2004).

1.8.1.1.a Filamentous fungi

1.8.1.1.a1 *Aspergillus fumigatus*

Asp. fumigatus is one of the most ubiquitous of the airborne saprophytic fungi (Fig. 12) (Vanden Bossche *et al*., 1988; Pitt, 1994; Haines, 1995).

Until recently, *Asp. fumigatus* was viewed as a weak pathogen responsible for allergic forms of the disease, such as farmer's lung, a clinical condition observed among individuals exposed repeatedly to conidia, or aspergilloma, an overgrowth of the fungus on the surface of preexisting cavities in the lungs of patients treated successfully for tuberculosis (Dixon *et al*., 1992; Kwon-Chung *et al*., 1992; Pennington, 1988). Over the past 10 years, *Asp. fumigatus* has become the most prevalent airborne fungal pathogen, causing severe and usually fatal invasive infections in immunocompromised hosts in developed countries (Bodey & Vartivarian, 1989; Andriole, 1993; Beck-Sagué & Jarvis, 1993; Dixon *et al*., 1996; Groll *et al*., 1996; Denning, 1998).

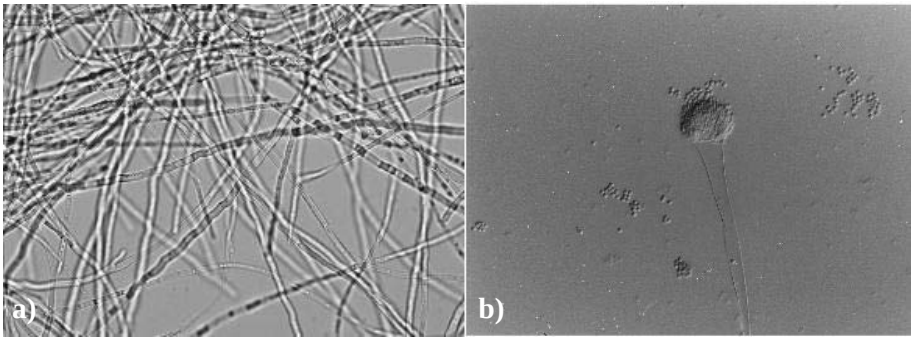

Figure 12 - *Aspergillus fumigatus*: a) hyphae; b) sporulating structures (After Vewer *et al*., 2012; Latgé, 1999).

1.8.1.1.a2 *Alternaria infectoria*

Alternaria species are increasingly found as etiologic agents of human disease, due to the growing number of immunocompromised patients (Fig. 13) (de Hoog *et al*., 2000). *A. infectoria* is a rare opportunistic agent of phaeohyphomycosis (Dubois *et al*., 2005; Gilaberte *et al*., 2005), a human infection that usually affects the sub-cutaneous tissue, in particular, the nervous system (Li & de Hoog, 2009).

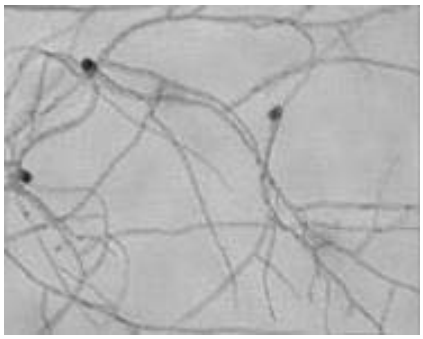

Figure 13 - *Alternaria infectoria* (After Fernandes *et al*., 2014).

1.8.1.1.b Yeasts

Yeasts are members of the phylum Ascomycota and can be found in plants, animals, soil, water and atmosphere, and also in extreme environments (e.g. osmophilic and halotolerant yeasts). As non-motile organisms, yeasts rely on aerosols, animal vectors and human activity for their natural scatter (Walker, 1998).

Yeasts from the genus *Candida*, particularly *Candida albicans*, are the most important agents causing yeast infection (Fig. 14). This opportunistic pathogen is normally associated with mucosal, cutaneous and nail infections (normally known as candidiasis), but can cause acute or chronic invasive infections in immunocompromised or debilitated individuals. This yeast is part of human flora and, in most cases, infections are derived from the individuals own reservoir in mouth, gastrointestinal tract, lower genital tract or skin. When there are serious impairments of host defenses, life-threatening invasive infections can occur (Richardson & Warnock, 2012).

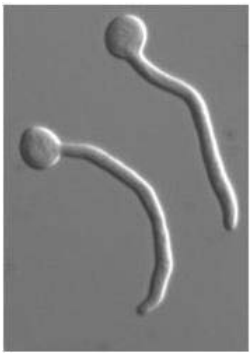

Figure 14 - *Candida albicans* hyphae [11].

1.8.1.2 Mechanism of antifungals' action

The study of antifungals' mechanism of action constitutes an important strategy for limiting the emergence of resistance to the commercially available agents, as well as to develop safer and more potent compounds in the future (Ghannoum & Rice, 1999; Thomas & Kim, 2011).

The cell membrane and cell wall of fungi are the most important targets for antifungal drugs. These physical and chemical barriers are responsible for the communication with the environment and, therefore, have a key role in metabolic processes (Hector, 1993; Richardson & Warnock, 2012).

1.8.1.2.a Cell membrane components

Ergosterol is the predominant sterol in fungal cell membranes, responsible for maintaining cell integrity, viability, function and normal growth (Ghannoum & Rice, 1999). The three major groups of antifungal agents in clinical use include azoles, polyenes and allylamines, which owe their antifungal activity to the interaction with ergosterol or to the inhibition of its synthesis (Ghannoum & Rice, 1999).

1.8.1.2.b Cell wall components

As it happens with the membrane, fungal cell wall is a target for antifungals action. Over the past decades a number of compounds able to affect fungal cell wall has been discovered, being active over the synthesis of chitin and β-glucans, which are essential cell wall components, responsible for fungal structure and normal cell growth. Among them, only echinocandins are commercially available. These compounds are able to inhibit β-glucans synthesis, which are unique compounds among the fungal kingdom (Hector, 1993).

1.8.1.3 Bacteria

Bacteria are considered the first inhabitants of earth and their number overcomes any other biological life form. These prokaryotic single celled microorganisms present a wide range of shapes and can be found alone, or forming groups or arranged as filaments (Srivastava, 2003). Bacteria live almost everywhere, from soil, water, waste, animals and plants to the deeper areas of earth (Whitman *et al*., 1998; Srivastava, 2003).

Bacteria can be divided into two main types, the Gram positive (Gram+) and the Gram negative (Gram─), according to their reaction to the Gram stain test. While Gram─ have a peptidoglycan cell wall surrounded by an external lipid membrane containing also LPS and lipoproteins, Gram+ only have a peptidoglycan cell wall (Srivastava, 2003; Paterson, 2006).

A high number of bacteria are harmless to humans and inhabit the human body without causing adverse effects. The great majority is found in skin and gut and most of them are beneficial. Nevertheless, there are also a large number of infectious bacteria capable of causing morbidity and mortality, especially in countries with ineffective health care conditions and in immunocompromised patients. Others, which seem less harmful, are largely responsible for serious infections including nosocomial infections. Among these are, for example, some serotypes of *Escherichia coli* (Spicer, 2008; Paterson, 2009) and *Staphylococcus aureus* (Archer, 1998).

1.8.1.3.a *Escherichia coli*

E. coli, a Gram─ rod, is a frequent cause of life-threatening bloodstream infections and other common infections, such as urinary tract infections (Kenedy *et al*., 2008; Spicer, 2008; Paterson, 2009). Extraintestinal invasive infections due to *E. coli* are the cause of considerable morbidity, mortality, and increased health care costs. Management is complicated by the increasing prevalence and spectrum of antimicrobial resistance (Russo & Johnson, 2003; Pitout & Laupland, 2008).

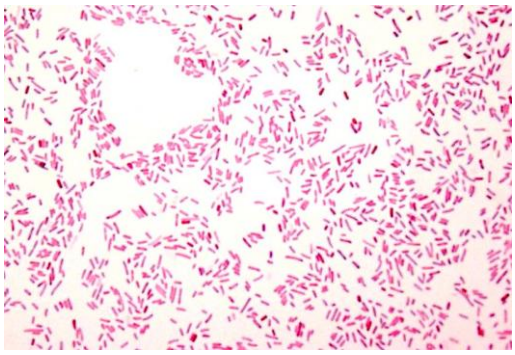

Figure 15 - Gram stain of *E. coli* [12].

1.8.1.3.b *Staphylococcus aureus*

S. aureus, a Gram+ coccus, is a virulent pathogen that is currently the most common cause of infections in hospitalized patients. *S. aureus* infection can involve any organ system. The success of *S. aureus* as a pathogen and its ability to cause such a wide range of infections are the result of its extensive virulence factors. The increase in the antimicrobial resistant profile of this virulent pathogen, coupled with its increasing prevalence as a nosocomial pathogen, is of major concern (Archer, 1998).

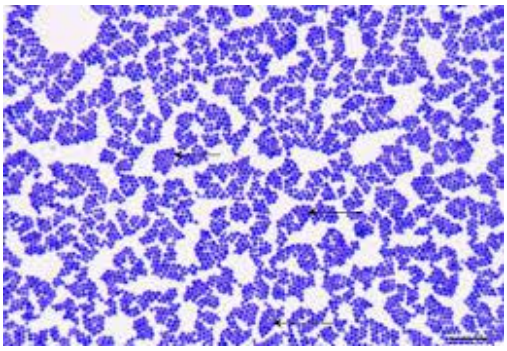

Figure 16- Gram stain of *S. aureus* [13].

1.8.1.4 Antifungal and antibacterial activity

There is an increasing interest in animal-pathogenic fungi, particularly in those species which are opportunistic pathogens in immunocompromised patients systems have been compromised, either as a result of disease (e.g. sufferers of Acquired Immune Deficiency Syndrome) or as a consequence of immunosuppressive drug therapy (Tariq, 1991). The search continues for naturally occurring compounds with antifungal

potential since the tools currently used are inefficient in multiple situations. Marine algae have received a lot of attention as potential sources of compounds possessing a wide range of biological activities, including antimicrobial properties. The antimicrobial activities of numerous algae species have been tested and reported, presenting an extended spectrum of action against bacteria and fungi **(**Guedes *et al*., 2012).

Carrageenans have proved to have effects against some bacterial strains such as *Salmonella enteritidis, S. typhimurium, Vibrio mimicus, Aeromonas hydrophila, E. coli, Listeria monocytogenes* and *S. aureus*. The growth of all the bacterial strains except *L. monocytogenes* was significantly inhibited by them, particularly by the iota-carrageenan. A growth inhibition experiment using *S. enteritidis* showed that the inhibitory effect of the carrageenans was not bactericidal but bacteriostatic. Removal of the sulfate residues eliminated the bacteriostatic effect of iota-carrageenan, suggesting that the sulfate residues in carrageenan play an essential role in this effect (Venugopal, 2008). In 2014, Sebaaly *et al*. reported that carrageenans isolated from the red alga *Corallina sp*. exhibited antibacterial activity against *Staphylococcus epidermis*. Infrared spectroscopy (IR) showed that the isolated carrageenan was of lambda-type.

Shanmughapriya *et al*. (2008) showed that extracts from *Gracilaria corticata* were found to be effective against *Pseudomonas aeruginosa* and *E. coli*. It was also effective against *Micrococcus luteus, S. epidermidis* and *Enterococcus faecalis*.

In 2007, Salvador *et al*. evaluated the antifungal and antibacterial activity of 82 Iberian macroalgae (18 Chlorophyta, 25 Phaeophyceae and 39 Rhodophyta) against three Gram-positive bacteria (*Bacillus subtilis*; *B. cereus*; *S. aureus*), two Gram-negative bacteria (*E. coli* and *P. aeruginosa*) and one yeast (*C. albicans*). The bioactivity was analyzed from crude extracts of fresh and lyophilized samples. Of the seaweeds analyzed, 67% were active against at least one of the six test microorganisms. The highest percentage of active taxa was found in Phaeophyceae (84%), followed by Rhodophyta (67%) and Chlorophyta (44%). Nevertheless, red algae had both the highest values and the broadest spectrum of bioactivity. In particular, *Bonnemaisonia asparagoides, B. hamifera, Asparagopsis armata* (and *Falkenbergia rufolanosa* phase) (Bonnemaisoniales) were the most active taxa. In this study, Ceramiales and Gigartinales had noteworthy antimicrobial activity, and Bonnemaisoniales was the order that had the highest bioactivity.

In another study, extracts from 44 species of seaweed from Canary Islands (Spain) were screened for the production of antifungal and antibacterial compounds

against a panel of Gram-negative and Gram-positive bacteria, mycobacterium, yeasts and fungi. A total of 28 species displayed antibacterial activity, of which six also showed antifungal activity. Regarding antifungal activity, six of the species tested - *Asparagopsis taxiformis* (Rhodophyta), *Cymopolia barbata* (Chlorophyta), *Caulerpa prolifera* (Chlorophyta), *Dictyota* sp. (Phaeophyta), *Enteromorpha muscoides* (Chlorophyta) and *Osmundea hybrid* (Rhodophyta), presented activity against the filamentous fungi *Asp. fumigatus*, and/or the yeasts *C. albicans* and *Saccharomyces cerevisiae*. *Asparagopsis taxiformis* and *Cymopolia barbata* were the species with the strongest activities against the broadest spectrum of target microorganisms. All the species with antibacterial activity were active against Gram-positive bacteria (*S. aureus* and *B. subtilis*), whereas only two species, *A. taxiformis* and *O. hybrida*, were active against mycobacterium. Only one species – *A. taxiformis* - showed activity against the whole panel of nine target microorganisms: *P. aeruginosa*, *Serratia marcescens*, *Enterococcus faecium*, *Mycobacterium smegmatis*, *S. aureus*, *B. subtilis*, *C. albicans*, *S. cerevisiae* and *Asp. fumigatus* (del Val *et al*., 2001). Genovese *et al*. (2012) also showed that *A. taxiformis* has antifungal activity against *Aspergillus fumigatus*, *A. terreus* and *A. flavus*.

In 2011, Stein *et al*. reported that five species of the red alga *Laurencia* (Rhodophyta) showed fungistatic (the lowest concentration of the agent that results in the maintenance or reduction of the *inoculum*) and/or fungicidal activity (the lowest concentration of the agent that results in no growth) against three strains of pathogenic fungi – *C. albicans*, *Candida parapsilosis* and *Cryptococcus neoformans*. Chloroform and methanol extracts of *L. dendroidea* showed fungistatic effects against *C. albicans*. Crude water extracts of the species showed a reasonable percentage of inhibition against *C. neoformans*; the chloroform extract of *Laurencia catarinensis* proved to have a fungistatic effect against *C. parapsilosis*, and fungicidal effect against *C. albicans* and *C. neoformans*; of all the extracts of *Laurencia intricata*, the chloroform extract was the most active; a fungistatic effect was observed for the methanol extract of *Laurencia aldingensis* against the three pathogenic fungi. The hexane and chloroform extracts of this species had fungicidal effects against *C. parapsilosis*. The same extracts were fungistatic against *C. albicans* and *C. neoformans*. Thus, *L. aldingensis* appears to be a particularly interesting alga, showing activity against all the three strains tested.

Tariq (1991) reported that extracts of *Dilsea carnosa*, *Laurencia pinnatifida*, *Odonthalia dentata* and *Vertebrata lanosa* (formerly *Polysiphonia lanosa*) reduced the

rate of colony extension in *Microsporum canis* and *Trichophyton verrucosum*, with seasonal variations in the levels of inhibitory activity.

1.8.2 Antiviral activity

1.8.2.1 Virus

Viruses can be considered obligate intracellular parasites, constituted by either a RNA or DNA genome surrounded by a protective protein coat (Baron, 1996).

Viruses are grouped on the basis of size and shape, chemical composition and structure of the genome, and mode of replication. Helical morphology is seen in nucleocapsids of many filamentous and pleomorphic viruses. Icosahedral morphology is characteristic of the nucleocapsids of many "spherical" viruses. Many viruses also have an outer envelope (Gelderblom, 1996).

The genome of a virus may consist of DNA or RNA, which may be single stranded (ss) or double stranded (ds), linear or circular. The entire genome may occupy either one nucleic acid molecule (monopartite genome) or several nucleic acid segments (multipartite genome). The different types of genome necessitate different replication strategies (Gelderblom, 1996).

The viruses that infect humans are currently grouped into 21 families, reflecting only a small part of the spectrum of the multitude of different viruses whose host ranges extend from vertebrates to protozoa and from plants and fungi to bacteria (Gelderblom, 1996). Of these, enteroviruses and lentiviruses can cause a large number of human diseases.

1.8.2.1.a Enteroviruses

Enteroviruses are a genus of the family *Picornaviridae*, a group of single-stranded RNA viruses, which includes *polioviruses*, *echoviruses*, group A and B *coxsackieviruses*, and numbered enteroviruses (Rueckert, 1996), have been implicated in a large variety of human diseases, ranging from mild illnesses to severe clinical

diseases such as myocarditis, meningitis, encephalitis, and paralysis (Fig. 17) (Melnick, 1996; Pallansch & Roos, 2001).

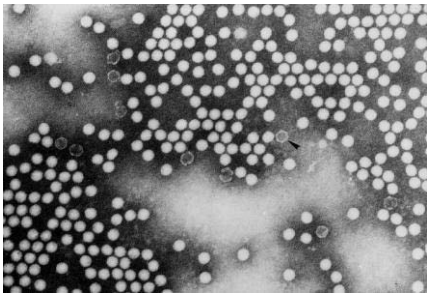

Figure 17 – *Picornavirus* [14].

1.8.2.1.b Lentiviruses

Lentiviruses are a subfamily of retroviruses, single-stranded RNA virus with a viral reverse transcriptase, that are characterized by long incubation periods between infection of the host and the manifestation of clinical disease (Fig. 18). Human immunodeficiency virus type 1, the causative agent of AIDS, is the most widely studied lentivirus. However, the lentiviruses that infect sheep, goats, and horses were identified and studied prior to the emergence of human immunodeficiency virus type 1 (Clements & Zink, 1996).

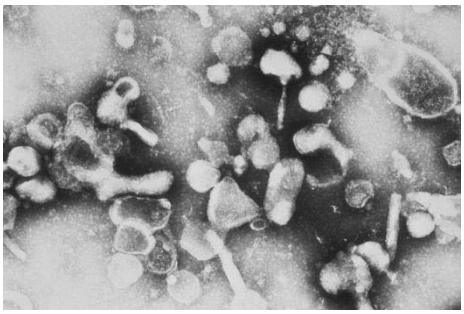

Figure 18 - Feline Leukemia Virus (Retroviridae) [15].

1.8.2.2 Main targets for antiviral drugs

Specific events in virus replication identified as targets for antiviral agents are viral adsorption, penetration, uncoating, and viral nucleic acid synthesis as well as viral protein synthesis. Specificity for infected cells may occur when virus-specified enzymes (e.g., thymidine kinase-induced by herpes simplex virus or varicella-zoster virus) activate drugs (e.g., acyclovir) (Gelderblom, 1996).

1.8.2.3 Antiviral activity of marine algal polysaccharides

In recent years, the constant outbreak of some emerging or remerging viral diseases has caused serious harm to human health. During the last decade, the number of antiviral products approved for clinical use has been increased from 5 to more than 30 drugs.

The potential antiviral activity of marine algal polysaccharides was first shown by Gerber *et al* (1958), describing that the polysaccharides extracted from *Gelidium robustum* (formerly *Gelidium cartilagenium*) (Rhodophyta) protected the embryonic eggs against *Influenza B* or *Mumps virus*. Many species of marine algae contain significant quantities of complex structural sulfated polysaccharides that have been shown to inhibit the replication of enveloped viruses including members of the *Flavivirus*, *Togavirus*, *Arenavirus*, *Rhabdovirus*, *Orthopoxvirus*, and *Herpesvirus* Families (Witvrouw & De Clercq, 1997). Polysaccharides extracted from Rhodophyta have been shown to exhibit antiviral activity against a wide spectrum of viruses including important human pathogenic agents such as *Human Immunodeficiency virus* (HIV), *Herpes simplex virus* (HSV), *Vesicular stomatitis virus* (VSV) and *Cytomegalovirus* (CMV) (Witvrouw & De Clercq, 1997). The chemical structure including the degree of sulfation, molecular weight, constituent sugars, conformation and dynamic stereochemistry are caused to determine the antiviral activity of algal sulfated polysaccharides (Luscher-Mattil, 2000; Damonte *et al.*, 2004; Adhikari *et al*., 2006). In addition, both the degree of sulfation and the distribution of sulfate groups on the constituent polysaccharides play an important role in the antiviral activity of these sulfated polysaccharides. Algal polysaccharides with low degrees of sulfation are generally inactive against viruses (Damonte *et al*., 2004).

Marine polysaccharides can either inhibit the replication of virus through interfering with the viral life cycle or improve the host antiviral immune responses to accelerate the process of viral clearance. The life cycle of viruses differs greatly between species but there are six basic stages in the life cycle of viruses: viral adsorption, viral penetration, uncoating of capsids, biosynthesis, viral assembly and viral release. Marine polysaccharides can inhibit viral life cycle at different stages or directly inactivate virions before virus infection. Specific antiviral mechanism of marine polysaccharides is commonly related to specific structure features of the polysaccharides and specific viral serotypes (Damonte *et al*., 2004).

Carrageenan might inhibit virus infection via direct actions on the virus surface by its negative charge (Wang *et al*., 2008). Several studies showed that carrageenan has a direct virucidal action on some enveloped viruses, which makes the viruses lose the ability to infect cells, thus effectively reducing the virus multiplication. Carlucci *et al*. (2002) found that lambda-type carrageenan could firmly bind to the *Herpes simplex virus* (HSV), leading to the inactivation of the HSV virion, thus inhibiting the replication of HSV. Their studies further suggest that carrageenan changes the structure of the glycoproteins gB and gC of HSV (Carlucci *et al*., 1999; Carlucci *et al*., 2002). Moreover, Harden *et al*. (2009) reported that carrageenan polysaccharides derived from red algae could directly inactivate HSV-2 at low concentrations. The virucidal activities increase with increased molecular weight of carrageenan polysaccharide up to 100 kDa, after which the virucidal activities level off. The direct virucidal actions of carrageenan may be due to the formation of a stable virion–carrageenan complex where binding is not reversible and hence the sites on the viral envelope required for virus attachment to host cells are occupied by the sulfated polysaccharide, which renders the virus unable to complete the subsequent infection process (Damonte *et al*., 2004).

Several studies have shown that carrageenan can mask the positive charge of host cell surfaces by the negative charge of its sulfate groups, so as to interfere with the adsorption process of viruses. Mazumder *et al*. (2002) obtained a high molecular weight sulfated galactan from red algae, and showed its antiviral activities against *Herpes simplex virus* 1 and 2 in bioassays, which is likely due to an inhibition of the initial viral attachment to the host cells. Carlucci *et al*. (1997; 1999) noted that lambda-carrageenan and partially cyclized mu/iota-carrageenan from *Gigartina skottsbergii* have potent antiviral effects against different strains of HSV types 1 and 2 during the virus adsorption stage. They subsequently confirmed the firm binding of carrageenan to

virus receptors on the host cell surface. Their studies demonstrate that lambda-carrageenan interferes with the adsorption process of the virus to the host cell surfaces.

Buck *et al*. (2006) found that carrageenan could directly bind to the HPV capsid, so as to inhibit not only the viral adsorption process but also the subsequent entry and uncoating process of the virus. They also found that the inhibition actions of carrageenan against HPV might be related to a mechanism that is independent of the heparan sulfate after viral adsorption (Buck *et al*., 2006). Moreover, Talarico and co-workers reported that lambda and iota-carrageenans could interfere with both DENV-2 adsorption and internalization into host cells and they are only effective if added together with the virus or shortly after infection (Talarico *et al*., 2007). The mechanism of this inhibition action may be due to that although DENV virus can enter into host cell in the presence of carrageenans, their subsequent uncoating and releasing from endosomes may be interfered by the carrageenans. The inhibitory action of iota-carrageenan on the uncoating process of dengue virus may be attributed to the direct interaction of carrageenans with the virus membrane glycoprotein E (gE) (Talarico *et al*., 2005; Talarico *et al*., 2007; Talarico *et al*., 2007a). Talarico and co-workers (Talarico *et al*., 2007; Talarico *et al*., 2011), also reported that iota-carrageenan could inhibit dengue virus (DENV) replication in mammalian and mosquito cells, and the mode of action of iota-carrageenan in both cell types is strikingly different.

In conclusion, the antiviral activities of carrageenans are very broad, which can suppress the replication of both enveloped and non-enveloped viruses. The antiviral effects of carrageenans are closely related to the molecular weights and the degree of sulfation of them. Moreover, the inhibitory actions of carrageenans on different viruses are usually different, which are associated with the types of carrageenans, the virus serotypes and the host cell itself (Damonte *et al*., 2004; Talarico *et al*., 2005).

1.9 Objectives

The biological activity of carrageenan as a natural occurring polysaccharide has been increasing widely for human applications and creates a strong position in the biomedical field. Due to their different chemical structure and physical properties this natural source can be used in the different applications.

The aim of this work is to study the cultivation methods (laboratory and IMTA culture) of the carrageenophyte alga *Chondracanthus teedei* var. *lusitanicus* (Rhodophyta, Gigartinales) and its antifungal, antibacterial and antiviral activity.

Chapter II - Materials and Methods

2.1 Harvesting sites

2.1.1 Carreço (Viana do Castelo)

Carreço beach is located at 41° 46’ North and 8° 52’ West. It has a dune area that surrounds the beach and has a diverse flora and fauna. A rocky area can also be seen in between tides (Fig. 19a) (Pereira 2015, MACOI, available online at: http://macoi.ci.uc.pt/local_detail.php?loc_id=34&searchSite=carre%E7o%2Fmontedor%2C+portugal).

2.1.2 Buarcos Bay (Figueira da Foz)

Buarcos Bay is located at 40 º 16' North and 8 ° 90' West, northern of Mondego Estuary and southern of Mondego Cape. It is dominated by rocky substrate, with some intertidal pools, little inclination and exposure to waves (Fig. 19b) (Pereira, MACOI, available online at http://macoi.ci.uc.pt/local_detail.php?loc_id=1).

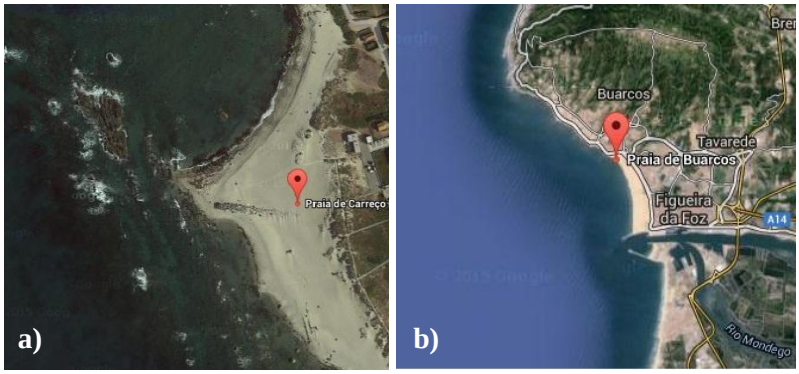

Figure 19 - Harvesting sites: a) Carreço beach, Viana do Castelo; b) Buarcos Bay, Figueira da Foz [16].

2.2 Harvesting and preparation of the algal material for cultivation

Specimens of *Chodracanthus teedei* var. *lusitanicus* were collected by hand in Carreço, Viana do Castelo, in July 2014 and in Buarcos Bay, in February 2015. In both times, samples were previously washed *in situ*, in order to remove sand and salts, put inside a plastic bag and stored in a cooler box. At the laboratory, all samples were rinsed in autoclaved distilled water and cleaned with a cotton swab in order to eliminate

salts, epiphytes and debris from the thallus surface. Following that, samples were separated into 3 groups, according to the different lifecycle phases - tetrasporophytes, fructified female gametophytes and non-fructified thalli (Fig. 20).

Figure 20 – *Chondracanthus teedei* var. *lusitanicus* separated according to its lifecycle phases.

2.2.1 Identification of *Chondracanthus teedei* var. *lusitanicus* lifecycle phases

Tetrasporophytes exhibit tetrasporangial sori with the appearance of dark red spots, which are prominent in the thallus, main axis and lateral branches (Fig 21a). Fructified female gametophytes present prominent spherical cystocarps, producing carpospores (Fig. 21b). Non-fructified thalli do not bear any cystocarps (Fig. 21c) (Pereira, 2012). The identification of the different lifecycle phases was carried out using a magnifying glass. Only thalli with more than 5cm were selected, since these were already well developed and, therefore, the chances of mistaken the different phases were reduced.

Figure 21 – Life phases of *Chondracanthus teedei* var. *lusitanicus*: a) Tetrasporophyte; b) fructified female gametophyte; c) Non-fructified thalli (Adapted from Pereira, 2012).

2.3 Cultivation of *Chondracanthus teedei* var. *lusitanicus*

The cultivation of this alga was performed in laboratory and in an IMTA system, both carried out at the facilities of the company ALGAplus (http://www.algaplus.pt/), in Ílhavo, from May to August 2014. Only the specimens collected in Viana do Castelo were used. For both laboratory and IMTA culture, the algal material was prepared as described previously.

For this study, only specimens belonging to tetrasporophytes and non-fructified thalli were used, in order to avoid germination of the spores and, therefore, allow its vegetative propagation.

2.3.1 Laboratory culture

2.3.1.1 Cultivation system

The aim of this experiment was to determine the best cultivation density for this alga.

To perform this study, sixteen 1L flasks were used to grow the seaweed. An air system was mounted in order to promote the movement of the seaweed inside the flasks. Each flask was put inside a cultivation chamber under controlled conditions of temperature (15 °C), irradiance (white fluorescence light) (100 μE m^{-2} s^{-1}), and photoperiod (16:8h) (light:dark) (Fig. 22).

Figure 22 – Laboratory culture of *Chondracanthus teedei* var. *lusitanicus*.

2.3.1.2 Experimental design

Four different cultivation densities were tested: 1, 2, 4 and 6 g L^{-1} (fresh weight). For each cultivation density, four replicates were used (3 maintaining initial cultivation density + 1 not maintaining initial cultivation density). The algal material was distributed in 1L flasks with salt water (filtered and autoclaved) enriched with 6 mL L^{-1} VSE medium (see appendix 1). Germanium dioxide was added at 2 mL L^{-1} every time the medium was renewed, in order to eliminate potential contaminations by diatoms.

This experiment consisted of 4 weeks (1 acclimation + 3 collecting data). Every 3-4 days the culture medium was renewed and salinity and pH levels were monitored using a multiparametric sensor. Once a week, the biomass was totally removed from the flaks and weighted on a scale. Flasks were changed for cleaned ones and restocked with the correspondent initial cultivation density. The excess biomass was dried. All measurements and medium exchanges were performed inside a flow chamber in order to avoid contaminations.

2.3.2 IMTA culture

2.3.2.1Cultivation system

This experiment took place in the outdoors facilities of the company ALGAplus and its purpose was to determine the best cultivation densities for this alga in an IMTA system.

In order to perform this experiment, nine 15 L (foot print 0, 0881 m^2) white tanks were used to grow the seaweed and were set to receive independent flows of water (water from fish effluent). The water flow was adjusted manually for each tank and the seaweeds were kept in constant movement by air diffusers placed in the bottom of the tanks (Fig. 23).

Figure 23 – IMTA system.

2.3.2.2 Experimental design

Three cultivation densities – 4, 6 and 8 g L^{-1} (fresh weight) were tested under a water flow of approximately 20 L h^{-1}. For each cultivation density, three replicates were used.

This experiment consisted of four weeks (1 acclimation + 3 collecting data). Once a week, the algal material was removed from the tanks and weighted. Tanks were cleaned and restocked with the correspondent initial cultivation density. Every day, salinity, pH levels, temperature and sun light irradiance were measured in different times of the day: in the morning and in the afternoon. Salinity, pH and temperature were measured with a multiparametric sensor and sun light irradiance was measured using a spherical light sensor.

2.4 Calculations of growth parameters

2.4.1 Dry Weight (DW)

The percentage of dry weight was determined by weighing one gram samples (n=4) and allowing it to dry for three days at 45 °C. Following drying, samples were removed and weighted. The dry weight was calculated by the formula: % Dry weight = Dry weight / Fresh weight (g).

2.4.2 Relative Growth Rate (RGR) and Productivity

The RGR of the seaweed at both laboratory and IMTA system was determined by the formula: RGR (% day^{-1}) = Ln (FW) − Ln (IW) / T * 100, where FW = Final fresh weight, IW = Initial fresh weight and T = Days in culture.

The productivity was calculated by the equation: (g (dw) m^{-2} wk^{-1}) = [(FW − IW) * % DW] / foot print], where wk = week and DW = dry weight.

2.5 Statistical analysis

A one-way ANOVA test was performed in order to observe if the cultivation densities influenced the relative growth rate and productivity of both laboratory culture and IMTA system. Tests were performed using GraphPad Prism 6 and confirmed in Statistica 8.

Since both IMTA and laboratory material was too deteriorated it could not be used for further studies. All the following studies were performed using *Chondracanthus teedei* var. *lusitanicus* collected in Buarcos Bay (Figueira da Foz).

2.6 Preparation of algal material for FTIR-ATR analysis

For FTIR-ATR *Chondracanthus teedei* var. *lusitanicus* was washed with autoclaved distilled water and cleaned with a cotton swab to remove epiphytes, sand and salts. The seaweed was then separated into three groups, according to its life cycle phases (tetrasporophytes, fructified female gametophyte and non-fructified thalli). The three groups were dried separately to constant weight for 48h at 60 °C. The dried seaweeds were finely grounded using a coffee grinder, in order to render the samples uniformly (Pereira 2004, Pereira 2006).

For FTIR-ATR, samples did not require any additional treatment (Pereira *et al*., 2003; Pereira & Mesquita, 2004).

2.7 Carrageenan extraction – Alkali extraction

For the extraction process, only samples belonging to the fructified female gametophyte and the tetrasporophytes were used, since there were not enough samples of non-fructified thalli to perform this assay.

For the alkali extraction the ground dry material was weighted in a Kern scale and one gram samples of each lifecycle phase were used (n=3). Before its extraction, the ground dry material (1 g) was rehydrated and pre-treated with a mixture of acetone and methanol (75 mL acetone and 75 mL methanol), at room temperature, for 12 h, to eliminate the organo-soluble fraction (Zinoun & Cosson, 1996) (Fig.24a). The samples were then placed in a solution of 150 mL g^{-1} of NaOH (1 M) in a hot bath, at 85-90 ºC, for 3 hours (Pereira & Mesquita 2004) (Fig. 24b). After that, the solutions were hot filtered, twice, under vacuum, through a cloth filter: first, the solutions were filtered using a Buchner funnel (Fig. 24c) coupled to a Buchner flask, and then it was filtered, again, using a G3 fritted funnel (Fig. 24d). The solutions were poured to a beaker and carrageenans precipitated by adding to the warm solution twice its volume of ethanol (96%). This resulted in the formation of a whitish clog (Fig. 24e). With a glass rod, carrageenans were pulled out and squeezed in order to drain the soaked liquid. Following that, carrageenans were put inside a clean flask and 100 mL of absolute alcohol was added during 12-24 hours in order to remove the excess water (Fig. 24f). Finally, the alcohol was removed and carrageenans were dried at 50-60 ºC, for 24 hours (Pereira and Mesquita 2004). This procedure was done under aseptic conditions, in order to avoid contaminations.

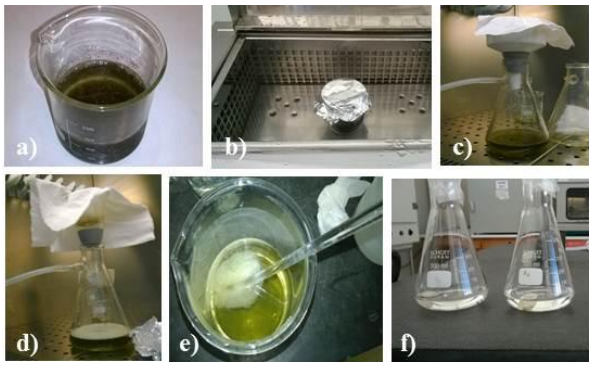

Figure 24 – Alkali extraction process: a) seaweed sample in acetone:methanol solution; b) seaweed sample in a hot bath; c) and d) filtration; e) carrageenan precipitation; f) immersion of the carrageenans in alcohol.

2.8 Determination of dry weight and carrageenan content

The percentage of dry weight was determined by weighing one gram samples (n=3) and allowing it to dry for three days at 45 °C. Following drying, samples were removed and weighted. The dry weight was calculated by the formula: % Dry weight = Dry weight / Fresh weight (g).

The determination of the carrageenan content was calculated using the formula: Carrageenan content = Dry weight (% fresh weight)/ Carrageenan weight * 100.

2.9 Carrageenan analysis by vibrational spectroscopy

2.9.1 FTIR-ATR

The FTIR spectra were obtained in a spectrophotometer IFS 55, using a "Golden Gate single reflection diamond ATR" system. The spectra represent the mean of two counts, each with 28 scans and a resolution of 2 cm^{-1} (Pereira *et al.*, 2003; Pereira & Mesquita, 2004).

2.10 Bioactivity assays

2.10.1 Preparation of the carrageenan extracts

In order to perform the bioactivity studies, the carrageenan extracts (fructified female gametophyte and tetrasporophyte) obtained previously were grinded using a mortar and a pestle. A stock solution of 10 g L^{-1} was prepared. Four working solutions were prepared from the stock solution, with concentrations of 5, 2.5, 1 and 0.1 g L^{-1}. Solutions were stored in 1.5 mL eppendorf tubes and kept at -20 ºC until further use. This procedure was carried out under aseptic conditions.

2.10.2 Antifungal activity

This study was performed following the standardized protocol M27-A2 for yeast and the M38A protocol for filamentous fungi developed by the Clinical and Laboratory Standards Institute (CLSI), with minor changes.

2.10.2.1 Tested fungi and media

Alternaria infectoria (CBS 137.90) was obtained from Centraalbureau voor Schimmelcultures (CBS) and was grown on potato dextrose agar medium (PDA; Difco) at 30 °C under 8 h-alternating light enriched with UV (lamp F15W T8BLB) and dark. For liquid culture, the medium used was YME: 4 g L^{-1} yeast extract, 10 g L^{-1} malt extract, 10 g L^{-1} glucose.

Asp. fumigatus was purchased from CBS (CBS 500.90) and was grown in PDA medium at 30. For liquid culture, the medium used was YME.

C. albicans (YP0037) was obtained from the Microbiology Pathogenic Yeast Collection, University of Coimbra and was grown in YPD medium (20 g L^{-1} glucose, 10 g L^{-1} peptone, 5 g L^{-1} Yeast extract, 20 g L^{-1} agar) at 30 °C.

All strains were stored at -80 ºC.

2.10.2.2 Microdilution test

2.10.2.2a Preparation of inocula and growth conditions

2.10.2.2.a1 *Alternaria infectoria*

A. infectoria was inoculated in PDA plates and incubated for fourteen days at 30 ºC with alternating 8:16h (light:dark) cycles under an ultraviolet (UV)-enriched lamp. Fourteen-day-old colonies were covered with sterile 0.85% saline solution and scraped with an inoculation loop. This resulted in a mixture of spores and hyphal fragments which was transferred to a sterile tube to allow the settlement of heavy particles. The upper homogeneous suspension was then transferred to another sterile tube and the optical densities (OD) were read and adjusted to match an OD of approximately 0.1 at

600 nm. The suspension was diluted 1:50 in RPMI-1640 medium (with glutamine, without bicarbonate, and with phenol red as pH indicator, Sigma) (see appendix 2) to obtain a final concentration of 0.4 x 10^4 to 5 x 10^4 CFU/mL.

2.10.2.2.a2 *Aspergillus fumigatus*

Asp. fumigatus was inoculated in PDA plates and incubated for seven days at 30 ºC. Seven-day-old colonies were covered with sterile 0.85% saline solution. One drop of Tween-20 was added in order to facilitate the preparation of the inoculum. This procedure led to a homogeneous conidia suspension. The OD was read in a spectrophotometer and adjusted between 0.09 and 0.11 at 600 nm (80-82% transmittance). Suspensions were diluted 1:50 in RPMI-1640 medium.

2.10.2.2.a3 *Candida albicans*

C. albicans was inoculated in YPD and incubated for 24 hours at 37 ºC. The inoculum was prepared by picking five colonies of approximately 1 mm in diameter from the 24-hour-old culture of *C. albicans.* Colonies were suspended in 0.85% saline solution, with an optical density adjusted to 0.5 McFarland. A working suspension was made by a 1:100 dilution followed by a 1:20 dilution of the stock suspension with RPMI 1640 broth medium, which resulted in 5.0 x 10^2 to 2.5 x 10^3 CFU/mL.

2.10.3 Determination the of antifungal activity of carrageenan extracts

The antifungal activity of the carrageenan extracts was evaluated by microdilution broth assay. The end point was the lowest concentration producing a significant change in hyphal morphology (minimum effective concentration, MEC) or a visible growth inhibition (minimum inhibitory concentration, MIC). In order to determine MEC and MIC, *A. infectoria, Asp. fumigatus* and *C. albicans* were inoculated in a sterile 96 multi-well plate and each seaweed extract was added at final concentrations of 10, 100, 250 and 500 μg/mL. Growth control wells contained inoculum and autoclaved distilled water.

2.10.3.1 Incubation

A. infectoria microdilution plates were incubated at 35 ºC, with constant orbital shaking at 120 rpm, with alternating 8:16 h (light:dark) cycles under an enriched-UV lamp. *Asp. fumigatus* microdilution plates were incubated at 35 ºC, without agitation. *C. albicans* microdilution plates were incubated at 35 ºC for 46 to 50 hours without agitation.

Fungal growth and morphology were evaluated by microscopy (Nikon Eclipse E400) after 48 hours of incubation. Digital images were captured with a microscope equipped with a digital camera (Nikon Digital Sight DS-L1).

2.11 Quantification of chitin and β-glucan content

This assay was only performed in *A. infectoria* and *Asp. fumigatus*.

To determine the quantification of chitin and glucan content of the cell walls, *A. infectoria* and *A. fumigatus* were grown on Erlenmeyer's flasks with YME medium.

2.11.1Preparation of inocula and growth conditions

A. infectoria and *Asp. fumigatus* spores suspension were prepared as described in section 2.10.2.2a, and used to inoculate Erlenmeyer flasks with YME.

The concentration of the extracts added to each Erlenmeyer was determined according to MEC and MIC results. For *A. infectoria*, the extract corresponding to the fructified female gametophyte was added with a final concentration of 150 µg/mL, and the extract corresponding to the tetrasporophyte was added with a final concentration of 100 µg/mL. For *Asp. fumigatus*, both extracts were added with a final concentration of 150 µg/mL.

2.11.2 Incubation

Erlenmeyer's containing *A. infectoria* were incubated for 72 hours at 30 ºC, with constant orbital shaking at 120 rpm, with alternating 8:16 h (light:dark) cycles under an

enriched-UV lamp. Erlenmeyer's containing *Asp. fumigatus* were incubated for 48 hours at 35 ºC, with constant orbital shaking at 120 rpm.

2.11.3 Quantification of chitin

The chitin content was determined by the glucosamine released through acid hydrolysis of the cell walls according to the described by Fernandes and co-workers (2014).

A. infectoria and *Asp. fumigatus* mycelia were collected to previously weighed microtubes (Fig. 25a) and washed 3 times with distilled water. Following that, samples were lyophilized (Fig. 25b) and the microtubes were weighed again, in order to determine the fungal dry weight. The pellet was sonicated in distilled water (Fig. 25c). Samples were then centrifuged and the water was discarded (Fig. 25d). The pellet was extracted in SDS-Mer-OH buffer at 100 ºC for 10 minutes using a heating Bio TDB-100 thermostat. Samples were centrifuged again and the supernatant was discarded. Then, HCl 6 M was added to the samples, which were then heated at 100 ºC in a heating thermostat for 18 hours. After that, samples were evaporated at 65 ºC and hydrated with distilled water (Fig. 25e). Then, a solution A (3.2 g of Na_2CO_3 3M in 10 mL of 4% acetylacetone) was added to the samples and incubated for 20 minutes in boiling water (Fig. 25f), followed by the addition of ethanol 96% and solution B (0.8 g of β-dimethylaminobenzaldehyde in 15 mL of HCl and 15 mL of absolute ethanol). Samples were incubated at room temperature for 1 hour (Fig. 25g). Following that, each sample was transferred to 96 multi-well plates and the absorbance was read in a spectrophotometer SPETRAmax PLUS 384 at 520 nm (Fig. 26h). A standard calibration curve was done with a glucosamine solution of 5 g L^{-1} (see supplemental material I).

Figure 25 – Chitin quantification: a) mycelia collection; b) lyophilization; c) sonication; d) centrifugation; e) evaporation; incubation in boiling water; samples at room temperature; samples inoculated in 96 multi-well plate.

2.11.4 Quantification of β-glucan

The β-glucan cell wall content was determined with aniline blue assay (Fernandes et al., 2014). Mycelia were collected and the fungal dry weight was determined. The pellet was sonicated in NaOH 1 M and incubated at 52 ºC for 30 minutes. Then, the samples were inoculated in 96 multi-well plates and aniline blue was added. The assay plates were incubated at 52 ºC for 30 minutes and then kept at room temperature for another 30 minutes. Results were read in a fluorometer SPECTRAmax GEMINI EM (λexc: 405 nm; λemiss: 460 nm). A standard curdlan titulation curve was done with a stock solution of 50 g L^{-1} with NaOH 1M (see supplemental material II).

2.12 Statistical analysis

A one-way ANOVA test was performed in order to observe if the increase/decrease of chitin and β-glucan upon exposure to carrageenan extracts was statistically significant ($P < 0.05$). A Tukey's test was performed in order to see if there were differences between treatments. Tests were performed using GraphPad Prism 6.

2.13 Antibacterial activity

2.13.1Tested bacteria and media

The carrageenan extracts were tested against *E. coli* and *S. aureus*, both obtained from Hospital dos Covões, Coimbra.

Both cultures were grown on Columbia Agar solid media at 37 °C. For liquid media, cultures were grown on Muller-Hinton at 37 °C.

Both cultures were stored at -80 °C.

2.13.2 Identification of bacteria

Before testing the carrageenan extracts against *S. aureus* and *E. coli*, a few tests were performed in order to confirm the identification of the bacteria. Thus, the Gram stain test was performed to allow the separation of bacteria into two large groups – Gram– and Gram+; the Catalase/Oxidase test was done to observe the presence of respiratory enzymes; API (Analitical Profile Index) tests, such as API ID 32 STAPH and API ID 32 GN were done to identify the bacteria species.

After the identification of bacteria, an antibiogram was performed in order to test the susceptibility of the bacteria to antibiotics.

2.13.3 Determination of the antibacterial activity of carrageenan extracts

This study was performed following the standardized protocol M07-A9 for bacteria that grow aerobically, developed by the Clinical and Laboratory Standards Institute (CLSI).

2.13.3.1 Inoculum preparation

Inoculum was prepared using the direct colony suspension method. Thus, a suspension was prepared by selecting 3 to 5 isolated colonies of *S. aureus* and *E. coli* from an 18 to 24-hour Columbia Agar plate. The suspension was adjusted to achieve a turbidity equivalent to a 0.5 McFarland standard.

2.13.3.2 Microdilution broth test

The antibacterial activity of the carrageenan extracts was evaluated by microdilution broth assay. Thus, a sterile 96 multi-well plate was inoculated with Muller-Hinton liquid media (100 µL) and each extract was added at final concentrations of 5, 2.5, 1, 0.5 and 0.1 µg/mL (50 µL). The bacterial suspension was diluted 1:20 in a 0.85% saline solution, in order to achieve a final concentration of 5 x 10^6 CFU/mL, and was added to the microdilution plate (10 µL).

Growth control wells containing liquid media and inoculum lacking extracts were done in duplicate.

2.13.3.3 Incubation

The microdilution plates were incubated at 35 ± 2 °C for 16-20 hours.

Results were read in a spectrophotometer at 600 nm.

2.14 Antiviral activity

2.14.1 Cells and viruses

HEK-293T cells and Caco-2 cells were obtained from American Type Culture Collection (ATCC). HEK-293T cells were grown on Dulbecco's Modified Eagle's Medium (DMEM) with 10% of heat inactivated Fetal Bovine Serum (FBS) and supplemented with 1% of penicillin-streptomycin. Caco-2 cells were grown on Dulbecco's Modified Eagle's Medium (DMEM) with 20% of heat inactivated Fetal Bovine Serum (FBS) and supplemented with 1% of penicillin-streptomycin. Both cells were stored in liquid nitrogen vapor until further use.

The strains of Lentivirus and Coxsackie virus A12 were obtained from ATCC and stored at -80 °C until further use.

2.14.2 Cytotoxicity assay

A stock solution of 10 mg/mL of each extract dissolved in H_2O MilliQ was prepared. The toxicity of the extracts was evaluated by seeding 100.000 of HEK-293T and Caco-2 cells in a 24 multi-well plate. Cells were incubated at 37 °C/ 5% CO_2 for 24 hours. The carrageenan extracts were diluted 1:10 and 1:100 and added to each well at concentrations of 1, 10, 50, 100 and 200 µg/mL, and incubated at 37 °C/ 5% CO_2 for 8 hours.

Cell viability was measured by ALAMAR blue method. Resazurin Sodium Salt (Sigma) was diluted 1:10 in DMEM media and added to each well (500 µL). Cells were then incubated at 37 °C/ 5% CO_2 until control wells turned violet (Fig. 26). After that, 80 µL of supernatant from each well were transferred to a 96 multi-well plate and read in a spectrophotometer at 570 and 600 nm.

Cells previously seeded in a 24 multi-well plate were washed with PBS and the carrageenan extracts were added again and incubated at 37 °C/ 5% CO_2 until performed 24 hours of incubation. Cell viability was measured again by ALAMAR blue method. This process was repeated until extracts were incubated for 48 hours.

Figure 26 – Citotoxicity assay.

2.14.3 Determination of the antiviral activity of carrageenan extracts

The antiviral activity of the carrageenan extracts was evaluated by testing FG and Tetra extracts against a strain of Lentivirus and a strain of Coxsackie virus A12. For both viruses, extracts were added at different times – before the viral infection (pre-treatment) and at the same time of the viral infection (virucidal assay). For Coxsackie virus, extracts were also added after the viral infection (post-treatment).

2.14.4 Infection with Lentivirus

2.14.4.1 Pre-treatment

A pre-treatment was done by seeding 500 µL of HEK-293T cells in a 12 multi-well plate and incubating it at 37 °C/ 5% CO_2 for 24 hours. Extracts were then added to the wells (200 µg/mL) and incubated at 37 °C/ 5% CO_2 for 2 hours. Following that, wells were washed with PBS, infected with 200 ng of Lentivirus and incubated for 72 hours at 37 °C/ 5% CO_2 (Fig.27). After that time, cells were fixed with 4% formaldehyde and then stained with DAPI. Results were evaluated by microscopy (Axio Imager z2) and quantified using Zen Lite program.

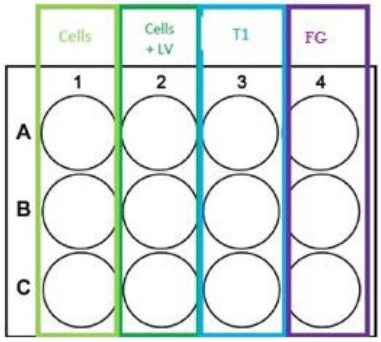

Figure 27 – Experimental design of the antiviral assay: Cells - HEK-293T cells without any treatment; cells + LV - HEK-293T cells and Lentivirus; T1 – Tetra extract; FG – Female Gametophyte extract.

2.14.4.2 Virucidal assay

A virucidal assay was performed by seeding 500 µL of HEK-293T cells in a 12 multi-well plate and incubating it at 37 °C/ 5% CO_2 for 24 hours. A mixture of virus (200 ng) and extract (200 µg/mL) was prepared. Cells were then infected with this mixture and incubated at 37 °C/ 5% CO_2 for 72 hours. After that time, cells were fixed with 4% formaldehyde and then stained with DAPI. Results were evaluated by microscopy (Axio Imager z2) and quantified using Zen Lite program.

2.14.5 Infection with Coxsackie virus A-12

2.14.5.1 Pre-treatment

A pre-treatment was done by seeding 400 µL of Caco-2 cells in a 24 multi-well plate and incubating it at 37 °C/ 5% CO_2 for 24 hours. Following that, 200 µg/mL of FG and Tetra extracts were added to the cells and incubated for 2 hours at 37 °C/ 5% CO_2. Cells were then washed with PBS, infected with 30 µL of Coxsackie virus A12 and incubated for 2 hours at 37 °C/ 5% CO_2. The content of the wells was then removed for RT-PCR analysis.

2.14.5.2 Virucidal asssay

A virucidal assay was done by seeding 400 µL of Caco-2 cells in a 24-multiwell plate and incubating it at 37 °C/ 5% CO_2 for 24 hours. Following that, a mixture of 30 µL of Coxsackie virus A12 and 200 µg/mL of extract was prepared. Cells were then infected with this mixture and incubated for 2 hours at 37 °C/ 5% CO_2. After that time, the content of the wells was washed with PBS and incubated for 48 hours. Following that, the content of the wells was removed for RT-PCR analysis.

2.14.5.3 Post-treatment

A post-treatment was performed by inoculating 400 µL of Caco-2 cells in a 24-multiwell plate and incubating it at 37 °C/ 5% CO_2 for 24 hours. Cells were then infected with 30 µL of Coxsackie virus A12 and incubated for 2h at 37 °C/ 5% CO_2. Wells were then washed with PBS and 200 µg/mL of extract were added to cells and incubated for 48 hours at 37 °C/ 5% CO_2 (Fig. 28). The content of the wells was then removed for RT-PCR analysis.

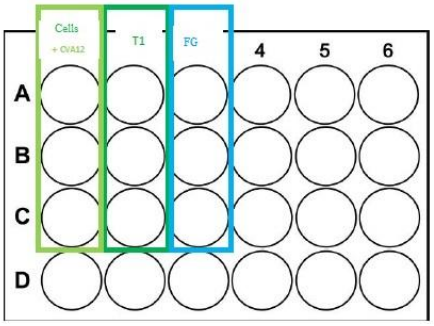

Figure 28 – Experimental design of antiviral assay: Cells + CVA12 – Caco-2 cells and Coxsackie virus A12; T1 – Tetra extract; FG – Female Gametophyte extract.

2.14.5.4 Quantification of Coxsackie virus A12 by RT-PCR

2.14.5.4.a Extraction and quantification of RNA samples

RNA extractions were carried out using MagNA Pure Compact Nucleic Acid Isolation Kit (Roche, USA) in a MagNa Pure Compact System and quantified in a nanodrop.

2.14.5.4.b Reverse Transcription (RT-PCR) and Quantitative Real-Time (qPCR) PCR

A reverse transcription PCR (RT-PCR) was done using a Transcriptor First Strand cDNA Synthesis Kit, according to the manufacturer's standards.

Quantitative assays were performed both for Coxsackie virus A12 and for the 18S gene, using the LightCycler 2.0 thermocycler (Rocha, USA) with the Light Cycler Fast Start DNA Master plus SYBR Green I, according to the manufacturer's standards.

Cell normalization was performed by the comparative C_T method, also referred to as the $2^{-\Delta\Delta CT}$. This method is done by using the following formula:

$2^{-\Delta\Delta CT}$ = [(C_T gene of interest - C_T internal control) sample A
- C_T gene of interest - C_T internal control) sample B]

Sample A corresponds to treated sample and sample B corresponds to untreated control.

Chapter III – Results

3.1 Cultivation of *Chondracanthus teedei* var. *lusitanicus*

3.1.1 Laboratory culture

3.1.1.1 Cultivation conditions

The laboratory culture was performed under controlled conditions of temperature (15 °C ± 1), light (~45 μE m^{-2} s^{-1}) and photoperiod (16:8) (light:dark). Although the initial light irradiance was determined to be 100 μE m^{-2} s^{-1}, the flasks containing the algae were only exposed to ~45 μE m^{-2} s^{-1}.

Salinity and pH values were monitored with a multiparametric sensor every time the medium was changed. Fig. 29 shows the temperature and salinity during this experience. Temperature values ranged between 15.3 °C (minimum) and 15.6 °C (maximum) and salinity ranged between 37.6 ‰ and 38‰, both with no significant changes.

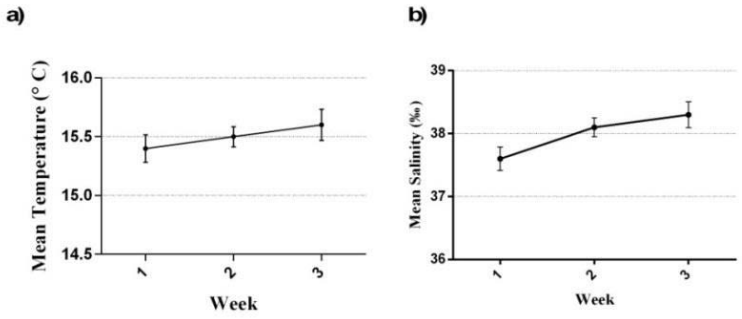

Figure 29 – Cultivation conditions: a) temperature; b) salinity (mean ± SE).

The monitoring of pH values over the weeks for each cultivation density is displayed in Fig. 30. Control columns represent the pH value when the medium was renewed (before algae have been added to the flasks). The other columns show the pH value 3 days after the medium was changed (with the algae still in the flasks). In the first week after the acclimation week, the pH value of all the cultivation densities, measured 3 days after the medium was renewed, registered an abnormal decrease when compared to the control (Fig. 30a, b, c and d). The opposite situation was observed in the following two weeks, where the pH values of the medium 3 days after its change increased in comparison with the control. The pH values of the control ranged between

8.1 (minimum) and 8.6 (maximum). The pH values of the medium 3 days after its change ranged between 8.1 (minimum) and 8.5 (maximum).

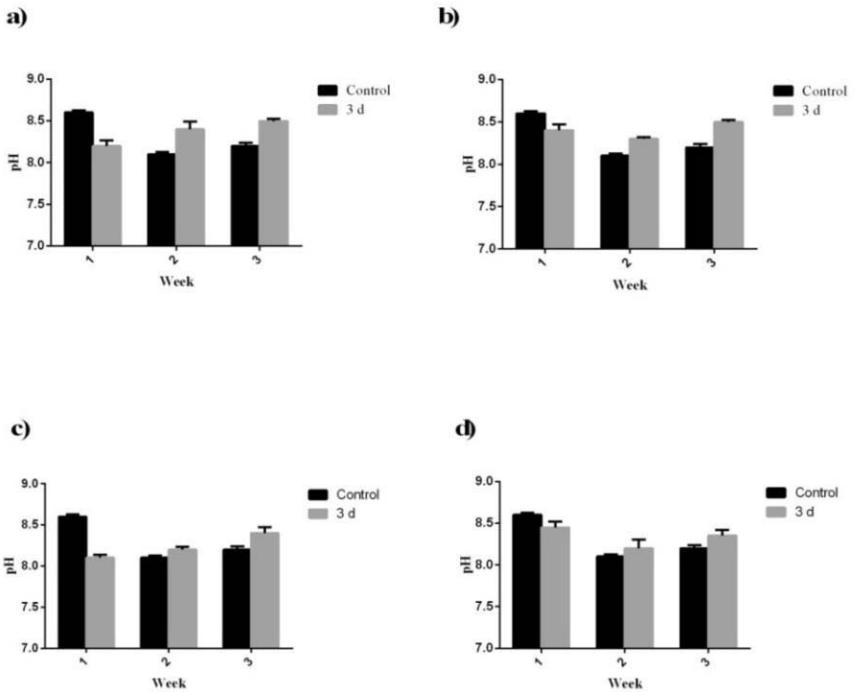

Figure 30 - pH values over the weeks (mean ± SE) for each cultivation density: a) pH variation for cultivation density 1 g L^{-1}; b) pH variation for cultivation density 2 g L^{-1}; c) pH variation for cultivation density 4 g L^{-1}; d) pH variation for cultivation density 6 g L^{-1}. Control represents the pH value measured when medium was renewed; 3 d represents the pH value of the medium 3 days later.

3.1.1.3 Relative Growth Rate (RGR) and Productivity

3.1.1.3.a Dry Weight (DW)

The calculation of the dry weight of *C. teedei* var. *lusitanicus* was essential to determine the percentage of water that composes this alga and also to calculate the productivity of this alga during its cultivation both at laboratory and in an IMTA system. This parameter was calculated using the following formula:

% Dry weight = Dry weight / Fresh weight (g).

The results are shown in Table 3. As it can be seen, this alga contains a great percentage of water in its constitution.

Table 3 – Percentage of dry weight of *C. teedei* var. *lusitanicus* (mean ± SE, n = 4).

Replicates	n = 1	n = 2	n = 3	n = 4
Fresh weight (g)	1	1	1	1
Dry weight (g)	0.21	0.20	0.21	0.21
% Dry weight	21%	20%	21%	21%
Mean % dry weight	21 ± 0.01%			
% water	79 ± 0.01%			

The relative growth rate and the productivity of *C. teedei* var. *lusitanicus* were measured during a period of 3 weeks after 1 week of acclimation. *C. teedei* var. *lusitanicus* was grown at different cultivation densities in 1 L flaks, with autoclaved salt water supplemented with VSE medium. The medium was changed every 3-4 days and the algal biomass was weighted once a week. The excess biomass was reduced to its correspondent initial cultivation density.

Figs. 31 and 32 show the relative growth rate and the productivity, respectively, calculated for each cultivation density.

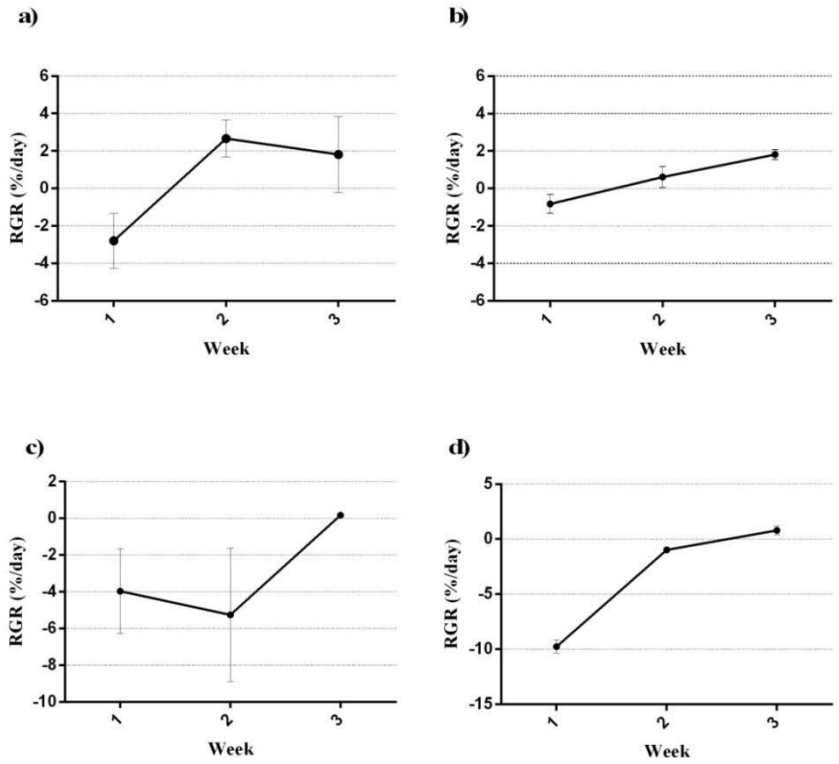

Figure 31 – RGR (mean ± SE) for each cultivation density over the weeks: a) RGR of cultivation density 1 g L^{-1}; b) RGR of cultivation density 2 g L^{-1}; c) RGR of cultivation density 4 g L^{-1}; d) RGR of cultivation density 6 g L^{-1}.

As it can be shown in Fig. 31, the different cultivation densities influence the relative growth rate of this alga. During this experiment, there was a week variation of the relative growth rate for all the cultivation densities. It can also be verified that in the first week after the acclimation week, there was a decrease in the biomass of all the cultivation densities, making its relative growth rate reach negative levels (Fig. 31a, b, c and d). In the second and third weeks of the experiment, there was an increase in the biomass of all the cultivation densities except for cultivation density 4 g L^{-1} (Fig. 31c). In fact, at cultivation densities of 4 g L^{-1} and 6 g L^{-1} the decrease in biomass in the first week was so significant that most of the algae in the flasks did not recover its initial weight. And even though there was an increase in the following weeks, its relative growth rate ended up reaching values close to zero (Fig. 31c and 31d). In cultivation densities of 1 g L^{-1} and 2 g L^{-1} (Fig. 31a and 31b), this species was capable of recovering its initial weight, and even increasing it (Fig. 31b). Even though negative, the cultivation density 2 g L^{-1} revealed to be the one with the highest relative growth rate (-0.8 ± 0.5 % day^{-1}). On the other hand, the cultivation density 6 g L^{-1} revealed to be the one with the lowest relative growth rate (-9.7 ± .5 % day^{-1}). Cultivation densities of 1 g L^{-1} and 4 g L^{-1} registered values of -2.8 ± 1.4 % day^{-1} and -3.9 ± 2.3 % day^{-1}, respectively.

The productivity was also registered over the weeks at different cultivation densities.

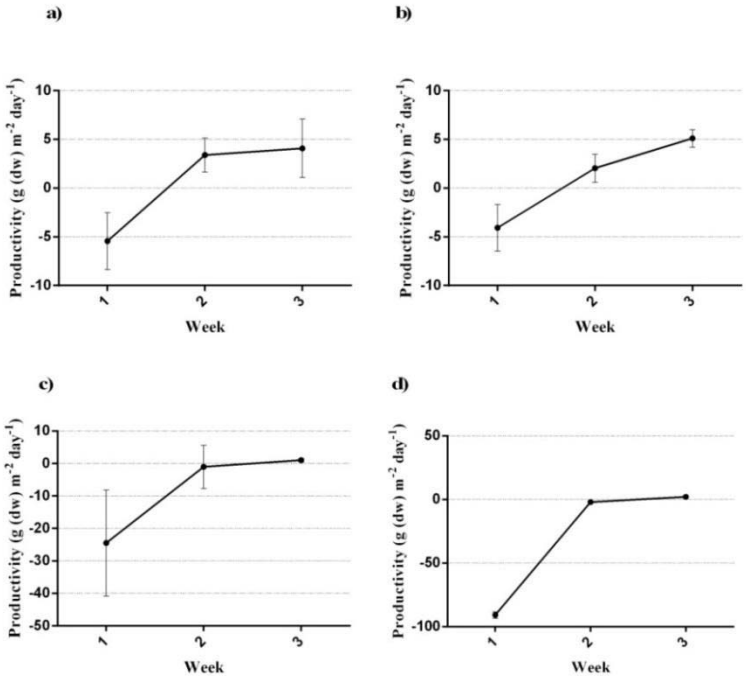

Figure 32 – Productivity (mean ± SE) for each cultivation density over the weeks: a) Cultivation density 1 g L^{-1}; b) Cultivation density 2 g L^{-1}; c) Cultivation density 4 g L^{-1}; d) Cultivation density 6 g L^{-1}.

During the experiment, there was a week variation of the productivity in all the cultivation densities (Fig. 32). It can also be observed that the productivity of all cultivation densities decreased in the first week after the acclimation week, reaching negative values (Fig. 32a, b, c and d). In the following two weeks it was registered an increase in the productivity, however, only cultivation densities of 1 g L^{-1} and 2 g L^{-1} reached positive values (Fig. 32a and b). Moreover, it can be observed that the cultivation density of 2 g L^{-1} (Fig. 32b) registered the highest value of productivity (even though negative) (-4.1 ± 2.4 (g (dw) m^{-2} day^{-1})), and the cultivation density 6 g L^{-1} (Fig. 32d) registered the lowest value (-90,82 ± 2.55 (g (dw) m^{-2} day^{-1})). Cultivation densities of 1 g L^{-1} and 4 g L^{-1} registered values of -5.44 ± 2.93 (g (dw) m^{-2} day^{-1}) and -24.45 ± 16.32 (g (dw) m^{-2} day^{-1}), respectively.

Figure 33 shows the relative growth rate and the productivity of *C. teedei* var. *lusitanicus* at different cultivation densities.

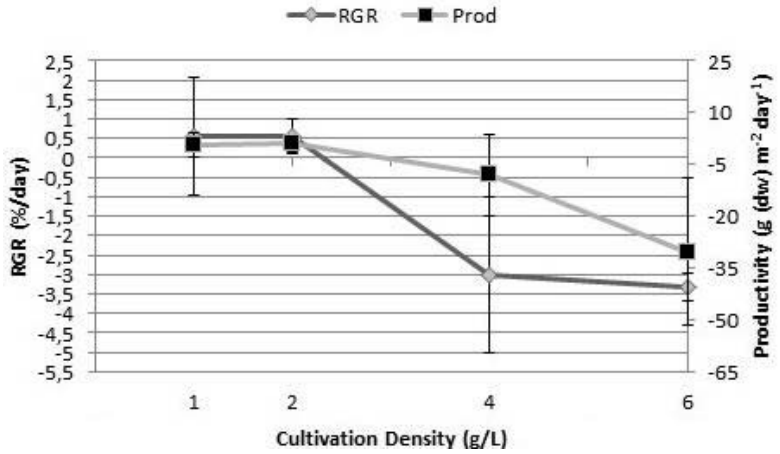

Figure 33 – RGR and Productivity (mean ± SE) of *C. teedei* var. *lusitanicus* at different cultivation densities.

Through the analysis of Fig. 33, it can be observed that the different cultivation densities influence both relative growth rate and productivity. For both parameters, the figure shows that cultivation densities of 1 g L^{-1} and 2 g L^{-1} were the ones with better results, contrary to what was verified for cultivation densities 4 g L^{-1} and 6 g L^{-1}, which achieved worse results. Moreover, it can also be seen that at higher cultivation densities (4 and 6 g L^{-1}), relative growth rate and productivity reached negative values.

The highest relative growth rate was achieved at cultivation density 1 g L^{-1} (0.56 ± 1.9 % day^{-1}) and the highest productivity was achieved at cultivation density 2 g L^{-1} (1.02 ± 2.55 (g (dw) m^{-2} day^{-1})). The lowest relative growth rate (-3.32 ± 2.3 % day^{-1}) and productivity (-30.27 ± 21.48 (g (dw) m^{-2} day^{-1})) were achieved at cultivation density 6 g L^{-1}. Cultivation densities 2 g L^{-1} and 4 g L^{-1} registered values of relative growth rate of 0.54 ± 0.7 % day^{-1} and -3.01 ± 2.7 % day^{-1}, respectively. Cultivation densities 1 g L^{-1} and 4 g L^{-1} registered values of productivity of 0.68 ± 3.4 (g (dw) m^{-2} day^{-1}) and -8.1 ± 11.7 (g (dw) m^{-2} day^{-1}), respectively.

It can also be seen that both parameters intersect with each other at cultivation density 2 g L^{-1}**,** which means that this is probably the optimal cultivation density for this alga to grown in laboratory.

3.1.1.4 Statistical analysis

Although it was observed that different cultivation densities influenced both RGR and productivity, it was necessary to verify if those differences were statistically significant. Results obtained reveal that cultivation densities did not statistically influence RGR and productivity (P>0.05) (see supplemental material III).

3.1.2 IMTA culture

3.1.2.1 Environmental parameters

During the cultivation of *C. teedei* var. *lusitanicus* in an IMTA system, environmental parameters such as temperature, pH, salinity and sunlight irradiance were monitored twice a day (morning and afternoon) over the weeks (Fig. 34). Temperature, pH and salinity were measured using a multiparametric sensor. Sunlight irradiance was measured using a spherical light sensor.

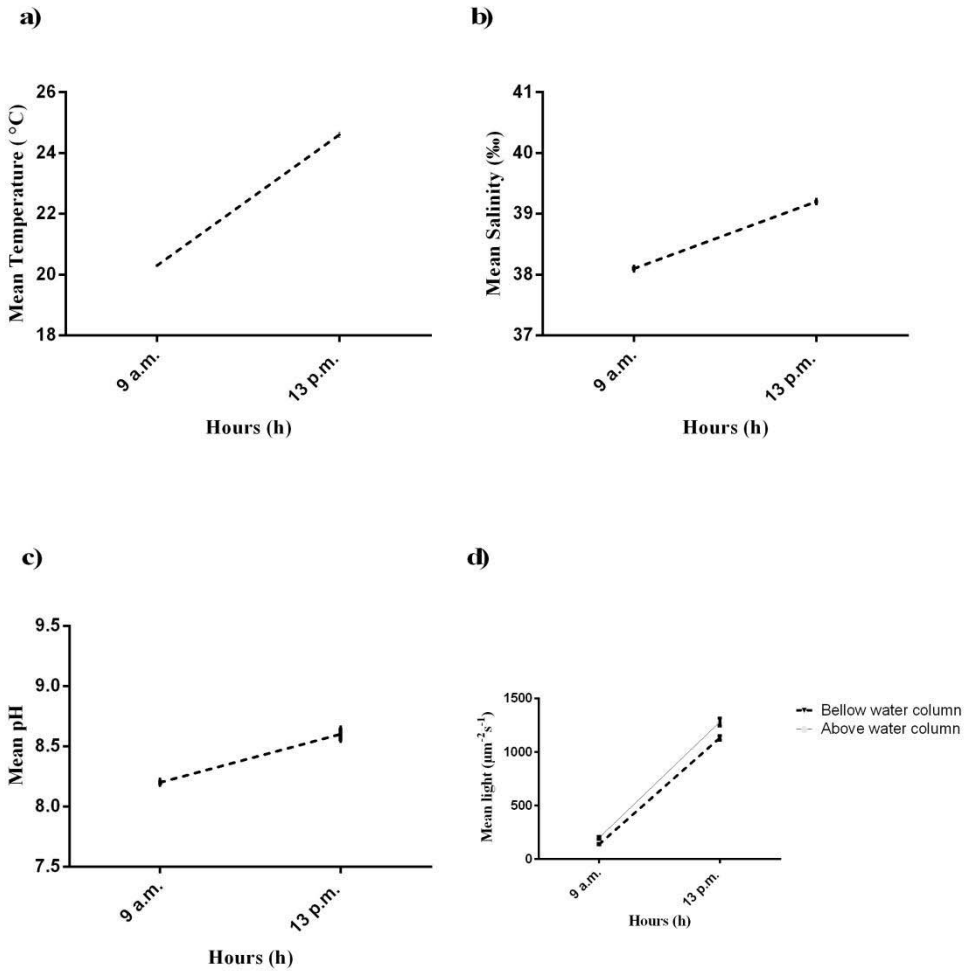

Figure 34 – Example of the daily variation of the environmental parameters in one week (mean ± SE), measured at 9 a.m. and 13 p.m.: a) temperature; b) salinity; c) pH; d) sunlight irradiance.

Fig. 34 shows an example of the daily variation of the environmental parameters over one week, measured at 9 a.m. and 13 p.m. The mean temperature in the morning and in the afternoon ranged between 20.3 ± 0.03 °C and 24.6 ± 0.01 °C, respectively. The mean salinity ranged between 38.1 ± 0.03 ‰ in the morning and 39.2 ± 0.03 ‰ in the afternoon. The pH values ranged between 8.3 ± 0.02 at 9 a.m. and 8.6 ± 0.01 at 13 p. m. Sunlight irradiance above water column varied between 199 ± 14.6 µE m^{-2} s^{-1} in the morning and 1279 ± 38 µE m^{-2} s^{-1} in the afternoon. The sunlight that irradiated directly in the algae (bellow water column) ranged between 137 ± 1.7 µE m^{-2} s^{-1} in the morning and 1132 ± 21.1 µE m^{-2} s^{-1} in the afternoon.

3.1.2.2 Relative Growth Rate and Productivity

As similar to what happened in laboratory, relative growth rate and productivity were also measured in an IMTA system during 3 weeks after 1 week of acclimation. *C. teedei* var. *lusitanicus* was grown at different cultivation densities on 15 L white tanks, which were set to receive independent flows of water from fish effluent. Water was renewed every 45 minutes, approximately. Once a week, the algal biomass was weighted and the excess was reduced to its correspondent cultivation density.

Figs. 35 and 36 display the relative growth rate and the productivity, respectively, at different cultivation densities.

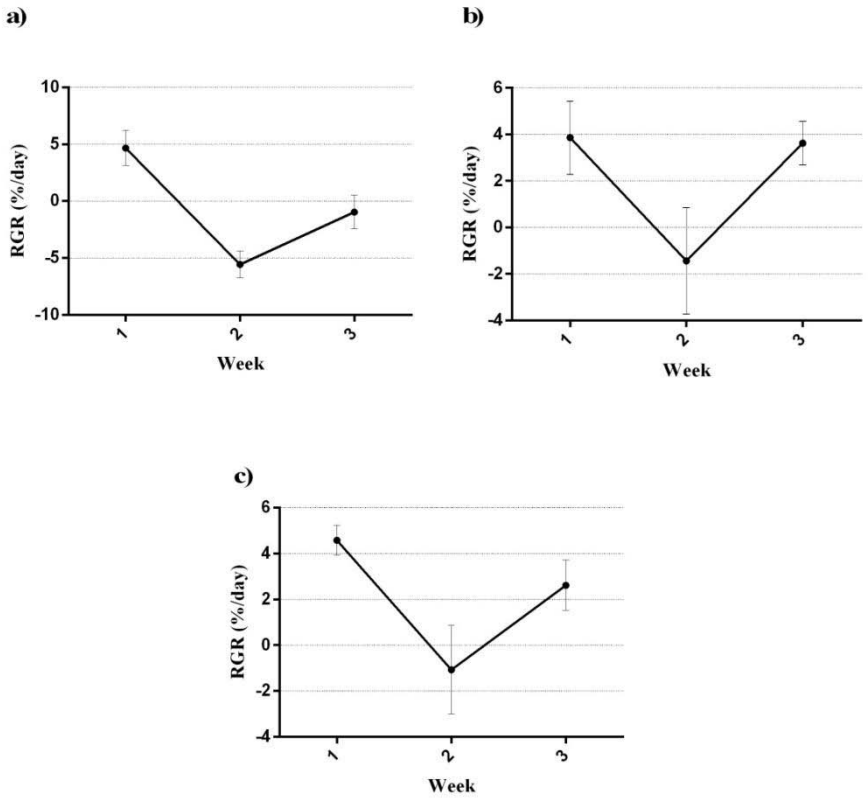

Figure 35 – RGR measured during 3 weeks at different cultivation densities (mean ± SE): a) RGR of cultivation density 4 g L^{-1}; b) RGR of cultivation density 6 g L^{-1}; c) RGR of cultivation density 8 g L^{-1}.

Fig. 35 represents the relative growth rate for each cultivation density over the weeks. Through the analysis of the graphs, it can be seen that the algal biomass of each cultivation density increased in the first week after the acclimation week, making its relative growth rate reach positive values (Fig. 35a, b and c). However, from the first to the second week it was noted a decrease in the algal biomass in all the cultivation

densities, and from the second to the third week it was noticed an increase. In all cultivation densities, the decrease in biomass registered from the first to the second week was recovered in the third week.

It can also be verified that the highest relative growth rate was achieved at cultivation density 4 g L^{-1} (4.7 ± 1.5 % day^{-1}) (Fig. 35a), followed by the cultivation density 8 g L^{-1} (4.6 ± 1.5 % day^{-1}) (Fig. 35c). This way, the cultivation density 6 gL^{-1} (Fig. 35b) presented the lowest relative growth rate (3.8 ± 1.6 % day^{-1}).

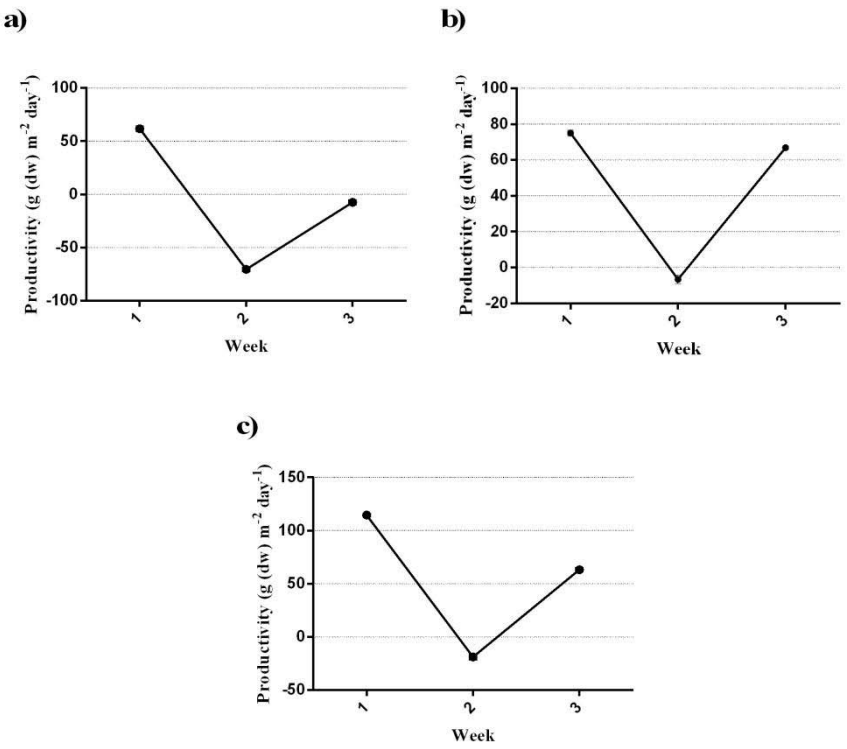

Figure 36 - Productivity (mean ± SE) for each cultivation density over the weeks.

Fig. 36 shows the productivity over the weeks at different cultivation densities. For all the cultivation densities it was observed a week variation of productivity and an increase in its values in the first week after the acclimation week. In the following two weeks it was noted a decrease followed by an increase in the values of productivity for all the cultivation densities. Moreover, it can be observed that the cultivation density of 8 g L^{-1} (Fig. 36c) registered the highest value of productivity (114,5 ± 0.7 (g (dw) m^{-2} day^{-1})), followed by the cultivation density 6 g L^{-1} (Fig. 36b) (75.0 ± 1.6 (g (dw) m^{-2}

day^{-1})); the cultivation density of 4 g L^{-1} (Fig. 35a) registered the lowest value of productivity (61,7 ± 1.5 (g (dw) m^{-2} day^{-1})).

Fig. 37 shows the relative growth rate and the productivity measured for each cultivation density.

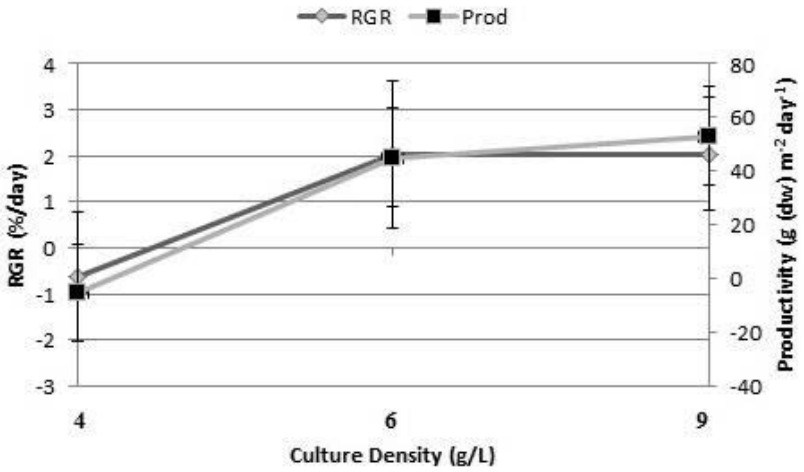

Figure 37 - RGR and Productivity (mean ± SE) of *C. teedei* var. *lusitanicus* at different cultivation densities.

Fig. 37 shows that the lowest values of relative growth rate (-0.62 ± 2.8 % day^{-1}) and productivity (-5.41 ± 1.4 g (dw) m^{-2} day^{-1})) were observed at cultivation density 4 g L^{-1}, and the highest relative growth rate (2.04 ± 1.9 % day^{-1}) and productivity (53.1 ± 1.2 g (dw) m^{-2} day^{-1})) at cultivation density 8 g L^{-1}. Cultivation density 6 g L^{-1} registered values of relative growth rate and productivity of 2.02 ± 2.2 % day^{-1} and 45.1 ± 1.6 g (dw) m^{-2} day^{-1})), respectively. It is noted that both parameters intersect with each other at cultivation density 6 g L^{-1}, which means that this is probably the optimal cultivation density for this species to grow in an IMTA system.

3.1.2.3 Statistical analysis

Although it was observed that different cultivation densities influenced both RGR and productivity, it was necessary to verify if those differences were statistically significant. Results obtained reveal that cultivation densities did not statistically influence RGR and productivity (P>0.05) (see supplemental material IV).

3.2 Determination of dry weight and carrageenan content

The results of the determination of the dry weight and the carrageenan content can be found on Table 4 and Table 5, respectively.

Table 4 – Determination of *C. teedei* var. *lusitanicus* dry weight (Mean ± SE, n = 3).

Replicates	n = 1	n = 2	n = 3
Fresh weight (g)	1	1	1
Dry weight (g)	0.19	0.18	0.19
% Dry weight	19%	18%	19%
Mean % dry weight	19 ± 0.002 %		
% Water	81 ± 0.002 %		

As seen on Table 4, this alga contains a great amount of water in its constitution (81%).

Table 5 - Determination of carrageenan content (mean ± SE, n = 3).

Lifecyle phase	**FG**	**FG**	**FG**	**Tetra**	**Tetra**	**Tetra**
Replicates	n = 1	n = 2	n = 3	n = 1	n = 2	n = 3
% carrageenan DW	31%	33%	35%	41%	36%	39%
Mean ± SE	33 ± 0.01 %			38 ± 1.1 %		

As seen on Table 5, the carrageenan extract belonging to the tetrasporophyte lifecycle phase revealed to be the one with the highest yield (38 ± 1.1 %). On the other hand, the extract belonging to the female gametophyte extract revealed to have a lower yield (33 ± 0.01 %).

3.3 Carrageenan analysis by vibrational spectroscopy

3.3.1 FTIR-ATR Spectra

The vibrational bands present in sulfated polysaccharides are related to the content of the S=O esters group present in the samples.

There are several vibrational bands characteristic of carrageenans. For example, the absorption band at 930 cm^{-1} is related to the vibrations of the 3,6 anhydrogalactose

bridges and, therefore, can be found in the spectra of kappa, iota and theta carrageenans. In addition, the absorption band at 845 cm^{-1}, which is associated with the vibrations of $C_{(4)}$-O-SO_3 (a fragment of the sulfated galactose), is characteristic of the kappa, mu, iota and nu spectra. On the other hand, the band at 805 cm^{-1}, which is associated with the vibrations of $C_{(2)}$-O-SO_3 (fragment of sulfated 3,4-anhydrogalactose), can only be observed in iota and theta carrageenan spectra. Thus, the relative intensity of the 805 cm^{-1} and 845 cm^{-1} peaks (805/845 cm^{-1} ratio) allows the determination of the iota/kappa ratio on hybrid carrageenans.

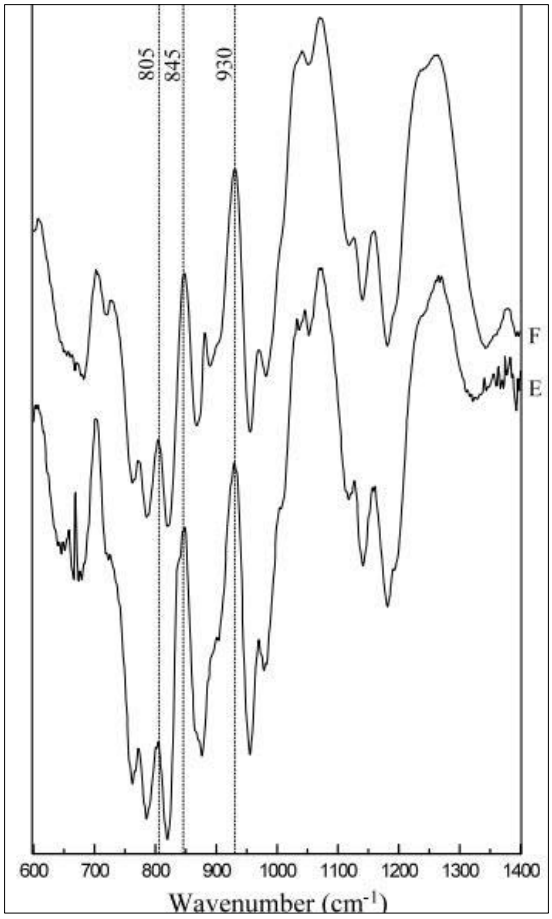

Figure 38 - FTIR-ATR spectra of alkali treated carrageenan: (E) *C. teedei* var. *lusitanicus* (non-fertile thalli); (F) *C. teedei* var. *lusitanicus* (female gametophytes).

Fig. 38 shows the FTIR spectra of *C. teedei* var. *lusitanicus* relative to non-fertile thalli and female gametophyte samples. It is noted the presence of a peak at 1070 cm^{-1}, which is present in kappa, iota an beta carrageenans, and a peak at 1240 cm^{-1}, which is the characteristic broad band of sulfate esters. It can also be observed the presence of diagnoses peaks, with strong bands at 930 and 845 cm^{-1}, characteristics of

kappa-carrageenan, and a medium band at 805 cm^{-1}, characteristic of iota-carrageenan. The presence of these three peaks is typical of κ-ι hybrid carrageenan.

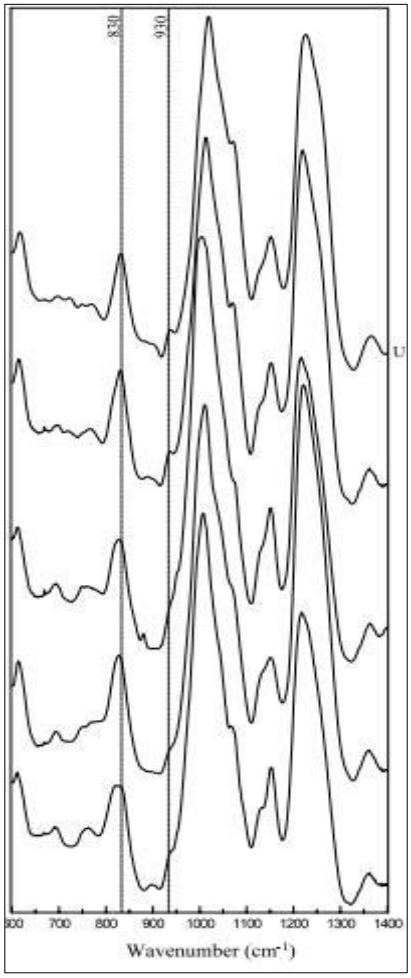

Figure 39 - FTIR spectra of alkali treated carrageenan: (U) *C. teedei* var.*lusitanicus* (tetrasporophytes).

Figure 39 shows the FTIR spectra of *C. teedei* var. *lusitanicus* relative to a tetrasporophyte sample. It can be observed a peak at 1240 cm^{-1}, which is the broad band characteristic of sulfate esters. A sharper peak is present at 830 cm^{-1} and a medium band is present at 820 cm^{-1}, which indicates the presence of ξ-carrageenan.

3.4 Bioactivity assays

3.4.1 Antifungal activity

3.4.1.1 Microdilution broth assay

The antifungal activity of the carrageenan extracts was determined using a microdilution broth assay. The data obtained allowed to observe the effect of these extracts - female gametophyte (FG) and tetrasporophyte (Tetra) - on hyphal morphology of *A. infectoria* and *Asp. fumigatus* upon exposure to concentrations of 10, 100, 250 and 500 µg/mL of each extract.

3.4.1.1.a *A. infectoria*

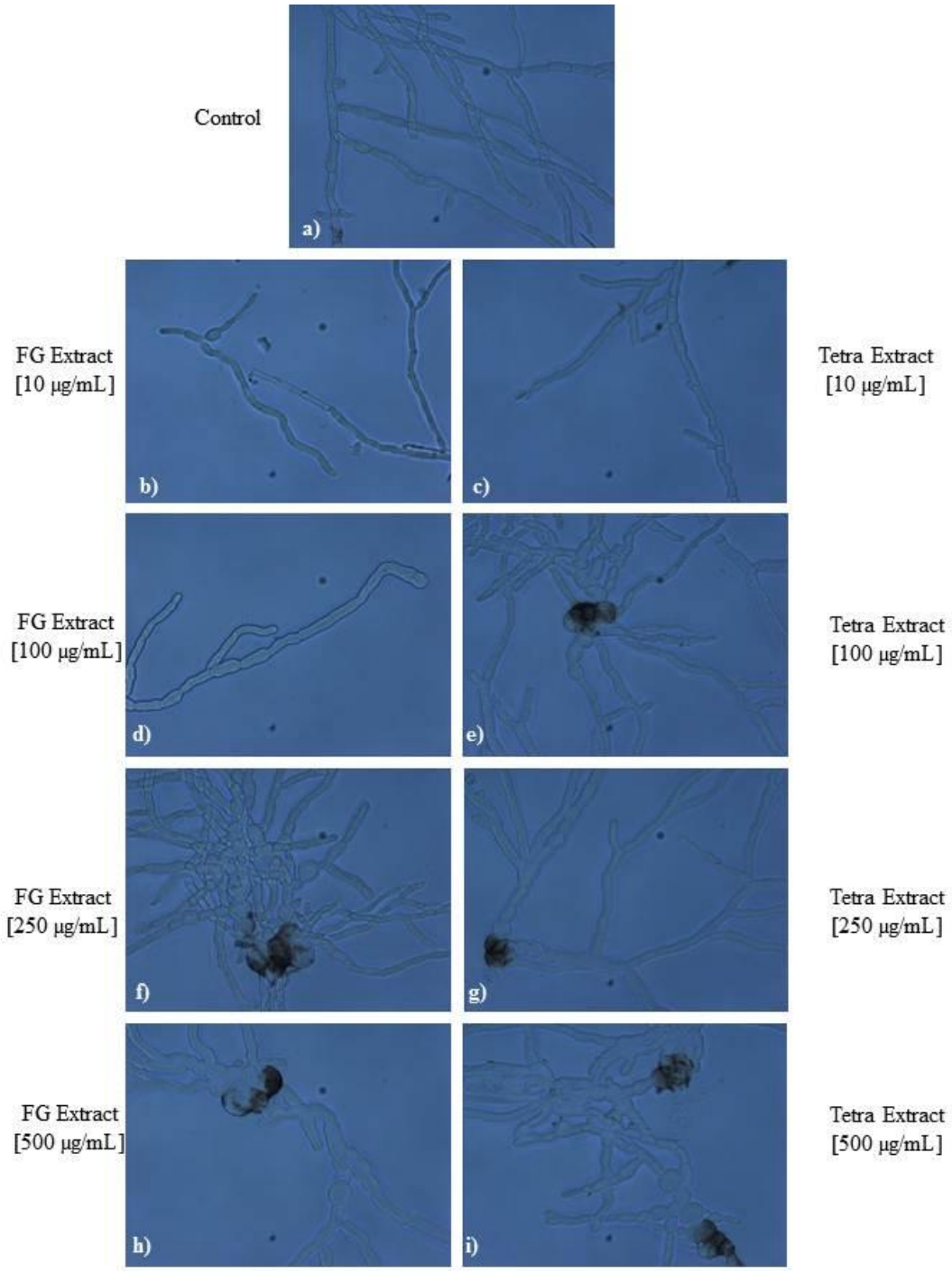

Figure 40 – Effect of FG and Tetra extracts on hyphal morphology of *A. infectoria*: a) Control; b), d), f) and h) *A. infectoria* upon exposure to FG extract at concentrations of 10, 100 250 and 500 µg/mL, respectively; c), e), g) and i) *A. infectoria* upon exposure to Tetra extract at concentrations of 10, 100, 250 and 500 µg/mL, respectively.

The microdilution broth assay allowed to observe that the carrageenan extracts, even at the highest tested concentration (500 µg/mL), did not inhibit the growth of *A. infectoria.* Nevertheless, both extracts were able to induced abnormal hyphal morphology (Fig. 40). In fact, after 48h of exposure to 250 and 500 µg/mL of FG

extract, hyphae of *A. infectoria* revealed morphological changes. Hyphae became swollen, exhibiting balloon-like structures (Fig. 40f and h). The same morphological changes were also observed after exposure to 100, 250 and 500 µg/mL of Tetra extract (Fig. 40e, g and i). These morphological features contrasted with the normal elongated hyphae in control cultures (Fig. 40a).

Since both extracts impact the *A. infectoria* hyphal morphology, the Minimum Effective Concentration (MEC) was determined to each extract. The tested concentrations ranged from 100 to 250 µg/mL of FG extract (Fig. 41) and 10 to 100 µg/mL of Tetra extract (Fig. 42).

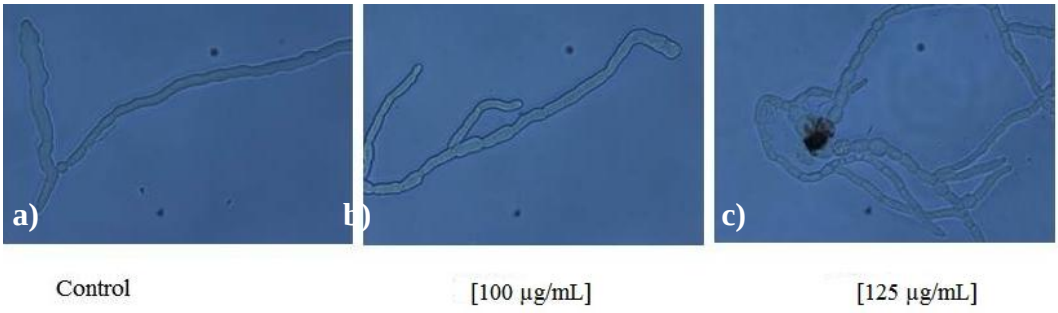

Figure 41 - *A. infectoria* morphology in a) the absence of any carrageenan extract (control); b) in the presence of 100 µg/mL and c) of 125 µg/mL of FG extract.

Alterations on hyphal morphology were observed after 48 hours of exposure to 125 µg/mL of FG extract (Fig. 41c). There were no significant changes upon exposure to 100 µg/mL (Fig. 41b) of the same extract when compared to control (Fig. 41a).

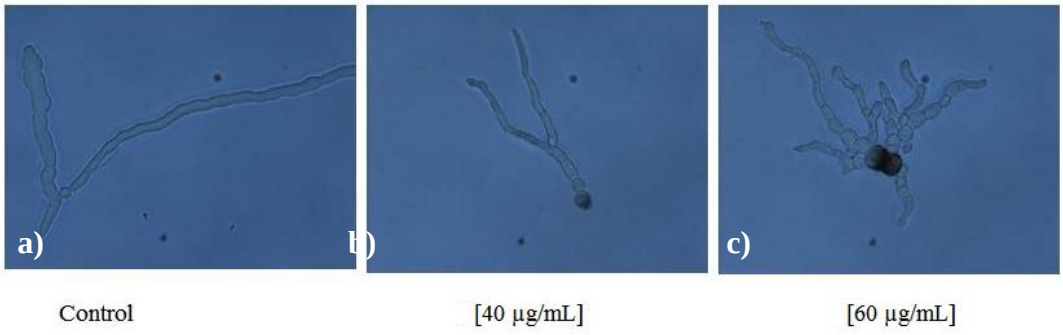

Figure 42 - *A. infectoria* morphology in a) the absence of any carrageenan extract (control); b) the presence of 40 µg/mL and; c) of 60 µg/mL of Tetra extract.

The morphology of *A. infectoria* remained unchanged after exposure to 40 µg/mL of Tetra extract (Fig. 42b). However, upon exposure to 60 µg/mL of the same extract, its hyphae became shortened and exhibited balloon-like structures (Fig. 42c), contrasting with the morphology observed on control culture (Fig. 42a).

3.4.1.1.b *Asp. fumigatus*

The microdilution broth assay allowed the observation of morphological changes in *Asp. fumigatus* upon exposure to carrageenan extracts. However, only the FG extract was able to induce these changes (Fig. 43).

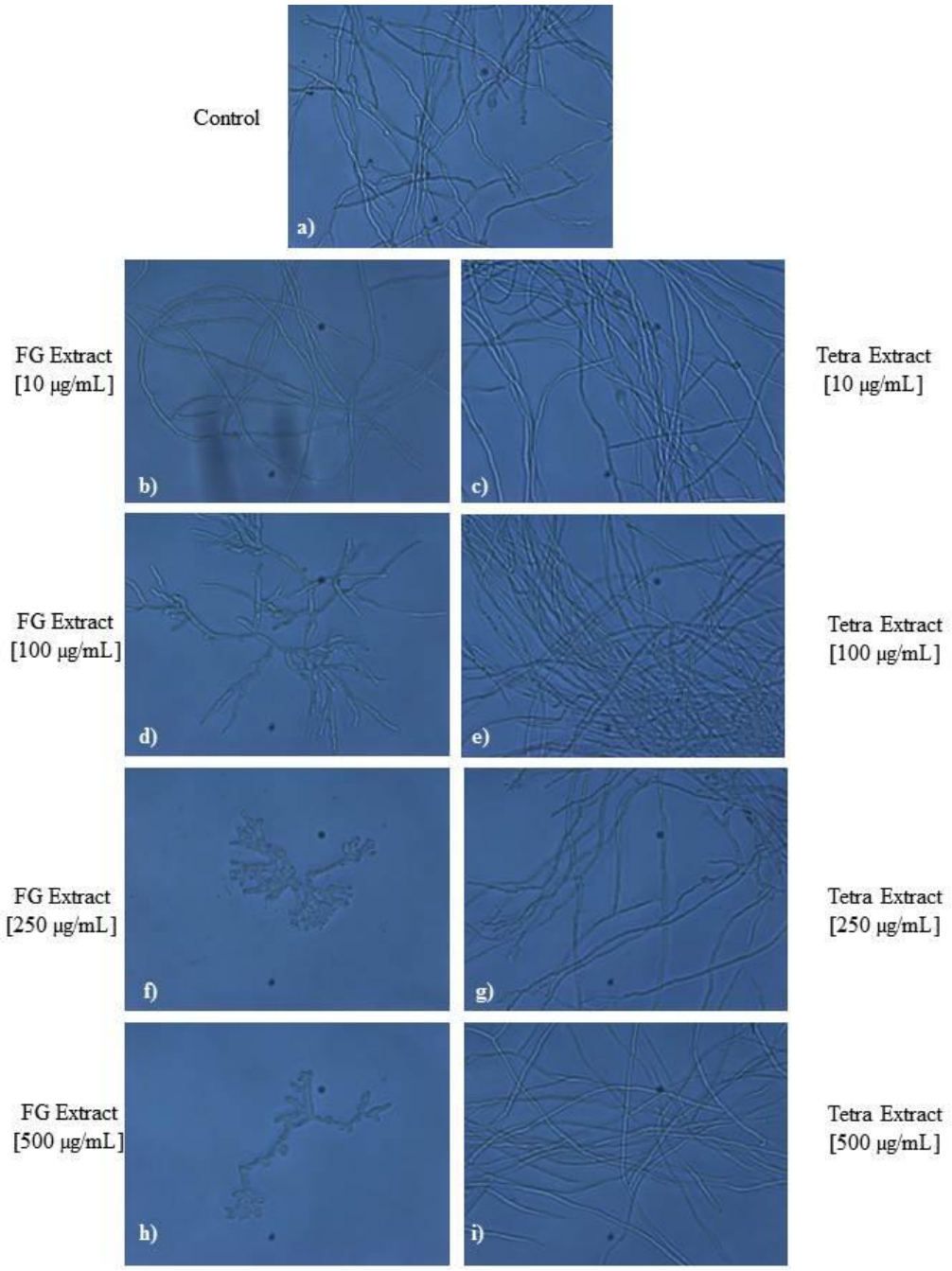

Figure 43 - Effect of FG and Tetra extracts on hyphal morphology of *Asp. fumigatus*: a) Control; b), d), f) and h) *Asp. fumigatus* upon exposure to FG extract at concentrations of 10, 100 250 and 500 µg/mL, respectively; c), e), g) and i) *Asp. fumigatus* upon exposure to Tetra extract at concentrations of 10, 100, 250 and 500 µg/mL, respectively.

After 48 hours of exposure to 100, 250 and 500 µg/mL of FG extract *Asp. fumigatus* mycelia were shortened, with a high degree of branching and rounded tips (Fig. 43d, f and h). These changes contrasted with the long and thin hyphae observed on control cultures (Fig. 43a). However, Tetra extract was not able to produce any morphological changes on the hyphae of *Asp. fumigatus* (Fig. 43c, e, g and i). For this

reason, a Minimum Effective Concentration was only determined to FG extract (Fig. 44).

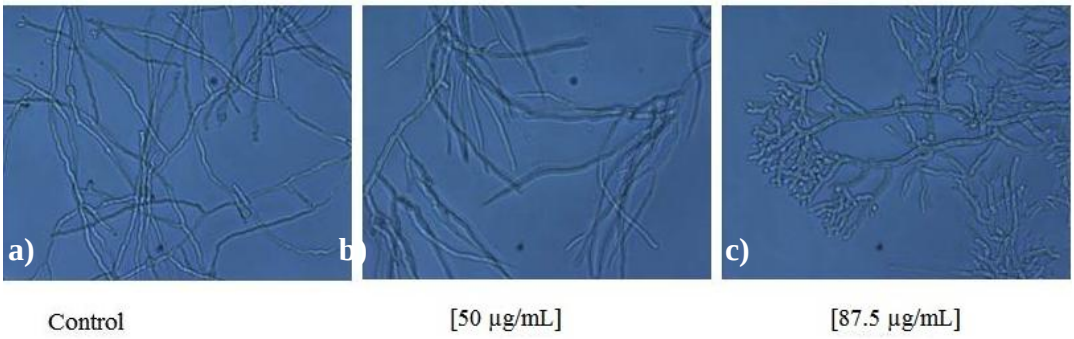

Figure 44 - *Asp. fumigatus* morphology with a) no exposure to any carrageenan extract and exposure to b) 50 µg/mL and c) to 87.5 µg/mL of Tetra extract.

The tested concentrations of FG extract ranged between 10 and 100 µg/mL. No significant changes were observed after 48h exposure to 50 µg/mL of extract (Fig. 44b). However, upon exposure to 87.5 µg/mL hyphae exhibited the same alterations described previously (rounded tips, high degree of branching) (Fig. 44c).

3.4.1.1.c *Candida albicans*

The microdilution broth assay revealed that carrageenan extracts did not prevent the growth of *C. albicans* in vitro and neither induced any morphological change.

3.4.1.2 Determination of the chitin and β-glucan cell wall content

The cell wall chitin and β-glucan contents of *A. infectoria* and *Asp. fumigatus* were determined in order to evaluate the impact of FG and Tetra extracts on the modulation of these cell wall components.

3.4.1.2.a *A. infectoria*

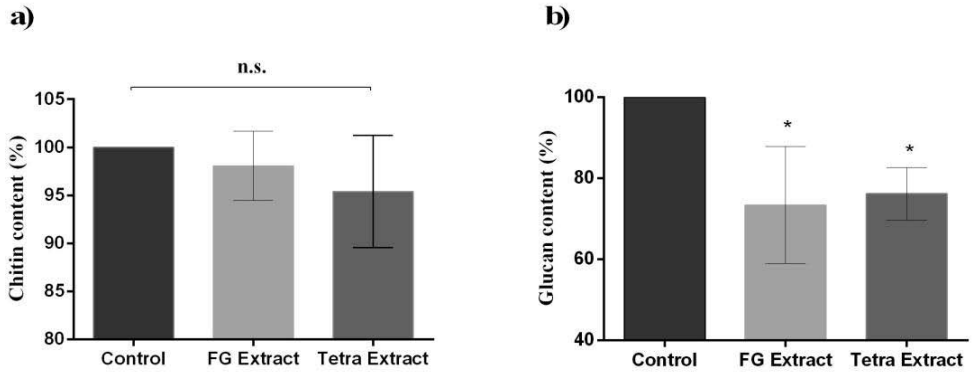

Figure 45 – Impact of FG (150 µg/mL) and Tetra (100 µg/mL) extracts in chitin (a) and β-glucan (b) content of *A. infectoria*. Results represent the mean ± SE of triplicates of two independent experiments (One-way ANOVA; Tukey's test). *, $P < 0.05$; n.s., non significant.

A. infectoria chitin cell wall content decreased slightly but not significantly upon exposure to 150 µg/mL of FG extract and 100 µg/mL of Tetra extract (Fig. 45a). On the other hand, β-glucan quantification revealed a significant decrease on glucan content of *A. infectoria* upon treatment with both extracts (Fig. 45b). A higher decrease in the *A. infectoria* β-glucan level was observed upon treatment with FG extract (73.3%).

3.4.1.2.b *Asp. fumigatus*

Even though only FG extract revealed morphological changes in *Asp. fumigatus*, Tetra extract was also tested for the quantification of chitin and β-glucan content. Both extracts were tested at concentrations of 150 µg/mL.

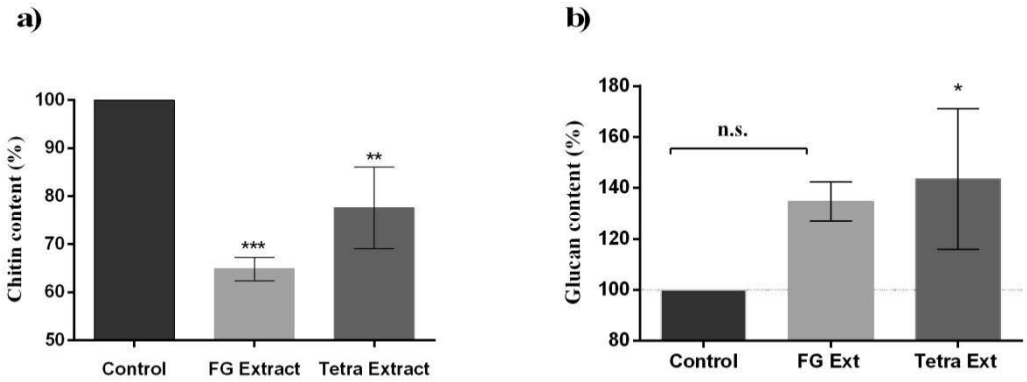

Figure 46 – Impact of FG and Tetra extract (150µg/mL) in chitin (a) and β-glucan (b) content of *Asp. fumigatus*. Results represent the mean ± SE of triplicates of two independent experiments (One-way ANOVA; Tukey's test). *, $P < 0.05$; **, $P < 0.03$; ***, $P < 0.01$.

The *Asp. fumigatus* cell wall chitin content decreased significantly upon treatment with both extracts. This decrease was more pronounced (64.8%) when the fungus was treated with FG extract (Fig. 46a). On the other hand, the β-glucan content increased in the presence of both extracts, although this difference was only statistically significant with Tetra extract treatment (Fig. 46b). Most probably this increase corresponds to a compensatory mechanism of the effect triggered by the extract – a decrease in the fungal cell wall chitin content.

3.4.1.3 Statistical analysis

A statistical analysis was performed using One-way ANOVA test. This test allowed observing if the increase/decrease in chitin and β-glucan cell wall content for both *A. infectoria* and *Asp. fumigatus* was statistically significant. As shown in Fig. 45a, the decrease in the chitin content of *A. infectoria* was not statistically significant. On the other hand, the decrease in glucan content was statistically significant (Fig. 45b). Thus, Tukey's test was done in order to determine if there were differences between treatments. The data obtained allowed to observe that both extracts were statistically significant in relation to the control, but were not statistically significant between each other (see supplemental material V and VI).

The same tests were performed for *Asp. fumigatus*. As shown on Fig. 46a, the decrease in chitin content revealed to be statistically significant. Tukey's test revealed that both extracts were statistically significant when compared to control. On the other hand, Tukey's test revealed that the increase in glucan content (Fig. 46b) was only statistically significant in Tetra extract (see supplemental material VII and VIII).

3.4.2 Antibacterial activity

The microdilution broth assay revealed that carrageenan extracts did not prevent the growth of *E. coli* and *S. aureus*. It was verified that both bacteria were able to grow in the presence of both extracts.

3.4.3 Antiviral activity

3.4.3.1 Cytotoxicity

The cytotoxicity of the extracts was evaluated by incubating each extract at different concentrations with Caco-2 and HEK-293T cell lines. Results were read after 8, 24 and 48 hours of incubation in a spectrophotometer (Fig. 47).

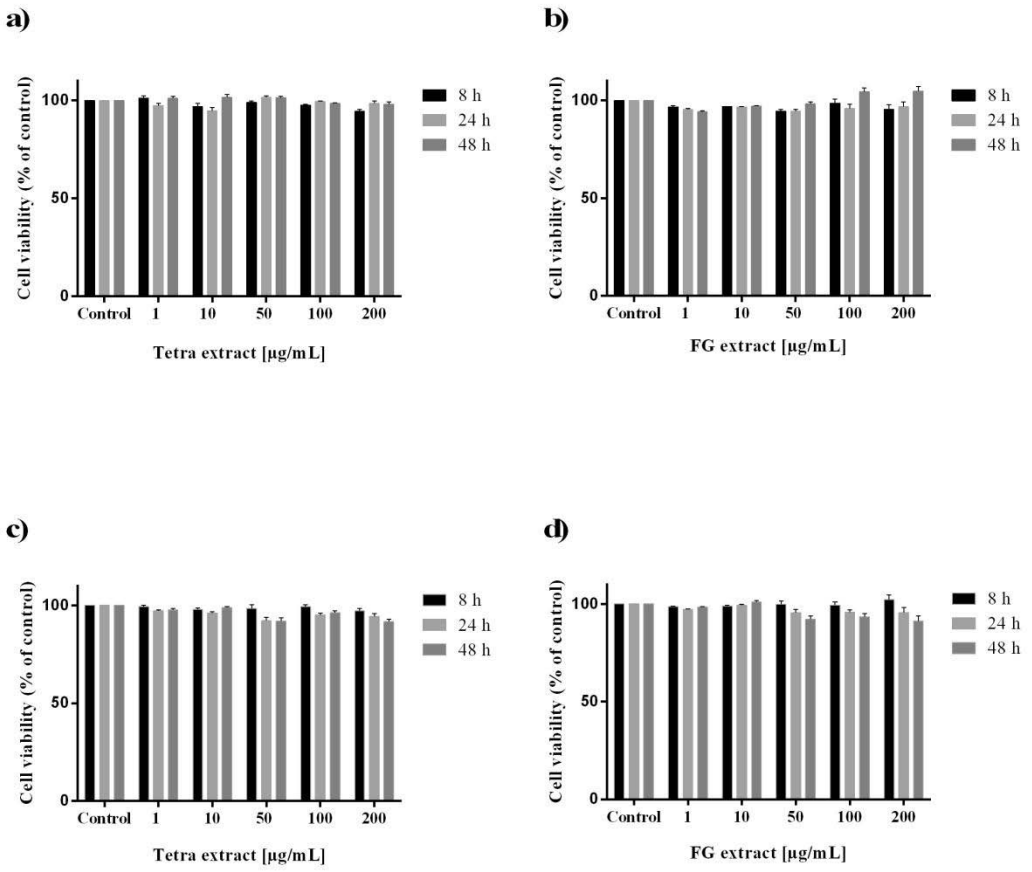

Figure 47 – Cell viability of Caco-2 cell line upon exposure to different concentrations of: a) Tetra and b) FG extract after 8, 24 and 48 hours of incubation; Cell viability of HEK-293T cell line upon exposure to different concentrations of: c) Tetra and d) FG extract after 8, 24 and 48 hours of incubation (Mean ± SE, n = 2).

The cytotoxicity of each extract was evaluated by determining its effect on cell viability. As seen in Fig. 47, none of the extracts caused a reduction below 80% on the cell viability of the two cell lines. Below this value, cell viability would be at risk and extracts would be considered toxic to the cells. For Caco-2 cell line, the lowest value of cell viability was observed upon exposure to 200 µg/mL of FG extract (94.16 ± 0.13%)

after 48 hours of incubation. For HEK-293T cell line, the lowest value was observed upon exposure to 1 µg/mL of FG extract (91.25 ± 2.35%) after 48 hours of incubation.

Since both extracts were dissolved in mili-Q water, its effect on the cell viability of HEK-293T and Caco-2 cells was also determined (Fig. 48 and Fig. 49).

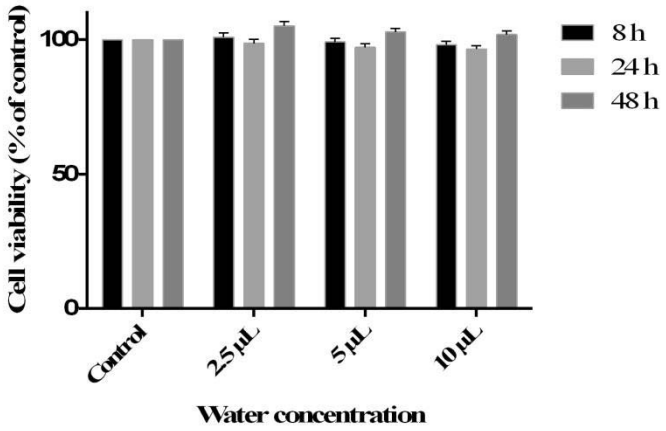

Figure 48 – Cell viability of HEK-293T cell line upon exposure to different concentrations of water (Mean ± SE, n = 4).

As seen in Fig. 48, water did not affect the cell viability of HEK-293T cell line. The lowest value (96.48 ± 1.31%) was observed upon exposure to 10 µL of the solvent after 24 hours of incubation.

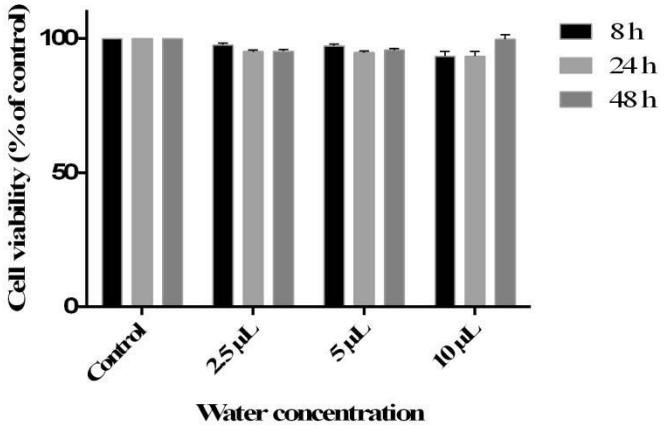

Figure 49 - Cell viability of Caco-2 cell line upon exposure to different concentrations of water (Mean ± SE, n = 2).

As seen in Fig. 49, water did not affect the cell viability of Caco-2 cell line. The lowest value (93.36 ± 1.72 %) was observed upon exposure to 10 µL of the solvent after 48 hours of incubation.

3.4.3.2 Antiviral activity of the carrageenan extracts against Lentivirus

3.4.3.2.a Pre-treatment

The antiviral activity of the carrageenan extracts was evaluated by inoculating 200 µg/mL of FG and Tetra extracts with HEK-293T cells before adding the Lentivirus (pre-treatment) (Fig. 50). Results were evaluated by cell counting using Zen lite program.

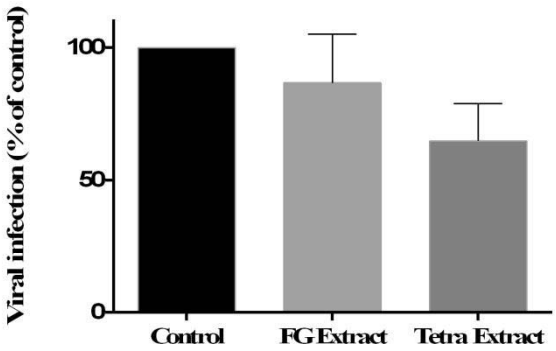

Figure 50 – Effect on Lentivirus infection upon pre-treatment with FG and Tetra extract (200 µg/mL). Results represent the mean ± SE of one independent experiment done in duplicate.

The impact of the pre-treatment with FG and Tetra extracts revealed that both extracts exhibited a decrease in the percentage of viral infection of Lentivirus. Although the impact was not statistically significant (P>0.05) (see supplemental material IX), Tetra extract showed the lowest value of viral infection when compared with control (64.74 ± 7.1 %). On the other hand, FG extract revealed a value of viral infection of 86.63 ± 9.2 % (Fig. 50).

3.4.3.2.b Virucidal assay

The virucidal assay was done by incubating the Lentivirus and the carrageenan extracts at the same time and infecting HEK-293T cells with this mixture (Fig. 51). Results were analyzed by cell counting using Zen lite program.

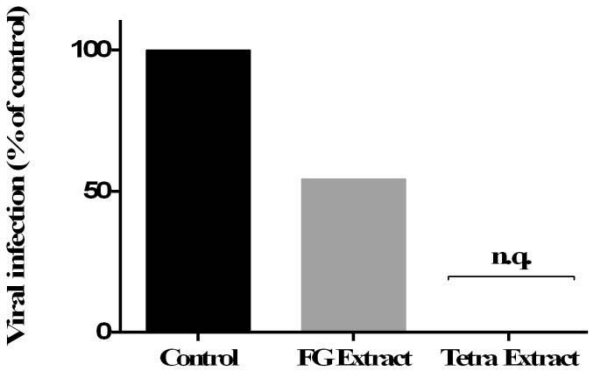

Figure 51 - Effect on Lentivirus infection upon virucidal treatment with FG and Tetra extract (200 µg/mL). Preliminary results of one independent experiment (n = 1). n.q.: Non quantifiable.

The preliminary data obtained revealed that only FG extract was able to reduce the Lentivirus infection (54.34 %). Tetra extract was not quantifiable since it did not allow the adhesion of HEK-293T cells to wells. Therefore, it was not possible to do cell counting and quantification.

3.4.3.3 Antiviral activity of the carrageenan extracts against Coxsackie virus A-12

3.4.3.3.a Pre-treatment

The antiviral activity of the carrageenan extracts was evaluated by inoculating 200 µg/mL of FG and Tetra extracts with Caco-2 cells before adding the Coxsackie virus A-12 (pre-treatment). Results were evaluated by quantitative Real-Time PCR and standardized by the C_T method, also referred to as the $2^{-\Delta\Delta CT}$ method, using the 18S gene. This method only provided a relative quantification of the results (Fig. 52).

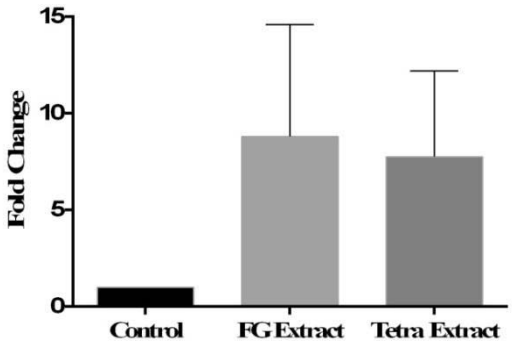

Figure 52 – Effect on Coxsackie virus A-12 infection upon pre-treatment with FG and Tetra extract (200 µg/mL). Results represent the mean ± SE of two independent experiment done in duplicate.

The data obtained revealed that none of the extracts were able to inhibit the viral infection in the pre-treatment. Results showed that the fold change in expression between the FG extract and the control was 8.8 ± 4.1, and the fold change in expression between the Tetra extract and the control was 7.75 ± 4.4 (Fig. 52).

3.4.3.3.b Virucidal assay

The virucidal assay was done by incubating the Coxsackie virus and the carrageenan extracts at the same time and infecting Caco-2 cells with this mixture. Results were analyzed by quantitative Real-Time PCR and standardized by C_T method (Fig. 53).

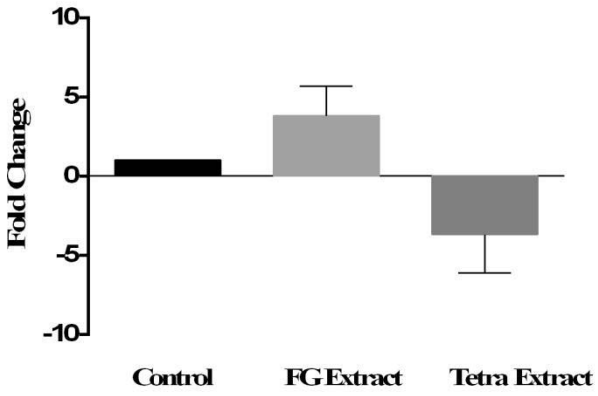

Figure 53 – Effect on Coxsackie virus infection upon virucidal treatment with FG and Tetra extract (200 µg/mL). Results represent the mean ± SE of two independent experiments done in duplicate.

The results obtained allowed to observe that FG extract was not able to inhibit the infection of Coxsackie virus. The fold change between this extract and the control was 3.8 ± 1.9. On the other hand, Tetra extract revealed to inhibit the infection of the virus. The fold change between this extract and the control was -3.7 ± 2.4 (Fig. 53).

3.4.3.3.c Post-treatment

The post-treatment was done by adding the carrageenan extracts after infection of the Caco-2 cells with Coxsackie A-12 virus. Results were also analyzed by Real-Time PCR and standardized by the C_T method (Fig. 54).

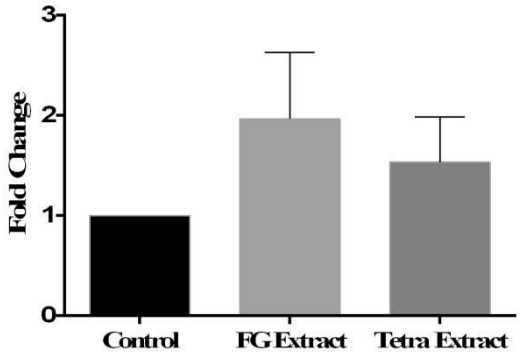

Figure 54 – Effect on the Coxsackie virus A-12 upon post-treatment infection with FG and Tetra extracts (200 µg/mL**).** Results represent the mean ± SE of two independent experiments done in duplicate.

The data obtained allowed to observe that none of the extracts exhibited activity against Coxsackie virus A-12. The fold change between the FG extract and the control was 1.9 ± 0.6 and the fold change between Tetra extract and the control was 1.5 ± 0.4.

Chapter IV - Discussion

4.1. Cultivation of Chondracanthus teedei var. lusitanicus

4.1.1 Laboratory culture

The cultivation of *C. teedei* var. *lusitanicus* through vegetative propagation revealed to be mostly unsuccessful. Over the first week of acclimation, this species started losing biomass and its color became green due to its deterioration. In the following weeks of cultivation, some of the ramifications were able to recover its color and started gaining biomass. However, this was only visible when this species was growing at lower cultivation densities (1 g L^{-1} and 2 g L^{-1}). At higher cultivation densities (4 g L^{-1} and 6 g L^{-1}) this species lost almost one third of its initial weight and was not capable of recovering it throughout the experiment. For this reason, the relative growth rates and productivity calculated for this species revealed to be higher at cultivation densities of 1 g L^{-1} and 2 g L^{-1} than at cultivation densities of 4 g L^{-1} and 6 g L^{-1}. For both relative growth rate and productivity, cultivation density 2 g L^{-1} revealed to be the one with better results and, therefore, this is most probably the optimal cultivation density for this species to grown on laboratory.

The reasons why *C. teedei* var. *lusitanicus* was so difficult to maintain at laboratory are probably due to the cultivation conditions to which it was exposed during the experiment, such as light irradiance, nutrient content and temperature. Although the initial light irradiance was determined to be 100 μE m^{-2} s^{-1}, the flasks containing the algae were only exposed to approximately 45 μE m^{-2} s^{-1}. Since light intensity is important to the photosynthesis process, a lower light irradiance decreases its rates. As this species was exposed to low light irradiance, most probably it was not able to perform photosynthesis correctly and, therefore, its respiratory rates became higher than its photosynthesis rates. This led to an abnormal decrease in the values of pH in the first week after the acclimation week, along with a loss in the biomass of this species, which was noticeable at all cultivation densities. The nutrient content of the medium and the temperature were also important limiting factors, since it was observable that *C. teedei* var. *lusitanicus* growth better when its nutrient content was limited (data not shown), and when exposed to higher temperatures.

According to Braga (1990), *Chondracanthus teedei* (formerly *Gigartina teedii*) collected in Brazil, was capable of reproducing itself by vegetative propagation when cultures were maintained under 50 to 90 μE m^{-2} s^{-1} of light intensity and PES medium

(Provasoli's Enriched Seawater) was changed every 15 days. In the same study, this species was able to develop better at 22 °C and 26 °C. When growing at 14 °C and 30 °C, this species ended up dying. Moreover, according to this author, under culture conditions, all plants with a minimum age of 2.0 —3.5 months and height > 0.6 cm, are able to become fertile if growing at temperatures between 18 °C and 26 °C (± 2 °C) and day length between 10:14h (light: dark) and 16:8 h (light: dark).

According to Guiry (1984), cultured isolates of *Chondracanthus teedei* (formerly *Gigartina teedii*) from England and from France started to reproduce after about the same time at 20 °C, 16:8 h; 15°C, 16:8 h; and 15 °C, 8:16 h, with half-strength PES medium. However, the morphology of the plants at the times of reproduction varied according to the regime. Plants grown at 15 °C, 8:16 h, were shorter and less branched than those grown at 15°C, 16:8 h, whilst plants grown at 20°C, 16:8 h, were less flattened, longer and more irregularly branched at the time of reproduction than those at 15 °C, 16:8h. A study performed by the same author (1987) shows that there were differences in tip elongation rates between *Chondracanthus teedei* strains. Strains from the Mediterranean grow 2-3 times faster at 15 °C, 16:8 h than Atlantic strains, which achieve an optimal growth at temperatures different than 15 °C. The author also reported that a very important feature of this species and other Gigartinaceae in culture is that crowded cultures tend to be non-fertile or show reduced reproduction. This feature might explain why *C. teedei* var. *lusitanicus* was mostly unsuccessful to grow at cultivation densities of 4 g L^{-1} and 6 g L^{-1}.

According to Bastos (2013), cultures of *Chondracanthus teedei* from Brazil growth better when maintained at 25 °C, 12:12 h (light: dark), with half-strength VSE medium changed every 7 days, and light intensity of 40 μE m^{-2} s^{-1}.

All studies mentioned before showed better results than those obtained in this work. Differences were substantially due to light intensity, higher temperatures and to a different medium (VSE *vs.* PES), used at different concentrations (half-strength *vs.* full-strength) and changed less frequently (3-4 days *vs.* 7-15 days).

It is important to refer that, to our knowledge, there is no data evidencing the cultivation of *Chondracanthus teedei* var. *lusitanicus* both at laboratory and at an IMTA system until this present moment.

4.1.2 IMTA culture

The cultivation of *C. teedei* var. *lusitanicus* in an IMTA system exhibited better results than that obtained at laboratory. In the first week after the acclimation week, there was an increase in the biomass of this species at all cultivation densities. However, from the first to the second week there was a decrease in its values, which were recovered in the following week. The same week variation was registered to relative growth rate and productivity calculated for this species. Thus, cultivation densities of 8 g L^{-1} and 6 g L^{-1} achieved higher relative growth rates and productivity values than that achieved at cultivation density 4 g L^{-1}. As shown in Fig 36, cultivation density 6 g L^{-1} revealed to be the one with better results and, therefore, this is most probably the optimal cultivation density for this species to grow in an IMTA system.

The decrease in biomass registered in the second week was due to excess biomass that was not removed from the previous week. As said previously, this species and other Gigartinaceae in crowded cultures tend to be non-fertile or show reduced reproduction (Guiry, 1984). Thus, the excess biomass allied to the size and conical shape of the tanks caused the deposit of this species at its bottom and, therefore, some of the samples were not able to capture sunlight, which is essential for its growth. However, it was noticeable that, when this species was growing in a round maintenance tank (100 L m^{-2}), where nutrients from fish effluent were firstly filtered by another tank containing *Ulva* sp. (Chlorophyta), it was able to gain biomass and maintain itself for more than a month without any special treatment. This means that *Ulva* sp. captured most part of the nutrients and that *C. teedei* var. *lusitanicus* is able to grow in medium not very rich in nutrients (data not shown).

By the end of the experience it was noticeable the presence of epiphytes, such as *Ulva* sp. and *Ceramium* sp. (Rhodophyta), which can decrease the host's growth and reproduction by limiting carbon uptake and reducing light penetration (Kraberg & Norton, 2007). And although there was a general increase in the biomass of this species from the second to the third week, some replicates showed loss of weight and exhibited a green color. This can be explained by the presence of these epiphytes.

As said previously, there is no data referring to the cultivation of *C. teedei* var. *lusitanicus* in an IMTA system, nevertheless, other carrageenophytes were already tested in this type of cultivation, such as the red algae *Mastocarpus stellatus*, *Chondrus crispus*, *Gracilaria vermiculophylla and Palmaria palmata*. In a study performed by

Domingues *et al*. (2014), *M. stellatus* reported values of productivity of 20.95 ± 3.29 (g (dw) m^{-2} day^{-1}) in spring, which were clearly larger when compared to *C. crispus* (8.4 ± 2.60 g (dw) m^{-2} day^{-1}) (Matos *et al*., 2006), but yet comparable to the ones obtained by Abreu *et al*. (2011) for *G. vermiculophylla* (23.3 ± 1.67 (g (dw) m^{-2} day^{-1})). However, in the same study, *M. stellatus* reported values of productivity of 40.6 ± 2.4 (g (dw) m^{-2} day^{-1}) in early summer (Domingues *et al*., 2014), which were comparable to those reported by Matos *et al*. (2006) for *G. vermiculophylla* (identified then as *G. bursa pastoris*) (31.2 ± 7.80 (g (dw) m^{-2} day^{-1})), *C. crispus* (36.6 ± 11.10 (g (dw) m^{-2} day^{-1})) and *Palmaria palmata* (40.2 ± 12.8 g (dw) m^{-2} day^{-1})). When compared to our results, it is evident that *C. teedei* var. *lusitanicus* exhibits values of productivity lower than the ones reported previously for other carrageenophytes. However, more studies must be performed before excluding the utilization of this alga in an IMTA system.

4.2 Carrageenan extraction

4.2.1 Determination of dry weight and carrageenan content

The alkali extractions performed in this study allowed to observe that the carrageenan content of *C. teedei* var. *lusitanicus* collected in February 2015 in Buarcos Bay, varied according to its different lifecycle phases. Thus, a higher percentage of carrageenan content was found in the tetrasporophyte phase (38 ± 1.1%) when compared to that obtained in the female gametophyte phase (33 ± 0.01%) (see Table 5). These results are below the ones reported for samples collect in Brazil (76%, Saito & Oliveira, 1990) and France (70% Zinoun, 1993; Zinoun *et al*., 1993a,b).

The biological precursors (mu and nu) of gelling carrageenans (kappa and iota-carrageenan) contain a sulfate ester group on the C_6 of the 4-linked α-D-galactose. This type of structure is responsible for the reduction of the ability of carrageenans to form a gel, due to the blockage of the sequence of repeating units of carrabiose, which are responsible for the formation of the double helix structures characteristics of gelling carrageenans. Most of the 6-sulfated units are converted into the 3,6 anhydro units after a long (30 to 48 hours) and highly alkali process (usually 0.1 M $CaCOH_2$). At an industrial level, the extraction processes used, aim to increase the profitability and to increment the gelling force of carrageenans. Alternatively, a less percentage of precursor 6-sulfated units are converted into the correspondent 3,6 anhydro units when

extraction processes take shorter periods of time (2-4 hours) and are less alkali (0.02 to 0.1 M NaOH), as the one used in this study. With this type of extraction process it is possible to extract carrageenans still containing its correspondent biological precursors. The mildly alkali extraction process is used in industry when the aim is to obtain carrageenans soluble at cold temperatures and/or with gelling capacity (Falshaw *et al*., 2003).

The comparison of the results obtained here with those reported by Pereira & Mesquita (2004), which studied the variation in dry weight and carrageenan content of *C. teedei* var. *lusitanicus* collected in Buarcos Bay, showed a difference, since the authors found that cystocarpic plants had the highest yield and non-fructified thalli plants had the lowest. Moreover, the study shows an average of the content in carrageenan of 37.9 ± 1.5 % (n = 15) in the female gametophytes and 35.4 ± 2.1% (n = 15) in the tetrasporophytes. These differences might probably be due to different seasonalities and different number of replicates, and to the fact that the authors did an annual study about this alga.

In relation to the dry matter of this species (see Table 4), expressed as percentage of fresh weight, it varied between 18 and 19%, with an average of 19 ± 0.002%. These results are similar to those reported by Pereira & Mesquita (2004), which revealed to be 17.5 ± 0.8% (n = 3) in December 2000.

4.3 Carrageenan analysis by FTIR-ATR

As seen on section 2.6, the characterization of the polysaccharides content can be analyzed directly from the ground dried seaweed by FTIR-ATR vibrational spectroscopy (Pereira & Mesquita, 2004).

The study of the carrageenans by vibrational spectroscopy (FTIR-ATR and FT-Raman) allowed determining the diagnostic bands for each of the main type of carrageenans (Pereira, 2004a). Thus, all carrageenan spectra present strong bands at 1210-1260 cm^{-1}, which corresponds to the S=O of sulfate esters (except for beta carrageenan) and strong bands at 1010-1080 cm^{-1}, which corresponds to the glycoside bond common to all carrageenans (Pereira *et al*., 2003). The other chemical groups are characteristic of each type of carrageenan: 3,6-anhydrogalactose (DA) at 925-935 cm^{-1};

galactose-4-sulfate (G4S) at 845-850 cm^{-1}; galactose-2-sulfate (G/D2S) at 820-830 cm^{-1}; galactose-6-sulfate (G/D6S) at 810-820 cm^{-1} and 3,6-anhydrogalactose-2-sulfate (DA2S) at 805 cm^{-1} (see Table 1 and Table 2) (Roberts & Quemener, 1999; Pereira *et al*., 2003; Pereira et al., 2015).

The spectroscopic analysis of the spectra relative to non-fructified thalli and female gametophyte obtained in this study shows the presence of strong bands at 930 and 845 cm^{-1}, characteristics of kappa-carrageenan, and a medium band at 805 cm^{-1}, characteristic of iota-carrageenan. The presence of these three peaks is typical of kappa/iota hybrid carrageenan. These results are in accordance with those presented for Zinoun (1993), Zinoun *et al*., (1993b) and Pereira & Mesquita (2004), which reported that *C. teedei* and *C. teedei* var. *lusitanicus* gametophytes, respectively, produced kappa/iota hybrid carrageenan. On the other hand, the spectrum relative to the tetrasporophyte phase shows the presence of a sharper peak at 830 cm^{-1} and a medium band at 820 cm^{-1}, which indicates the presence of xi carrageenan.

The data obtained in this study is in agreement with that showed by Chopin (1999) and Pereira & Mesquita (2004), which demonstrated that the two phases in the life cycle of *C. teedei* var. *lusitanicus* seem to present a variation similar to that existing in other species of *Chondracanthus* genus: the gametophytes stages produce carrageenans of the kappa family (hybrid kappa/iota/mu/nu carrageenan), while the tetrasporophyte stages produce carrageenans of lambda family (hybrid xi/tetha carrageenan).

The results obtained in this study are also in accordance with that stated by McCandless *et al*. (1982, 1983), who studied the carrageenan patterns in the Phyllophoraceae and the Gigartinaceae, respectively. In the Phyllophoraceae, gametophytes produce iota or iota/kappa carrageenan hybrids, while in the Gigartinaceae they produce kappa or kappa/iota carrageenan hybrids. In both families, tetrasporophytes produce lambda carrageenan.

4.4 Bioactivity assays

4.4.1 Antifungal activity of the carrageenan extracts

The antifungal activity of the carrageenan extracts of *Chondracanthus teedei* var. *lusitanicus* belonging to the female gametophyte (FG) and tetrasporophyte (Tetra) life cycle phases was evaluated by microdilution broth assay, with determination of Minimum Effective Concentrations (MEC), against *Alternaria infectoria* and *Aspergillus fumigatus*. With respect to *A. infectoria*, both extracts have proved to cause an alteration on the hyphal morphology of this fungus, causing the formation of balloon-like structures. The FG extract caused this alteration at concentrations of 125 µg/mL and the Tetra extract at concentrations of 60 µg/mL. In what regards to *Asp. fumigatus*, only one extract (FG extract) have proved to cause an alteration on its hyphae morphology. Thus, *Asp. fumigatus* hyphae became shortened and with a high degree of branching after exposure to 87.5 µg/mL of FG extract.

Until the present moment, there is no data evidencing the activity of carrageenan extracts from *C. teedei* var. *lusitanicus* against fungi. However, there is data evidencing the activity of other seaweeds against *Alternaria* and *Aspergillus* species. In fact, Galal *et al*., (2011) reported that crude ethyl acetate extract of *Padina gymnospora* (Ochrophyta) and methanolic extract of *Codium fragile* (Chlorophyta) exhibited strong activity against most of the tested fungi including *Fusarium oxysporum*, *Alternaria alternata*, and *Alternaria brassicicola*. In 2015, Khallil *et al*., studied the antifungal potential of five brown seaweeds against eight fungal species. These authors reported that cyclohexanic extracts of *Sargassum vulgar*, *Cystoseira barbata* and *Colpomenia sinuosa* showed antifungal activity against *A. alternata*.

As mentioned in section “Introduction”, del Val (2001) reported the activity of six seaweeds - *Asparagopsis taxiformis* (Rhodophyta), *Cymopolia barbata* (Chlorophyta), *Caulerpa prolifera* (Chlorophyta), *Dictyota* sp. (Phaeophyta), *Enteromorpha muscoides* (Chlorophyta) and *Osmundea hybrid* (Rhodophyta) - which presented activity against the filamentous fungi *Asp. fumigatus*. Another study performed by Genovese *et al*., (2012), reported the activity of *Asparagopsis taxiformis* against *Aspergillus* species, such as *Asp. fumigatus, Aspergillus terreus* and *Aspergillus flavus*. It is important to refer that these studies were conducted using methanol extracts and not carrageenan extracts.

Although there is no evidence of previous studies reporting that seaweed extracts can cause alteration on the morphology of *A. infectoria*, the results obtained in this study revealed morphological alterations similar to those reported by Fernandes and co-authors (2014) using antifungal drugs. The authors reported that, upon exposure to the antifungal nikkomycin Z (2 µg/mL), *A. infectoria* suffered structure abnormalities in its hyphae, such as the formation of swollen, balloon-like cells. These abnormalities were not evident upon exposure to caspofungin, however, significant structural alterations were observed during hyphal development from the onset of spore germination, where short, stubby and highly branched hyphae were observed. Authors also described that the frequency of cell swelling upon exposure to nikkomycin Z was higher near the germinated spore and decreased as hyphal growth progressed, which suggested the existence of an adaptive mechanism that might progressively increase drug resistance upon hyphal growth. The data obtained in this study, using seaweed extracts revealed that the frequency of the cell swelling was, sometimes, higher near the germinated spores, but in some cases it was also evident towards the middle of the hyphae (Fig. 39 h and i).

The results obtained here with *Asp. fumigatus* showed morphological changes similar to the ones observed by Verwer *et al*., (2011), who reported that when *Asp. fumigatus* was exposed to 4 µg/mL of caspofungin, its hyphae became short, stubby and highly branched. These results are also similar to those described by Bowman *et al*., (2002), except that no cell lysis was detected at the hyphal tips.

Hereupon, it can be shown that both carrageenan extracts of *C. teedei* var. *lusitanicus* revealed to have similar morphological effects as the ones induced by caspofungin and nikkomycin Z.

In what regards to *C. albicans*, results obtained in this study revealed that none of the carrageenan extracts prevented its growth or induced any morphological alteration. However, there is data evidencing the activity of seaweed extracts against this fungus. In fact, del Val *et al*., (2001) reported the activity of methanol extracts of *Asparagopsis taxiformis* (Rhodophyta) and *Cymopolia barbata* (Chlorophyta), which were effective against *C. albicans*. Ballantine *et al*., (1987) also reported the activity of *Asparagopsis taxiformis* against *C. albicans*.

In 2011, Stein *et al*. studied the activity of five species of the red alga *Laurencia* (Rhodophyta) against *C. albicans*, *Candida parapsilosis* and *Cryptococcus neoformans*.

Chloroform and methanol extracts of *Laurencia dendroidea* showed MIC values of < 31.25 μg/mL against *C. albicans*. The chloroform extract of *Laurencia catarinensis* proved to have the same effect against *C. parapsilosis*. The methanol extract of *Laurencia aldingensis* showed MIC values of < 31.25 μg/mL against *C. parapsilosis* and 65.2 μg/mL against *C. albicans*. The hexane and chloroform extracts of this species against *C. parapsilosis* were indicated by MIC values of 49.0 and 57.8 μg/mL, respectively.

Since carrageenan extracts of *C. teedei* var. *lusitanicus* did not show any antifungal activity against *C. albicans*, this might be due to the use of different strains of *C. albicans* and to the fact that the extracts and methods used by others revealed to be different from the ones used in this study.

4.4.2 Quantification of chitin and β-glucan

Since the carrageenan extracts studied here proved to induce morphological changes in *A. infectoria* and *Asp. fumigatus*, this could indicate that, most probably, these alterations occur in the cell wall of this fungi. So, the chitin and β-glucan contents of *A. infectoria* and *Asp. fumigatus* were determined in order to evaluate the impact of the FG and Tetra extracts on the modulation of these cell wall components.

The data obtained in this study revealed that *A. infectoria* chitin cell wall content decreased slightly but not significantly upon exposure to 150 μg/mL of FG extract and 100 μg/mL of Tetra extract (Fig. 44a). On the other hand, β-glucan quantification revealed a significant decrease on glucan content of *A. infectoria* upon treatment with both extracts. A higher decrease was observed upon treatment with FG extract (73.3%) (Fig. 44b).

In relation to *Asp. fumigatus*, results obtained showed that *Asp. fumigatus* cell wall chitin content decreased significantly upon treatment with 150 μg/mL of both extracts, and this decreased revealed to be more pronounced (64.8%) when the fungus was treated with FG extract (Fig. 45a). On the other hand, the β-glucan content increased in the presence of both extracts, although this difference was only statistically significant with Tetra extract treatment. Most probably this increase corresponds to a compensatory mechanism of the effect triggered by the extract – a decrease in the fungal cell wall chitin content.

To date, there is no evidence of the activity of red seaweed extracts on the modulation of these cell wall components. However, one study with phlorotannins from brown algae revealed its impact on these cell wall components (Lopes, 2014). In this study, *Fucus spiralis* purified phlorotannins extract significantly reduced the amount of chitin in the dermatophyte *Trichophyton rubrum*. However, none of the tested phlorotannins extracts affected the β-glucan content of the studied microorganisms (Lopes, 2014).

The data obtained in this study regarding the modulation of *A. infectoria* chitin and β-glucan cell wall components revealed to be in agreement with those described by Fernandes and co-authors (2014). The authors reported that *A. infectoria* β-glucan synthesis was inhibited upon treatment with 1 µg/mL of caspofungin. However, chitin levels upon the same treatment remained almost unchanged.

The results obtained in the present study regarding the decrease in *Asp. fumigatus* chitin content and the increase in β-glucan content upon exposure to both extracts are very similar to those described by Verwer *et al*., (2011). The authors reported the impact of the antifungals caspofungin and nikkomycin Z on the modulation of *Asp. fumigatus* cell wall chitin and β-glucan content. They stated that upon exposure to nikkomycin Z, chitin concentrations decreased when a concentration of 16 µg/mL or greater was used, while β-glucan concentrations increased when a concentration of 0.5 µg/mL or greater was used. The opposite occurred when *Asp. fumigatus* was exposed to caspofungin, which caused an increase in chitin and decrease in β-glucan content. Authors suggested that the inhibition of the synthesis of a single cell wall component (either chitin or β-glucan) results in a subsequent increase in synthesis of the other cell wall component. This is the result of a compensatory mechanism and would explain the altered morphology after exposure to FG and Tetra extracts.

4.4.3 Antibacterial activity

The results obtained in this study revealed that none of the extracts was capable of inhibiting the growth of *E. coli* and *S. aureus*. However, there is data evidencing the effect of seaweed extracts against these and other bacteria. As reported by Shanmughapriya *et al*. (2008) the extracts from *Gracilaria corticata* were found to be

effective against *P. aeruginosa*, *E. coli*, *Micrococcus luteus*, *S. epidermidis* and *E. faecalis*.

In 2014, Sebaaly *et al*. reported that the lambda-carrageenan isolated from the red alga *Corallina sp*. exhibited antibacterial activity against *Staphylococcus epidermis*.

The differences between the results reported by Shanmughapriya and collaborators (2008) and the ones obtained in this study may be related to different seaweed extracts (methanol *vs*. carrageenans), different bacteria strains and species (*S. epidermis* vs. *S. aureus*). The differences between the results described by Sebaaly and collaborators (2014) and the results obtained in this study might be due to the use of different bacteria strains, different carrageenan extractions (aqueous *vs*. alkali) and to the fact that seaweeds were collected from different places (Asia *vs.* Portugal) at different times of the year (April-July *vs*. February).

4.4.4 Antiviral activity

4.4.4.1 Cytotoxicity assay

The cytotoxicity assay, evaluated by the ALAMAR blue method, revealed that none of the carrageenan extracts were toxic to HEK-293T and Caco-2 cell lines.

These results are in agreement with those described by Matsuhiro *et al*., (2005), who reported that the carrageenan extracts from *Schizymenia binderi* (Gigartinales, Rhodophyta) did not show any cytotoxic effects on Vero cells viability. The results are also in accordance with those described by Bouhlal *et al*., (2011) who reported that viability assay showed no destruction of cell layer.

4.4.4.2 Antiviral activity of the carrageenan extracts against Lentivirus

4.4.4.2.a Pre-treatment

The data obtained in this study revealed that the viral infection by Lentivirus was reduced upon exposure to a pre-treatment with FG and Tetra extracts. Although the inhibitions were not statistically significant, FG extract was able to reduce 14 % of the

virus infection, and the Tetra extract was able to reduce, approximately, 35 % of the virus infection.

Damonte *et al*., (2004) reported that marine polysaccharides can either inhibit the replication of virus by interfering with its viral life cycle (viral adsorption, viral penetration, uncoating of capsids, biosynthesis, viral assembly and viral release) at different stages or directly inactivate virions before virus infection.

The results relative to the effects of the pre-treatment with FG and Tetra extracts might be explained by the interference of these extracts with the adsorption process of the virus. In fact, Carlucci *et al*. (1997; 1999) noted that lambda-carrageenan (which is the same carrageenan type found in Tetra extract), and partially cyclized mu/iota-carrageenan from *Gigartina skottsbergii* (Rhodophyta) showed potent antiviral effects against different strains of HSV types 1 and 2 during the virus adsorption stage. They subsequently confirmed the firm binding of carrageenan to virus receptors on the host cell surface, and demonstrated that lambda-carrageenan interferes with the adsorption process of the virus to the host cell surfaces.

Furthermore, Mazumder *et al*. (2002) described that a sulfated galactan extracted from a red alga showed antiviral activity against *Herpes simplex virus* 1 and 2, which was likely due to an inhibition of the initial viral attachment to the host cells.

Moreover, Buck *et al*. (2006) found that carrageenan could directly bind to the HPV capsid, so as to inhibit not only the viral adsorption process but also the subsequent entry and uncoating process of the virus.

4.4.4.2.b Virucidal effect

Although preliminary, the data obtained in this study showed that the FG extract proved to have a virucidal effect on Lentivirus. These results might be explained by the interference of these extracts in the multiplication of the virus. In fact, Damonte *et al*., (2004) reported that the direct virucidal actions of carrageenan may be due to the formation of a stable virion–carrageenan complex where binding is not reversible and, therefore, the sites on the viral envelope required for virus attachment to host cells are occupied by the sulfated polysaccharide. This results in an inability of the virus to complete the subsequent infection process.

Several studies showed that carrageenans have a direct virucidal action on some enveloped viruses, which makes the viruses lose the ability to infect cells, thus effectively reducing the virus multiplication. Carlucci *et al*. (2002) found that lambda-type carrageenan could firmly bind to the *Herpes simplex virus* (HSV), leading to the inactivation of the HSV virion, thus inhibiting the replication of HSV. Their studies further suggest that carrageenan changes the structure of the glycoproteins gB and gC of HSV (Carlucci *et al*., 1999; Carlucci *et al*., 2002).

Moreover, Talarico and co-workers (2007) reported that lambda and iota-carrageenans could interfere with both Dengue virus type 2 (DENV-2) adsorption and internalization into host cells and that they are only effective if added together with the virus or shortly after infection. And although DENV can enter into host cell in the presence of carrageenans, the mechanism of this inhibition action may be due to their interference in the uncoating process and releasing from endosomes.

In what regards to the Tetra extract, its quantification was not possible due to a technical limitation, i. e. the inability of the infected HEK-293T cells to bind to the microplate.

4.4.4.3 Antiviral activity of the carrageenan extracts against Coxsackie virus A-12

Although preliminary, the data obtained in this study showed that none of the extracts was able to reduce the viral infection in the pre and post-treatment. One can speculate that this might be due to the inability of the extracts to interfere with the virus adsorption to the host cells and to its further replication. The results obtained here differ from those described by Chiu and collaborators (2012), which reported that kappa carrageenan showed a strong and effective anti-EV 71 (Enterovirus-71) activity, and was able to reduce plaque formation and prevent viral replication before or during viral adsorption. In virus binding assay, kappa carrageenan showed the ability to bind to EV 71 firmly, forming carrageenan-viruses complexes, leading to the disruption of the virus-receptor interaction. These differences might be due to different virus subtypes used (EV-71 vs. Coxsackie virus A-12) and different antiviral assays.

The results obtained in our study were evaluated by Real-Time RT-PCR and standardized by comparative C_T method ($2\text{-}\Delta\Delta C_T$), using the 18S gene as reference

gene (Schmittgen & Livak, 2008). This method was used in order to allow a uniformity of the results and, therefore, this only provides a relative analysis of the Real-Time RT-PCR data. The quantitative endpoint for real-time qPCR (quantitative real-time PCR) is the threshold cycle (C_T). The C_T is defined as the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold. Advantages of the comparative C_T method include ease of use and the ability to present data as 'fold change' in template. Disadvantages of the comparative C_T method include the assumptions of PCR efficiency must hold or the PCR must be further optimized.

Although the C_T method only provides a relative quantification, the results presented here regarding the virucidal effect, showed that the Tetra extract was able to decrease almost 4 fold the viral infection. This result is in accordance with that described by Eccles and collaborators (2010) which described the antiviral efficacy of the iota carrageenan in volunteers with early symptoms of the common cold. The study revealed that viral load in the placebo group increased almost 6- fold (579%), while it dramatically decreased by 92% in the iota-carrageenan group. The result indicated that the treatment of patients with iota carrageenan nasal spray leads to a highly statistically significant reduction of viral load in the nasal cavity, while placebo treatment has no influence on viral replication at all.

Chapter V – Conclusions and Future Perspectives

5.1 - Conclusions and Future Perspectives

The data obtained in this study is the first one referring to the antifungal, antibacterial and antiviral activity of *Chondracanthus teedei* var. *lusitanicus*. It is also the first one regarding its cultivation in an IMTA system.

The present study allowed to understand that this species reveals to be difficult to maintain itself through vegetative reproduction, both at laboratory and at an IMTA system. It requires special cultivation conditions, such as light, temperature and nutrient content.

Due to its high carrageenan content and its predominance in Portugal and Galiza, *C. teedei* var. *lusitanicus* can constitute a potential industrial source of carrageenans. Since there is a risk of over-harvesting its populations that may lead to its loss, more studies are needed before discarding this alga from its utilization in an IMTA system.

In what regards to the antifungal activity, the carrageenan extracts of this species revealed to have an impact on hyphal morphology of *A. infectoria*, causing the formation of balloon-like structures similar to those induced by nikkomycin Z, and *Asp. fumigatus*, causing the induction of shortened and highly branched hyphae similar to that observed upon exposure to caspofungin.

The alterations on hyphal morphology of both fungi led to the study of the impact of both carrageenan extracts on chitin and β-glucan cell wall content. It was concluded that both extracts were able to decrease the β-glucan content of *A. infectoria*. It was also verified that both extracts reduced significantly the chitin content in *Asp. fumigatus* and increased the β-glucan content. This highlights the anti-mold potential of the algal extracts and prompts to future studies to unravel the mechanism by which the carrageenans change the molds' cell wall.

The carrageenan extracts did not inhibit the growth of *C. albicans* and neither inhibited the growth of *E. coli* and *S. aureus*. This allowed concluding that the carrageenans extracted from *C. teedei* var. *lusitanicus* do not possess antibacterial activity and might not be effective against some yeasts.

In what regards to the antiviral activity of this species, although it was noted a tendency of the carrageenan extracts to inhibit the Lentivirus infection, at both pre-treatment and virucidal assay, these were preliminary results. More confirmatory studies are needed. The understanding of which step of the viral infection could be affected by the extracts would further increase the potential of the extracts as an antiviral drug.

Chapter VI – References

6.1 References

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[5]http://macoi.ci.uc.pt/spec_list_detail.php?spec_id=142&searchSpecies=porphyra+umbilicalis+%28linnaeus%29+k%FCtzing

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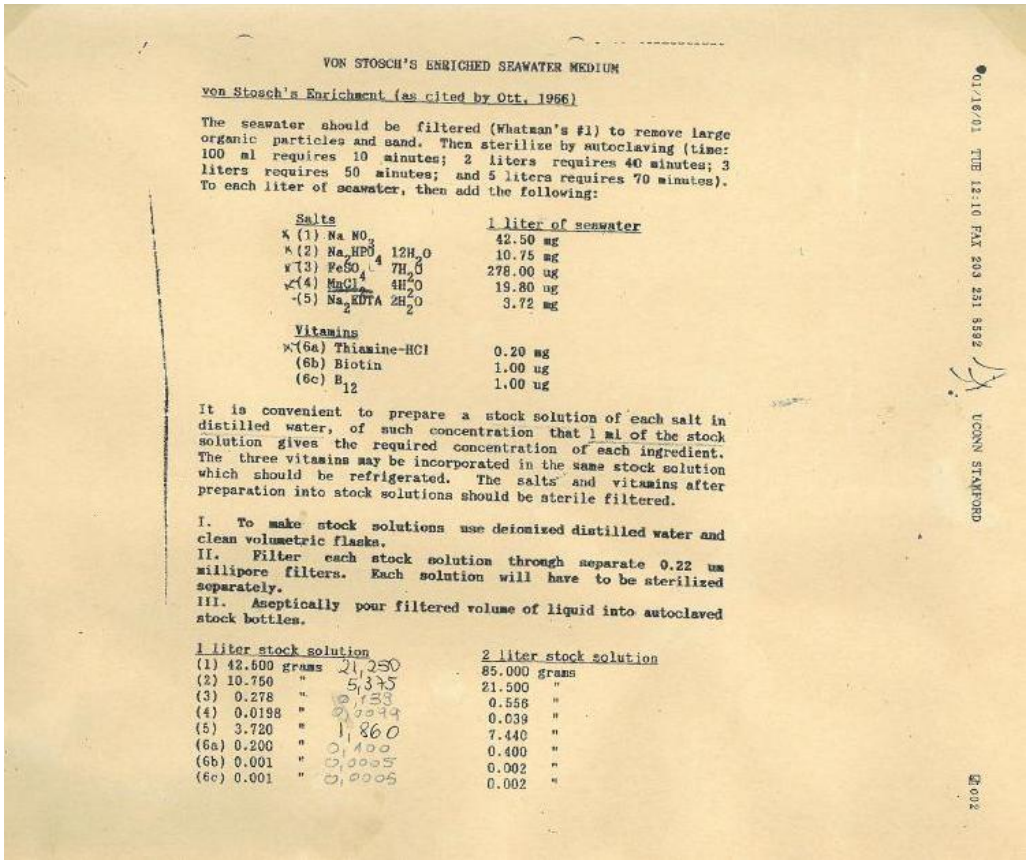
Appendix I – VSE culture medium



VON STOSCH'S ENRICHED SEAWATER MEDIUM

von Stosch's Enrichment (as cited by Ott, 1966)

The seawater should be filtered (Whatman's #1) to remove large organic particles and sand. Then sterilize by autoclaving (time: 100 ml requires 10 minutes; 2 liters requires 40 minutes; 3 liters requires 50 minutes; and 5 liters requires 70 minutes). To each liter of seawater, then add the following:

Salts	1 liter of seawater
(1) $NaNO_3$	42.50 mg
(2) $Na_2HPO_4 \cdot 12H_2O$	10.75 mg
(3) $FeSO_4 \cdot 7H_2O$	278.00 ug
(4) $MnCl_2 \cdot 4H_2O$	19.80 ug
(5) $Na_2EDTA \cdot 2H_2O$	3.72 mg
Vitamins	
(6a) Thiamine-HCl	0.20 mg
(6b) Biotin	1.00 ug
(6c) B_{12}	1.00 ug

It is convenient to prepare a stock solution of each salt in distilled water, of such concentration that 1 ml of the stock solution gives the required concentration of each ingredient. The three vitamins may be incorporated in the same stock solution which should be refrigerated. The salts and vitamins after preparation into stock solutions should be sterile filtered.

I. To make stock solutions use deionized distilled water and clean volumetric flasks.
II. Filter each stock solution through separate 0.22 um millipore filters. Each solution will have to be sterilized separately.
III. Aseptically pour filtered volume of liquid into autoclaved stock bottles.

1 liter stock solution	2 liter stock solution
(1) 42.500 grams 21,250	85.000 grams
(2) 10.750 " 5,375	21.500 "
(3) 0.278 " 0,139	0.556 "
(4) 0.0198 " 0,0099	0.039 "
(5) 3.720 " 1,860	7.440 "
(6a) 0.200 " 0,100	0.400 "
(6b) 0.001 " 0,0005	0.002 "
(6c) 0.001 " 0,0005	0.002 "

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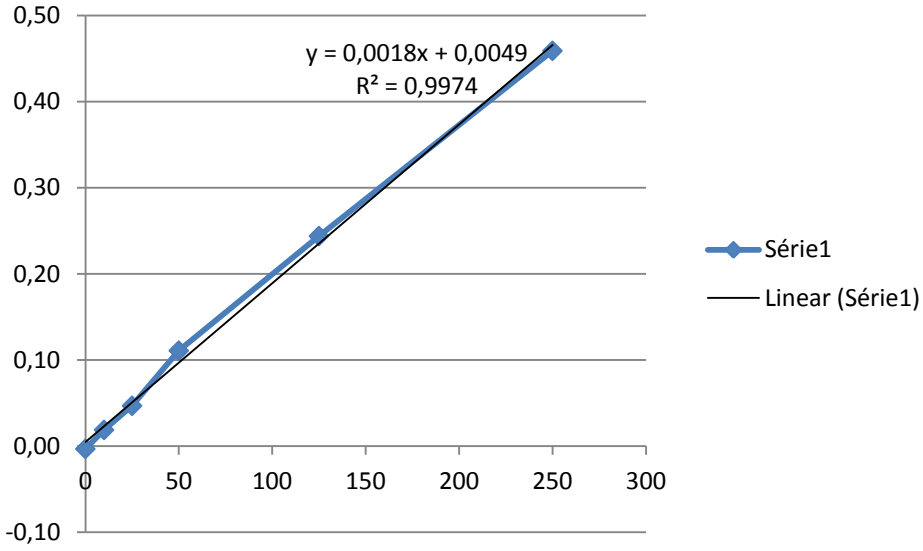
Appendix II – RPMI-1640 Medium

Component	R0883 [1x] g/L	R1145 [10x] g/L	R1383 g/L	R1780 [1x] g/L	R2405 [1x] g/L	R6504 g/L	R8758 [1x] g/L
Inorganic Salts							
Calcium Nitrate • $4H_2O$	0.1	1	0.1	0.1	0.1	0.1	0.1
Magnesium Sulfate (anhydrous)	0.04884	0.4884	0.04884	0.04884	0.04884	0.04884	0.04884
Potassium Chloride	0.4	4	0.4	0.4	0.4	0.4	0.4
Sodium Bicarbonate	2	—	—	2	2	—	2
Sodium Chloride	6	60	6	6	6	6	6
Sodium Phosphate Dibasic (anhydrous)	0.8	8	0.8	0.8	0.8	0.8	0.8
Amino Acids							
L-Alanyl-L-Glutamine	—	—	—	—	0.4344	—	—
L-Arginine	0.2	2	0.2	—	0.2	0.2	0.2
L-Asparagine (anhydrous)	0.05	0.5	0.05	0.05	0.05	0.05	0.05
L-Aspartic Acid	0.02	0.2	0.02	0.02	0.02	0.02	0.02
L-Cystine • 2HCl	0.0652	0.652	0.0652	0.0652	0.0652	0.0652	0.0652
L-Glutamic Acid	0.02	0.2	0.02	0.02	0.02	0.02	0.02
L-Glutamine	—	—	0.3	0.3	—	0.3	0.3
Glycine	0.01	0.1	0.01	0.01	0.01	0.01	0.01
L-Histidine	0.015	0.15	0.015	0.015	0.015	0.015	0.015
Hydroxy-L-Proline	0.02	0.2	0.02	0.02	0.02	0.02	0.02
L-Isoleucine	0.05	0.5	0.05	0.05	0.05	0.05	0.05
L-Leucine	0.05	0.5	0.05	—	0.05	0.05	0.05
L-Lysine • HCl	0.04	0.4	0.04	—	0.04	0.04	0.04
L-Methionine	0.015	0.15	0.015	0.015	0.015	0.015	0.015
L-Phenylalanine	0.015	0.15	0.015	0.015	0.015	0.015	0.015
L-Proline	0.02	0.2	0.02	0.02	0.02	0.02	0.02
L-Serine	0.03	0.3	0.03	0.03	0.03	0.03	0.03
L-Threonine	0.02	0.2	0.02	0.02	0.02	0.02	0.02

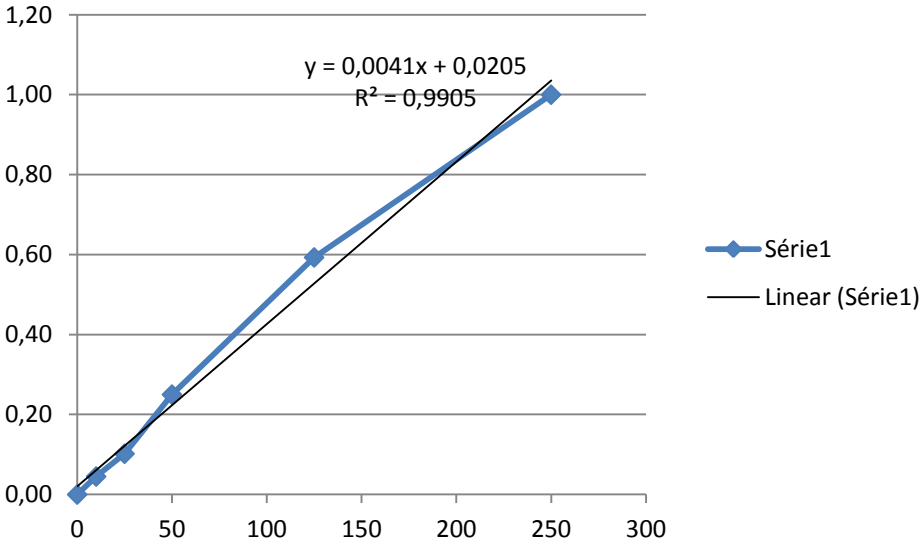
L-Tryptophan	0.005	0.05	0.005	0.005	0.005	0.005	0.005
L-Tyrosine • 2Na • $2H_2O$	0.02883	0.2883	0.02883	0.02883	0.02883	0.02883	0.02883
L-Valine	0.02	0.2	0.02	0.02	0.02	0.02	0.02
Vitamins							
D-Biotin	0.0002	0.002	0.0002	0.002	0.002	0.0002	0.0002
Choline Chloride	0.003	0.03	0.003	0.003	0.003	0.003	0.003
Folic Acid	0.001	—	0.001	0.001	0.001	0.001	0.001
myo-Inositol	0.035	0.35	0.035	0.035	0.035	0.035	0.035
Niacinamide	0.001	0.01	0.001	0.001	0.001	0.001	0.001
p-Aminobenzoic Acid	0.001	0.01	0.001	0.001	0.001	0.001	0.001
D-Pantothenic Acid (hemicalcium)	0.00025	0.0025	0.00025	0.00025	0.00025	0.00025	0.00025
Pyridoxine • HCl	0.001	0.01	0.001	0.001	0.001	0.001	0.001
Riboflavin	0.0002	0.002	0.0002	0.0002	0.0002	0.0002	0.0002
Thiamine • HCl	0.001	0.01	0.001	0.001	0.001	0.001	0.001
Vitamin B_{12}	0.000005	0.00005	0.000005	0.000005	0.000005	0.000005	0.000005
Other							
D-Glucose	2	20	—	2	2	2	2
Glutathione (reduced)	0.001	0.01	0.001	0.001	0.001	0.001	0.001
Phenol Red • Na	0.0053	0.053	0.0053	—	0.0053	0.0053	0.0053
Add							
L-Glutamine	0.3	0.3 at 1×	—	—	—	—	—
Sodium Bicarbonate	—	2.0 at 1×	2	—	—	2	—

Supplemental material I – Glucosamine calibration curves

Alternaria infectoria

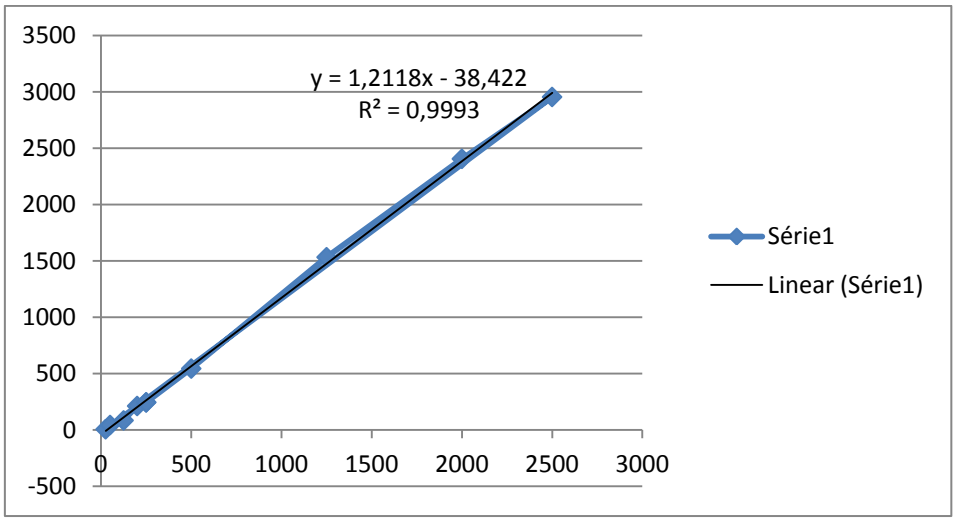


Aspergillus fumigatus

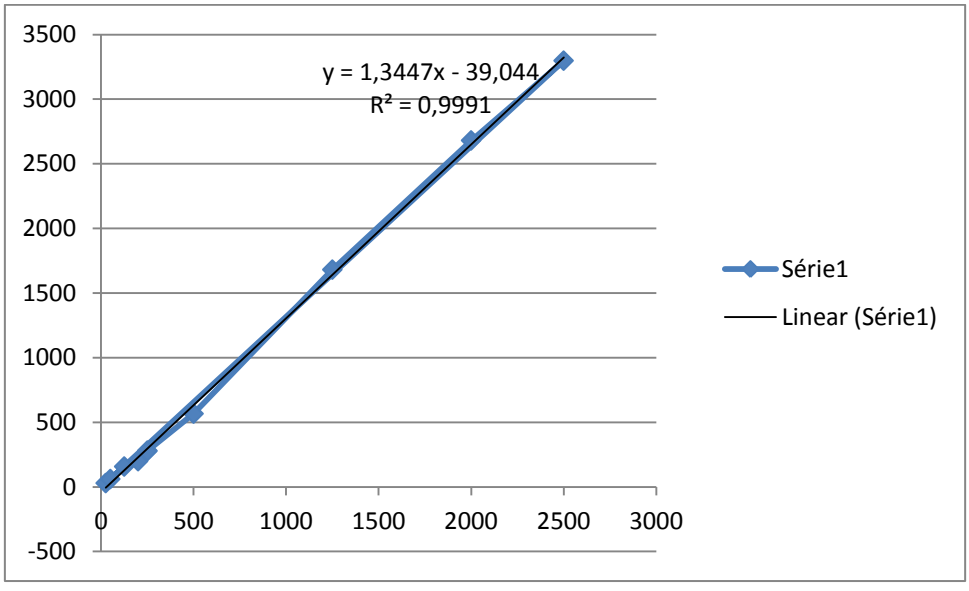


Supplemental material II – Curdlan calibration curves

Alternaria infectoria



Aspergillus fumigatus



Supplemental material III – Statistical analysis (ANOVA table) relative to RGR and Productivity in laboratory culture, respectively.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	20,5509	1	20,55091	1,631212	0,237354
"Var1"	41,4592	3	13,81972	1,096929	0,404869
Error	100,7884	8	12,59855		

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	1012,078	1	1012,078	1,347453	0,279188
"Var1"	1941,552	3	647,184	0,861643	0,499277
Error	6008,839	8	751,105		

Supplemental material IV – Statistical analysis (ANOVA table) relative to RGR and Productivity in IMTA system, respectively.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	11,82941	1	11,82941	0,817778	0,400681
"Var1"	14,00577	2	7,00289	0,484116	0,638390
Error	86,79186	6	14,46531		

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	8594,84	1	8594,835	2,362863	0,175167
"Var1"	6031,63	2	3015,813	0,829097	0,480922
Error	21824,80	6	3637,466		

Supplemental material V – Statistical analysis (ANOVA and Multiple comparisons tables) relative to *Alternaria infectoria* chitin content.

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	32.00	2	16.00	F (2, 6) = 1.016	P = 0.4169
Residual (within columns)	94.50	6	15.75		
Total	126.5	8			

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Control vs. FG Extract	1.934	-8.009 to 11.88	No	ns
Control vs. Tetra Extract	4.600	-5.343 to 14.54	No	ns
FG Extract vs. Tetra Extract	2.666	-7.277 to 12.61	No	ns

Supplemental material VI - Statistical analysis (ANOVA and Multiple comparisons tables) relative to *Alternaria infectoria* β-glucan content.

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	1280	2	640.1	F (2, 6) = 7.672	P = 0.0222
Residual (within columns)	500.6	6	83.44		
Total	1781	8			

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Control vs. FG Extract	26.60	3.717 to 49.48	Yes	*
Control vs. Tetra Extract	23.76	0.8785 to 46.65	Yes	*
FG Extract vs. Tetra Extract	-2.838	-25.72 to 20.05	No	ns

Supplemental material VII - Statistical analysis (ANOVA and Multiple comparisons tables) relative to *Aspergillus fumigatus* chitin content.

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	1896	2	948.0	F (2, 6) = 36.49	P = 0.0004
Residual (within columns)	155.9	6	25.98		
Total	2052	8			

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Control vs. FG Extract	35.11	22.34 to 47.88	Yes	***
Control vs. Tetra Extract	22.39	9.622 to 35.16	Yes	**
FG Extract vs. Tetra Extract	-12.72	-25.49 to 0.05014	No	ns

Supplemental material VIII - Statistical analysis (ANOVA and Multiple comparisons tables) relative to *Aspergillus fumigatus* β-glucan content.

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	3180	2	1590	F (2, 6) = 5.810	P = 0.0395
Residual (within columns)	1642	6	273.6		
Total	4821	8			

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Control vs. FG Ext	-34.64	-76.08 to 6.795	No	ns
Control vs. Tetra Ext	-43.58	-85.02 to -2.144	Yes	*
FG Ext vs. Tetra Ext	-8.939	-50.38 to 32.50	No	ns

Supplemental material IX - Statistical analysis (ANOVA and Multiple comparisons tables) relative to Lentivirus infection

Pre-treatment

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	1268	2	634.0	F (2, 3) = 3.516	P = 0.1635
Residual (within columns)	540.9	3	180.3		
Total	1809	5			

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Control vs. FG Extract	13.37	-42.75 to 69.48	No	ns
Control vs. Tetra Extract	35.27	-20.85 to 91.38	No	ns
FG Extract vs. Tetra Extract	21.90	-34.21 to 78.01	No	ns