DEPARTAMENTO DE CIÊNCIAS DA VIDA

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UNIVERSIDADE DE COIMBRA

Screening of biotechnological potential of *Osmundea pinnatifida:* cultivation trials and biological activities

Paulo Jorge Ferreira da Silva

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biodiversidade e Biotecnologia Vegetal, realizada sob a orientação científica do Professor Doutor Leonel Carlos dos Reis Tomás Pereira (Universidade de Coimbra) e do Professor Doutor Rui Filipe Pinto Pedrosa (Instituto Politécnico de Leiria)

Paulo Jorge Ferreira da Silva

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The real voyage of discovery consists

not in seeking new landscapes,

but in having new eyes.

Marcel Proust

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Scientific publications in the framework of this dissertation

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Silva, P. J., Alves, C., Pinteus, S., Silva, J., Pedrosa, R., & Pereira, L. Antioxidant and antitumor potential of wild and IMTA-cultivated *Osmundea pinnatifida*. (Submitted)

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Rego, A., **Silva, P. J.**, Silva, R., Pereira, J., Pereira, L., & Abreu, M. H. (2014). Cultivation trials in land-based IMTA conditions of macroalgae with pharmacological potential: *Osmundea pinnatifida*, *Codium tomentosum* and *Bifurcaria bifurcata*. In *Aquaculture Europe*, Donostia-San Sebastián, Spain, 14-17 October 2014.

Abstract

Wild and integrated multi-trophic aquaculture (IMTA) samples of the Portuguese edible seaweed *Osmundea pinnatifida* were screened for antioxidant, antitumor and antimicrobial (antibacterial and antifungal) activities. Extracts were obtained using solvents with different polarity, namely methanol, dichloromethane and *n*-Hexane.

The antioxidant activity was assessed through the 2,2-diphenly-1-picrylhydrazyl (DPPH) radical scavenging activity and oxygen radical absorbance capacity (ORAC) methods. Total phenolic content (TPC) was also estimated. The antitumor activity was evaluated in HepG-2, MCF-7 and SH-SY5Y cell lines through the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay. The antibacterial activity was assessed against *Escherichia coli* and *Staphylococcus aureus* through a standardized broth microdilution assay. In turn, antifungal activity against *Candida albicans* was studied using the standardized disk diffusion and broth microdilution; and also against *Alternaria infectoria* and *Aspergillus fumigatus* through radial growth inhibition and standardized broth microdilution assays, specific to filamentous fungi. Light, confocal and fluorescence microscopy observation, along with cell wall chitin and β-glucan quantification were also used to study the effect of the extracts on the *A. infectoria* and *Asp. fumigatus* fungal cell wall and morphology.

Among the solvents used for extraction, dichloromethane was the most effective to extract phenolic compounds in both samples. It were accounted 46.82 mg gallic acid equivalents (GAE)/g of dry extract in dichloromethane extract of wild sample (Dw) and 43.48 mg GAE/g in dichloromethane extract of IMTA-cultivated (Dc). The highest ORAC was also verified in the dichloromethane extracts (Dw: 414 μmol trolox equivalents (TE)/g; Dc: 499.9 μmol TE/g), evidencing a high correlation with the TPC content ($r=0.9499$, $P<0.01$) which was also sustained by the principal components analysis (PCA). The tested extracts were weak DPPH radical scavengers due to the high EC_{50} values obtained (>900 μg/mL). However, the effect of some fractions was dose-dependent. Dichloromethane extracts promoted a cytostatic effect on MCF-7 cells. Additionally, they shown cytotoxicity in SH-SY5Y cells and inhibited its proliferation. Dc presented higher cytotoxicity (IC_{50}: 923.6 μg/mL) whereas Dw was more effective (IC_{50}: 508.8 μg/mL) in inhibiting SH-SY5Y cells proliferation. All this effects were dose-dependent. Overall, there

were no statistically significant differences (*P*>0.05) in the antioxidant and antitumor activities shown by the wild and IMTA-cultivated samples.

The *n*-Hexane fraction of the wild seaweed (Hw) inhibited the sporulation of *A. infectoria* in RPMI medium at 30 μg/mL and also induced a statistically significant (*P*< 0.01) decrease in β-glucan content in mycelium grown in YME at the same concentration. All the studied extracts inhibited the radial growth of *Asp. fumigatus* in PDA. *n*-Hexane fractions at 100 μg/mL were the most effective (Hw: 14.64%; Hc: 19.83%). Furthermore, liquid cultures in YME supplemented with 100 μg/mL of Dw and 30 μg/mL of Hw showed abnormal conidial heads, completely devoid of conidia. *Asp. fumigatus* grown in YME supplemented with 100 μg/mL of Dw and with 100 μg/mL of Dc induced a statistically significant (*P*<0.01 and *P*<0.03, respectively) reduction in chitin content. *Asp. fumigatus* treated with 30 μg/mL of Hw also presented decreased chitin contents, although not significant.

Thus, the present study reveals *Osmundea pinnatifida* as a promising source of biologically active compounds with interest for further biotechnological applications.

Keywords: edible seaweed, antioxidant activity, antitumor activity, antifungal activity, *Aspergillus fumigatus*

Resumo

A *Osmundea pinnatifida* é uma espécie de alga vermelha edível abundante na costa portuguesa. Amostras selvagens e cultivadas em aquacultura multitrófica integrada (AMTI) foram avaliadas quanto à suas atividades antioxidante, antitumoral e antimicrobiana (antibacteriana e antifúngica). Os extratos foram obtidos através um processo de extração sequencial, no qual foram usados solventes orgânicos de diferentes polaridades, nomeadamente metanol, diclorometano e *n*-Hexano.

A atividade antioxidante foi avaliada através de métodos *in vitro*. Foram usados os métodos do DPPH (*2,2-diphenly-1-picrylhydrazyl*) e índice ORAC (*Oxygen Radical Absorbance Capacity*). O conteúdo em polifenóis foi também estimado através do método de Folin-Ciocalteu. Por sua vez, a atividade antitumoral foi avaliada em três linhas celulares, HepG-2, MCF-7 e SH-SY5Y, através do método MTT (*3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide*). A atividade antibacteriana foi avaliada contra *Escherichia coli* e *Staphylococcus aureus* através do método standardizado da microdiluição em meio líquido. A atividade antifúngica contra *Candida albicans* foi estudada usando métodos standardizados – difusão em disco e microdiluição em meio líquido. Foi também aferida a atividade antifúngica contra os fungos filamentosos *Alternaria infectoria* e *Aspergillus fumigatus* através da inibição do crescimento radial e do método standardizado de microdiluição em meio líquido, específico para fungos filamentosos. Observações em microscopia ótica, confocal e de fluorescência, juntamente com a quantificação dos conteúdos em quitina e em β-glucano, foram também usados para estudar o efeito dos extratos na parede celular de *A. infectoria* e *Asp. fumigatus*.

Entre os solventes usados na extração, o de diclorometano foi o mais eficaz na extração de compostos fenólicos em ambas as amostras. Para o extrato de diclorometano da amostra selvagem (Dw) foram determinados 46,82 mg de equivalentes em ácido gálico (EAG)/g de extrato seco, enquanto que para o extrato de diclorometano da amostra cultivada em AMTI (Dc) o conteúdo em fenóis foi estimado em 43,48 mg EAG/g. O índice ORAC mais elevado também foi verificado nos extratos de diclorometano (Dw: 414 µmol de equivalentes de trolox (ET)/g; Dc: 499,9 µmol ET/g), evidenciando uma elevada correlação com o conteúdo total em polifenóis (r=0,9499; P<0,01), que foi corroborada pela análise de componentes principais. Os extratos testados apresentaram uma fraca capacidade de reduzir o radical DPPH devido aos valores elevados de EC_{50} obtidos (>900

μg/mL). No entanto, o efeito de algumas frações dependente da concentração. Os extratos de diclorometano promoveram um efeito citostático nas células MCF-7. Para além disso, estes extratos exibiram citotoxicidade em células SH-SY5Y e inibiram a sua proliferação. Dc apresentou uma citotoxicidade maior (IC_{50}: 923,6 μg/mL), enquanto que Dw foi mais eficaz (IC_{50}: 508,8 μg/mL) na inibição da proliferação em células SH-SY5Y. Estes efeitos demonstraram ser dependentes da concentração. No geral, não se verificaram diferenças estatisticamente significativas ($P>0,05$) entre a amostra selvagem e a cultivada em AMTI ao nível das atividades antioxidante e antitumoral.

A fração de *n*-Hexano da alga selvagem (Hw) inibiu a esporulação de *A. infectoria* em meio RPMI a 30 μg/mL. Para além disso, induziu um decréscimo estatisticamente significativo ($P<0,01$) no conteúdo em β-glucano no micélio crescido em meio YME, à concentração referida anteriormente. Todos os extratos em estudo inibiram o crescimento radial de *Asp. fumigatus* em PDA. Entre eles, as frações de *n*-Hexano a 100 μg/mL foram as mais eficazes (Hw: 14,64%; Hc: 19,83%). Para além disso, culturas líquidas em meio YME suplementado com 100 μg/mL de Dw e 30 μg/mL de Hw evidenciaram cabeças aspergilares anormais, completamente desprovidas de conídios. *Asp. fumigatus* crescido em meio YME suplementado com 100 μg/mL de Dw e com 100 μg/mL de Dc induziram uma redução estatisticamente significativa ($P<0,01$ e $P<0,03$) nos conteúdos de quitina. *Asp. fumigatus* tratados com 30 μg/mL de Hw também apresentou um decréscimo nos conteúdos de quitina, apesar de não ser significativo.

Assim, o presente estudo revelou a *Osmundea pinnatifida* como uma fonte bastante promissora de compostos biologicamente ativos com interesse para futuras aplicações biotecnológicas.

Palavras-chave: macroalga edível, atividade antioxidante, atividade antitumoral, atividade antifúngica, *Aspergillus fumigatus*

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Table 4. Effect of the different *O. pinnatifida* extracts, tested at 10 µg/mL and 100 µg/mL, on colony diameter (CD; mm) and on radial growth inhibition (RGI; %) of *Asp. fumigatus*. 64

Abbreviations

AAPH	2,2′-azobis(2-methylpropionamidine) dihydrochloride
Dc	Dichloromethane extract of the IMTA-cultivated seaweed
DMSO	Dimethyl sulfoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
Dw	Dichloromethane extract of the wild seaweed
FCR	Folin-Ciocalteu reagent
GAE	Gallic acid equivalent
Hc	*n*-Hexane fraction of the IMTA-cultivated seaweed
HepG-2	Carcinoma model of a human hepatocellular cancer
Hw	*n*-Hexane fraction of the wild seaweed
IMTA	Integrated multi-trophic aquaculture
Lab TI	First laboratory *in vitro* trial
Lab TII	Second laboratory in vitro trial
Mc	Methanol fraction of the IMTA-cultivated seaweed
MCF-7	Human breast adenocarcinoma model
MEC	Minimum effective concentration
MHB	Mueller-Hinton broth
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
Mw	Methanol fraction of the wild seaweed
ORAC	Oxygen radical absorbance capacity
PDA	Potato dextrose agar

RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI – 1640	Roswell Park Memorial Institute (RPMI) 1640 medium
SH-SY5Y	Model of human neuroblastoma
TPC	Total phenolic content
YME	Yeast malt extract medium
YNB	Yeast nitrogen base
YPD	Yeast-extract-peptone-dextrose agar

INTRODUCTION

1. Marine environment as a source of bioactive compounds

The earth appears to be almost completely covered with water when viewed from a certain point in outer space. Saltwater covers almost 71% of the earth surface and contain 97% of the earth's water (Miller Jr. & Spoolman, 2012). As a consequence, the marine biodiversity is extremely vast, containing about 2.2 million eukaryotic marine species globally. It is estimated that this number is only a small percentage of the total number of species that are yet to be described (Mora, Tittensor, Adl, Simpson, & Worm, 2011). The marine environment has proved to be a rich source of biologically active natural products, many of which have not been found in terrestrial sources (Carté, 1996). In fact, marine organisms are able to synthesize secondary metabolites to cope with ecological pressures such as microbial infection, predation, and competition for space, nutrients and resources (Garson, 2001). These natural compounds offers a great scope for the discovery and/or development of new drugs (Pomponi, 2001). Indeed, some natural products have made it into clinical routines, mainly in the cancer therapeutic area, and many more are in all phases of clinical testing (Martins, Vieira, Gaspar, & Santos, 2014).

The immense diversity of marine organisms, concomitant with their biotechnological potential, has attracted the attention of both science and industry. Although marine macroalgae inhabit the oceans for over 2 billion years, human beings only began to understand its enormous potential in recent decades, which goes far beyond its primary and direct use as bio-fertilizer, as was typical especially in the North of Portugal. Today, seaweeds are used in many countries for different purposes, including direct consumption as food or supplements (by animals and humans) and as a feedstock for the extraction of phycocolloids (e.g., alginate, agar, carrageenan). Due to its unique structural and biochemical composition, as well as its various biological activities (e.g., antibacterial, antifungal, antitumoral and antiviral), seaweeds are a valuable source to the pharmaceutical industry and to the emerging cosmetics industry and well-being (Pereira & Correia, 2015). It is presumed that the unique features of the marine environment, where the seaweeds are grown, are mainly responsible for most of its properties (Rajapakse & Kim, 2011). Moreover, macroalgae have also attracted attention as a possible renewable feedstock to biorefinery applications, including the production of biofuels such as bioethanol and biogas (Dahiya, 2014).

2. Seaweeds

Seaweeds (or macroalgae) are photosynthetic marine organisms belonging to the Eukaryota domain and the kingdoms Plantae (the green and red algae) and Chromista (the brown algae). Although classification systems have changed over time, it is generally accepted that the green algae are included in the phylum Chlorophyta and their pigmentation is identical to that of terrestrial plants (*i.e.* chlorophylls a, b and carotenoids); the red algae belong to the phylum Rhodophyta and their photosynthetic pigments are chlorophyll a, phycobilins (*i.e.* R-phycocyanin, R-phycoerythrin) and carotenoids, mostly β-carotene, lutein and zeaxanthin; and that the brown algae are included in the phylum Ochrophyta (or the Heterokontophyta), Class Phaeophyceae and their pigments include the chlorophylls a and c as well as carotenoids, dominated by fucoxanthin (Cardoso et al., 2014).

Seaweeds are fundamental to the food chain of all aquatic ecosystems. As primary producers, they produce oxygen and organic compounds which serve as the basic trophic level or food for many other living beings (Cardoso et al., 2014). Indeed, seaweeds are consumed in a daily basis in some parts of the world, namely in the East Asia where the direct use as food has strong roots (Cardoso et al., 2014), and are now being spread to the western cousins, where they are considered healthy delicacies. In fact, and in comparison with many common vegetables and animal-based foods, edible seaweed are rich in some health-promoting molecules such as dietary fiber, minerals, polyunsaturated fatty acids (mainly omega-3 fatty acids), essential amino acids, and vitamins A, B, C and E (Rajapakse & Kim, 2011).

2.1. *Osmundea pinnatifida* (Hudson) Stackhouse

Despite the phylogeny and taxonomy of the genus *Osmundea* have been somewhat controversial in the past, it is now strongly supported that this genus is a monophyletic group within the *Laurencia* complex. Indeed, the genus *Osmundea* Stackhouse, resurrected by Nam et al. (1994), is segregated from the other four genera of the *Laurencia* complex (*Laurencia sensu stricto*, *Chondrophycus*, *Palisada* and *Yuzura*) by a set of vegetative and reproductive characters. Besides, *Osmundea* has been considered the only genus within *Laurencia* complex that shows a disjunct distribution, occurring in the North-East and

South-West Pacific, the Atlantic and Indian oceans and the Mediterranean sea (Machín-Sánchez et al., 2012).

Osmundea pinnatifida belongs to the phylum Rhodophyta, class Florideophyceae, order Ceramiales, family Rhodomelaceae and tribe Laurenciae (Guiry & Guiry, 2015). It is a small red alga (up to 15 cm in length) with a flattened, cartilaginous in texture, branched and erect thallus. Branching is alternate and occurs in one plane only, with branches becoming shorter towards their apex and broadly rounded. *O. pinnatifida* is perennial, being attached to the substratum (mainly rocky) by stoloniferous branches and a basal crust (Figure 1A & B). The plant is highly variable in size and coloration depending upon its location on the shore. Higher shore plants are generally dwarfed and yellow-green in colour (Figure 1C), owing to exposure to high levels of sunlight while on the lower shore they are reddish-brown (Figure 1D). Fresh plants have a peculiar smell and flavour (Machín-Sánchez et al., 2012; Pereira, 2009; 2014).

Figure 1. Morphology of *Osmundea pinnatifida* (A & B) and variations in coloration upon the location in the coast: C – higher shore seaweed; D – lower shore seaweed (A – retrieved from Vestjens & Frijsinger (2011); B – retrieved from Gaspar (2002)).

As referred above, *Osmundea* species have a disjunct geographical distribution. Indeed, as shown in Figure 2, the occurrence of *O. pinnatifida* has been recorded in Europe

(Britain, Bulgaria, Faroe Islands, France, Ireland, Norway, Portugal, Spain) and Atlantic Islands (Canary Islands, Madeira, Savage Islands); South America (Brazil); Africa (Cape Verde, Ghana, Mauritania, Morocco, São Tomé and Príncipe, Senegal, Western Sahara) and Asia (Cyprius, India, Indonesia, Korea, Oman, Pakistan, Philippines, Taiwan, Turkey,Yemen) (Guiry & Guiry, 2015). Thus, it is a fairly resilient seaweed with a noteworthy ability to thrive in a wide range of environmental conditions.

O. pinnatifida figures among the dominant intertidal flora of the north-western zones of the Iberian Peninsula (Cardoso et al., 2014), being quite abundant throughout the year in the intertidal rocky shores on the north and centre of Portugal.

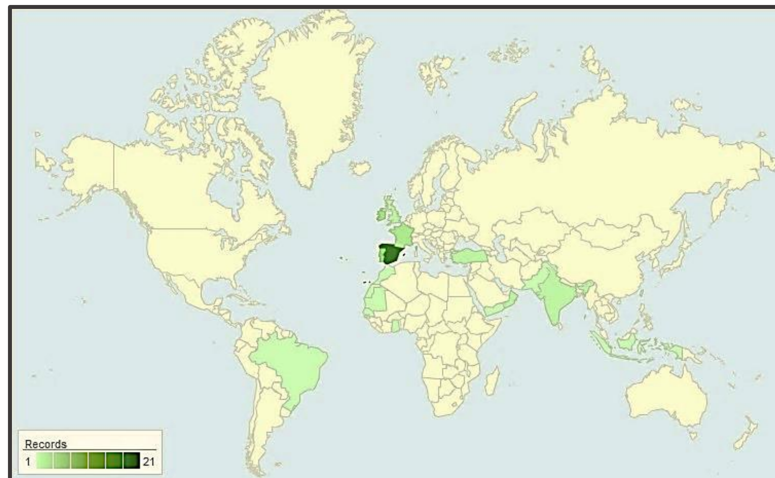


Figure 2. Global distribution of *Osmundea pinnatifida* (adapted from Guiry & Guiry (2013)).

2.1.1. Economic importance: current and potential uses

O. pinnatifida is commonly known as pepper dulse (Guiry & Guiry, 2015) or *erva malagueta* (in Portuguese), due to its slightly spicy flavour. Both designations indicate their utility in the traditional diet. This aromatic seaweed is dried and used as a pepper- or curry-flavoured spice in Scotland, Ireland and Portugal, namely in the Azores islands (Pereira, 2014). Besides, it is also pickled in vinegar with onions and eaten with fried fish by the Azoreans (Patarra, Paiva, Neto, Lima, & Baptista, 2011).

Recently, the nutritional value of *O. pinnatifida* has been evaluated, along with some other species. The great majority of these studies were carried out with samples collected from Azores islands (Paiva, Lima, Patarra, Neto, & Baptista, 2014; Patarra, Leite, Pereira,

Baptista, & Neto, 2013; Patarra, Paiva, Neto, Lima, & Baptista, 2011). Patarra et al. (2011) revealed that seaweed is a good source of fiber and protein. They were also found to be an important source of dietary essential fatty acids, namely linoleic acid, which prevent deficiency symptoms and cannot be synthesized by humans (Patarra et al., 2013). Paiva et al (2014), in addition to corroborate *O. pinnatifida* as a rich source of protein, also indicates this seaweed as a valuable source of vitamins A, E (α-tocopherol and γ-tocopherol) and K (K1 and K2), polyunsaturated fatty acids, and minerals (sodium, potassium, magnesium, cesium and calcium). High ash contents, which can contribute with important microelements to both human and animal nutrition, and acceptable amounts (as compared to terrestrial foodstuffs) of 9 out of 10 essential amino-acids, were also found. The antioxidant properties, fatty acid profile, low omega-6/omega-3 and hypocholesterolemic (h)/hypercholesterolemic (H) ratios observed, along with the non-animal nature of the nutrients can have potential health benefits, turning it useful for food supplement and/or pharmaceutical industries (Paiva et al., 2014). In the investigations carried out with *O. pinnatifida* collected in Portugal, it was found that this specie is rich in mannitol (Andrade et al., 2013), a naturally occurring polyol (sugar alcohol) widely used in the food, pharmaceutical, medical, and chemical industries (Saha & Racine, 2011). Furthermore, an inhibitory potential against α-glucosidase was showed by Rodrigues, Sousa, et al (2015). Alpha-glucosidase inhibitors (AGIs) are widely used in the treatment of patients with type 2 diabetes because they delay the absorption of carbohydrates from the small intestine and, thus, have a lowering effect on postprandial blood glucose and insulin levels (van de Laar et al., 2005).

There is also some investigation on biological activities of *O. pinnatifida* extracts. Antioxidant activity has been screened *in vitro*, revealing that *O. pinnatifida* is a potential source of biological active compounds with antioxidant properties (Andrade et al., 2013; Barreto et al., 2012; Jiménez-Escrig, Gómez-Ordóñez, & Rupérez, 2012; Paiva, Patarra, Neto, Lima, & Baptista, 2012; Rodrigues, Freitas, et al., 2015; Rodrigues, Sousa, et al., 2015), although a further characterization is required. Preliminary studies revealed that ethanol extracts have strong *in vitro* antileishmanial activity against the promastigote form of *Leishmania major* (Sabina et al., 2005), a protozoa that can cause disease (leishmaniasis) in humans (Pearson & Sousa, 1996).

The studies mentioned above were carried out using *O. pinnatifida* collected in different sites and periods and subjected to distinct pre-treatments. As reported by Paiva et al. (2014), the variability in the chemical composition of macroalgae may be the result of

its development stage, geographical distribution, habitat, season and nutrient content of the growth medium, among other environmental factors. Furthermore, it is also known that different sampling methodologies and drying procedures can also affect the biochemical composition, and consequently, the nutritional value (Paiva et al., 2014) and the biological activities of algae.

Seaweeds are rich in polysaccharides, with concentrations that can range from 4 to 76% of dry weight. Of all the polysaccharides, macroalgal hydrocolloids (*i.e.*, high molecular weight polysaccharides that typically forms colloidal solutions), also known as phycocolloids, are by far the most relevant in terms of their industrial commercialization (Cardoso et al., 2014). It is also important to emphasize that phycocolloids are secondary metabolites. Red seaweeds synthesize a great variety of sulfated galactans, which are the major components of the extracellular matrix (Souza et al., 2012), being agarans (agar) and carrageenans the most common (Cardoso et al., 2014). *O. pinnatifida* is mostly an agar producer (Rodrigues, Freitas, et al., 2015). Agar is approved in European Union for use in varied food industries as natural additive, being therefore encoded as E406. About 90% of the agar produced globally is for food applications. Agar is tasteless and has remarkable thickening, stabilizing and gelling properties, which turns it in a crucial ingredient in the preparation of processed foods such as fruit jellies, dairy products, chewing gum and other confectionary, baked goods, icings, soups, canned meat and frozen fish. In addition to food applications, about 10% of all agar is currently used for biotechnological applications, including the preparation of inert, solidified culture media for bacteria, microalgae, fungi, tissue culture as well as for separation of macromolecules by electrophoresis. Although, agar applications are expected to increase in the near future, mainly because of their health-associated properties (Cardoso et al., 2014).

3. Biological activities

As previously referred, several biological activities have been attributed to secondary metabolites isolated from marine organisms, including macroalgae (Mayer, Rodríguez, Taglialatela-Scafati, & Fusetani, 2013). These chemicals have displayed, among others, antimicrobial (antibacterial, antifungal, antimalarial, antiprotozoal) antidiabetic, anti-inflammatory, antioxidant (Mayer et al., 2013) and antitumor properties (Sithranga Boopathy & Kathiresan, 2010). Among them, the antimicrobial and antitumor activities are especially important, since there is a need for novel compounds with potential to replace

those currently used in therapeutics, for which there are already troublesome issues such as drugs resistance and side effects. Nevertheless, antioxidant activity is by far the most investigated since a host of diseases seem to be mediated, at least in part, by oxidative stress (Finkel & Holbrook, 2000).

3.1. Antioxidant activity

In recent years, it has been a growing interest in healthy lifestyles. The demand for natural antioxidants (either from foods and/or supplements) has increased due to the recognition by the population of the benefits that these compounds bring to human health, including free radical neutralization and the so-called anti-aging effect. Hence, the antioxidant potential of both plant crude and purified extracts, as well as isolated compounds, have been extensively assessed while antioxidants have gained increasing reputation among the scientific community and the general public.

3.1.1. Reactive species and oxidative stress

The formation of oxygen-derived free radicals is a consequence of aerobic metabolism since oxygen is the terminal acceptor in the respiration of aerobic organisms (Gombart, 2013). Free radicals are molecules or molecular fragments containing one or more impaired electrons in atomic or molecular orbitals, which give them a considerable degree of reactivity. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are products of normal cellular metabolism. Radicals derived from oxygen represent the most important class of radical species generated in living system, encompassing, , among others, superoxide anions ($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$) and peroxyl radicals ($ROO^{\bullet}$) (Singh, Arseneault, Sanderson, Murthy, & Ramassamy, 2008). ROS are well recognized for playing a dual role as both beneficial and deleterious species. Beneficial effects of ROS occur at low/moderate concentrations and involve physiological roles in cellular responses to noxia, as for example in defence against infectious agents, in the function of a number of cellular signalling pathways, and the induction of a mitogenic response. In contrast, oxidative stress may ensue when there is an overproduction of ROS on one side and a deficiency of enzymatic and non-enzymatic antioxidants on the other. Cellular lipids, proteins, or DNA can be damaged and their normal function inhibited by excessive ROS. Because of this, oxidative stress has been implicated in a host of human diseases (e.g.

cancer, diabetes mellitus and neurodegenerative disorders) as well as in the ageing process (Valko et al., 2007).

3.1.2. Antioxidants

Exposure to free radicals from a variety of sources has led organisms to develop a series of defence mechanisms. These defences against free radical-induced oxidative stress involve preventative and repair mechanisms, and physical and antioxidant defences. Enzymatic antioxidant defences include superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase, while non-enzymatic antioxidants are represented by ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione, carotenoids, flavonoids, and other antioxidants (Valko et al., 2007). Antioxidants can decrease the oxidative damage directly *via* reacting with free radicals, or indirectly by inhibiting the activity or expression of free radical generating enzymes or enhancing the activity or expression of intracellular antioxidant enzymes (Lü, Lin, Yao, & Chen, 2010). Under normal conditions, there is a balance between both the activities and the intracellular levels of these antioxidants, which is essential for the survival of organisms and their health (Valko et al., 2007).

3.1.3. Evaluation of antioxidant capacity

Since seaweeds are photosynthetic organisms, free radicals and other oxidative reagents are produced when they are exposed to high oxygen concentrations and light. The apparent lack of structural damage in seaweeds is considered to be related with their ability to synthesize the compounds required for their protection against oxidative injury. Hence, algae are likely to be a source of potent antioxidant compounds that could also be suitable for protecting human bodies against the damaging effects of ROS. Algal antioxidants include fat-soluble compounds, such as carotenoids and vitamin E, and water-soluble compounds, such as other vitamins, phycobiliproteins and polyphenols (Shalaby, 2014).

Depending upon the reactions involved, the antioxidant capacity assays can be classified into two types: assays based on hydrogen atom transfer (HAT) reactions and assays based on electron transfer (ET). The majority of HAT-based assays apply a competitive reaction scheme, in which antioxidant and substrate compete for thermally generated peroxyl radicals through the decomposition of azo compounds. These assays

include, among others, the oxygen radical absorbance capacity (ORAC). ET-based assays measure the capacity of an antioxidant in the reduction of an oxidant, which changes colour when reduced. The degree of colour change is correlated with the sample's antioxidant concentrations. ET-based assays include, among others, the total phenols assay by Folin-Ciocalteu reagent (FCR) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Huang, Ou, & Prior, 2005).

Total phenols assay by FCR and DPPH radical scavenging capacity assay figure among the most popular assays to assess the antioxidant capacity of plant-derived materials, including seaweed extracts. Along with other ET-based assays, these methods involve two components in the reaction mixture – antioxidants and oxidant (also the probe) – and are based on the following ET reaction: probe (oxidant) + e^- (from antioxidant) → reduced probe + oxidized antioxidant. The probe itself (FCR or DPPH) is an oxidant that abstracts an electron from the antioxidant (seaweed extract, in this case), causing colour change of the probe. The degree of the colour change is proportional to the antioxidant concentrations. The reaction end point is reached when colour change stops. For total phenols assay by FCR, the change of absorbance is plotted against the antioxidant concentration to give a linear curve. The slope of the curve reflects the antioxidant's reducing capacity, which may be expressed as gallic acid equivalent (GAE). Total phenols by FCR resemble the redox titration in classical chemical analysis (Huang, Ou, & Prior, 2005). For DPPH radical scavenging capacity assay, the change of absorbance is compared with a control, in which the antioxidant is absent, being the antioxidant's reducing capacity usually expressed as percentage of control. Total phenols assay by FCR and DPPH radical scavenging capacity assay were already used to evaluate the antioxidant capacity of *O. pinnatifida* extracts (Andrade et al., 2013; Barreto et al., 2012; Paiva et al., 2014; Paiva et al., 2012; Rodrigues, Sousa, et al., 2015).

The ORAC assay has emerged as a convenient method to determine antioxidant capacity of a range of samples. This assay applies a thermal radical generator to give a steady flux of peroxyl radicals in air-saturated solution. Added antioxidant competes with the probe (substrate in this case) for the radicals and inhibits or retards the probe oxidation (Huang, Ou, & Prior, 2005). This assay have the following components: an azo radical initiator, normally AAPH (2,2′-azobis(2-methylpropionamidine) dihydrochloride); a molecular probe (fluorescence in this case – fluorescein) for monitoring reaction progress; antioxidant (seaweed extract or reference compound in this case); and reaction kinetic parameters collected with a fluorescence microplate reader for antioxidant capacity

quantitation (Huang, Ou, & Prior, 2005). ORAC assay was already used to investigate the antioxidant capacity of both plant (Wojcikowski, Stevenson, Leach, Wohlmuth, & Gobe, 2007) and seaweed extracts (Wang, Jónsdóttir, & Ólafsdóttir, 2009).

3.2. Antitumor activity

Cancer figures among the leading cause of morbidity and mortality worldwide. According to the estimates from the International Agency for Research on Cancer (IARC), 14 million new cancer cases and 8.2 million cancer deaths were accounted in 2012 (Ferlay et al., 2013). The global cancer burden is growing at an alarming pace, reaching about 21.7 million new cancer cases and 13 million cancer deaths by 2030 (American Cancer Society, 2015).

Cancer is the generic term for a group of diseases, also known as malignant tumors that can affect any part of the body (WHO, 2002). Most cancers are named by the cells that give rise to them. Breast cancer, for example, is the name attributed to a malignant tumor whose origin is breast cells (Oncologiacuf, n.d.). Malignant tumors of the brain, lung, breast, prostate, skin, and colon are among the diseases known as cancer. They have different causes, evolution and treatments for each type, but there is a common feature to all of them: the uncontrolled division and growth of cells (Portal da Saúde, 2005). The process of cell turnover is normally well controlled throughout life by basic biological mechanisms. In cancer, however, the control mechanisms are disrupted. Cells in the affected part of the body grow beyond their usual boundaries, invade adjoining tissues, and may spread to secondary organs or tissues as metastases (WHO, 2002).

Several approaches on targeted therapies have significantly changed the treatment of cancer over the last years (Brannon-Peppas & Blanchette, 2004; Chari, 2008; Kreitman, 2006; Mathew & Verma, 2009). Cisplatin, carboplatin, and oxaliplatin are still involved in nearly 50% of all anticancer therapies worldwide (Liu et al., 2014). However, problems of platinum resistance and undesirable side effects are limiting their future use, emphasizing the urgent need for new anticancer agents.

3.2.1. Red seaweeds as sources of anticancer compounds

In the past three decades, many researchers have worked on the antitumor activity of seaweeds. Extracts or even isolated compounds from green, brown and red seaweeds have

exhibited antitumor properties, especially *in vitro*, against human tumor cells (Sithranga Boopathy & Kathiresan, 2010). Regarding the red seaweeds, extracts from the edible *Palmaria palmata* inhibited epithelial cancer cell (HeLa) proliferation *in vitro* (Yuan, Carrington, & Walsh, 2005). The compound chondriamide-A from *Chondria* sp. exhibits cytotoxicity towards human nasopharyngeal and colorectal cancer cells. The alcoholic extract of the *Acanthophora spicifera* exhibited tumoricidal activity on Ehrlich's ascites carcinoma cells developed in mice (at 20 mg/kg), comparable to the standard drug 5-fluorouracil; which was further evidenced by increase in the mean survival time, decrease in tumor volume, and viable cell count (Sithranga Boopathy & Kathiresan, 2010). It is important to emphasize that *Chondria* sp. and *A. spicifera* are phylogenetically closed to *O. pinnatifida*, since they belong to the family Rhodomelaceae.

3.2.2. Evaluation of *in vitro* cytotoxicity

The in vitro cytotoxicity testing, like other in vitro trials for evaluation of biological activities, are the first step for the evaluation of the antitumor capacity of any kind of compounds, whether of natural, synthetic or semi-synthetic nature. The numerous methods used and endpoints measured in cytotoxicity determination can be grouped into the following categories of evaluation: assessments of cell damage by morphological means, measurements of cell damage, measurements of cell growth and measurements of specific aspects of cellular metabolism. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) cytotoxicity test is based on the measurement of the viability of cells via metabolic activity. Yellow water-soluble MTT is metabolically reduced in viable cells to a blue-violet insoluble formazan. The number of viable cells correlates to the colour intensity determined by photometric measurements after dissolving the formazan in alcohol (ISO, 2009).

3.3. Antimicrobial activity

3.3.1. Bacteria and antibiotic treatments

Bacteria comprise a large Domain of unicellular prokaryotic organisms, being therefore morphologically characterized for the absence of a membrane-bound nucleus and other intracellular organelles, such as mitochondria. Prokaryotes are the ancestors of all life

forms, having developed an impressive adaptability that allows them to thrive in almost all available ecological habitats. The Bacteria are an extremely diverse group of organisms differing in size, shape, habitat and metabolism (Rogers, 2011). Species of bacteria can be divided into two major groups, called gram-positive and gram-negative, based on the Gram stain reaction. The differences in cell wall structure are at the heart of the Gram stain: gram-positive bacteria have very thick cell walls consisting primarily of peptidoglycan (as much as 90% of the cell wall), whereas gram-negative bacteria cell wall is chemically complex and consists of at least two layers – a thinner layer of peptidoglycan (about 10% of the total cell wall) and an outer membrane that also contains polysaccharides linked (Madigan, Martinko, Bender, Buckley, & Stahl, 2010).

Some bacteria live in symbiotic relationships with plants and animals, where they carry out important functions for the host, such as nitrogen fixation and cellulose breakdown. Additionally, they play a vital role in several ecosystems, contributing to the degradation of organic matter in soil, increasing its fertility and, thus, sustaining higher life forms. Some bacteria can cause disease in plants, animals and humans, while others are harmless (Rogers, 2011). This is the case of normal microbiota, which comprise a great variety of microorganisms, including bacteria including bacteria and unicellular fungi, that colonizes the skin and mucous membranes (e.g. oral cavity, respiratory tract, gastrointestinal tract and urogenital tract) of every healthy human. These organisms are estimated to outnumber human cells by a factor of 10. It has been proven that normal microbiota provides a first line of defense against microbial pathogens, assists in digestion, is involved in toxin degradation and contributes to the maturation of the immune system. However, shifts in the normal microbiota or stimulation of inflammation by these commensals may cause diseases such as inflammatory bowel disease (Carroll, 2013). On the other hand, bacteria have been the cause of some of the most deadly diseases and widespread epidemics of humans, including plague, cholera, dysentery, diphtheria, typhoid fever, typhus, pneumonia and tuberculosis. Water purification, immunization (vaccination) and antibiotic treatment have reduced the morbidity and the mortality of bacterial disease at least in the developed countries (Isbary & Stolz, 2012).

In fact, antibiotics have revolutionized medicine in many respects (Davies & Davies, 2010). Antibiotics are synthetic, natural or semisynthetic molecules and can be classified according to the bacterial cellular component or system they affect, in addition to whether they induce cell death (bactericidal drugs) or merely inhibit cell growth (bacteriostatic drugs) (Kohanski, Dwyer, & Collins, 2010; Walsh, 2003). Most current bactericidal

antibiotics are natural products or semisynthetic derivatives that inhibit DNA (quinolones), RNA (rifamycins), cell wall (β-lactams and glycopeptides) or protein synthesis. Drugs that inhibit protein synthesis are among the broadest classes of antibiotics and can be divided into two subclasses according to the ribosomal subunit they target − 50S inhibitors (macrolides, lincosamides, streptogramins, amphenicols and oxazolidinones) and 30S inhibitors (tetracyclines and amino cyclitols) (Kohanski et al., 2010). However, their effectiveness and ease of access led to a serious threat: the rise of antibiotic resistance in hospitals, communities, and the environment concomitant with their use (Davies & Davies, 2010).

3.3.2. Antibiotic resistance and need for new antibiotics

The extraordinary genetic capacities of microbes, namely their ability to exchange small packages of genetic information on plasmids, have benefited from man's overuse of antibiotics to exploit every source of resistance genes and every means of horizontal gene transmission (i.e., from one cell to another) to develop multiple mechanisms of resistance for each and every antibiotic introduced into practice clinically, agriculturally, or otherwise (Davies & Davies, 2010; Morse & Meitzner, 2013). In May 2015, a global action plan was endorsed by World Health Assembly to tackle antimicrobial resistance (including antibiotic resistance). The increase of the investment in new medicines is part of the strategic objectives delineated to achieve the action plan goal, which is to ensure continuity of successful treatment and prevention of infectious diseases with effective and safe medicines (World Health Organization, 2015).

3.3.3. *E. coli* and *S. aureus*: from commensalism to lethal infections

Escherichia coli is a rod-shaped, gram negative bacteria that colonizes the gastrointestinal tract of human infants within a few hours after birth, being the most abundant facultative anaerobe of the human intestinal microflora. Usually, *E. coli* coexists with its human host in good health and with mutual benefit for decades, rarely causing disease except in immunocompromised hosts or where the normal gastrointestinal barriers are breached (as in peritonitis, for example). The niche of commensal *E. coli* strains is the mucous layer of the mammalian colon, where they are a highly successful competitor (Kaper, Nataro, & Mobley, 2004). This could be due to its ability to utilize gluconate in the

colon in a more efficient way than other resident species, allowing it to occupy a highly specific metabolic niche (Sweeney et al., 1996). Nevertheless, several highly adapted *E. coli* clones have acquired specific virulence attributes, which confers an increased capacity to adapt to new niches. Only the most successful combinations of virulence factors have persisted to become specific patotypes of *E. coli* that are capable of causing disease in healthy individuals, such as enteric/diarrhoeal disease, urinary tract infections or sepsis/meningitis (Kaper et al., 2004).

Staphylococcus aureus is a round-shape, facultative anaerobic gram-positive bacteria with the ability to asymptomatically colonize healthy individuals. The ecological niches of *S. aureus* strains are the anterior nares. Over time, three patterns of carriage can be distinguished in healthy subjects: about 20% of people are persistent carriers (carrying almost always one type of strain), 60% are intermittent carriers, and approximately 20% almost never carry *S. aureus* (Kluytmans, Van Belkum, & Verbrugh, 1997). However, *S. aureus* also constitutes a versatile and dangerous pathogen in humans (Lowy, 1998). Although it be naturally susceptible to almost every antibiotic that has ever been developed, the treatment of infections caused by *S. aureus* has become more difficult due to its notorious capacity to become resistant to antibiotics (Chambers & DeLeo, 2009).

Escherichia coli and *Staphylococcus aureus* are the main causes of bloodstream infections (BSIs) in humans. A remarkably increase in the number of BSIs caused by *E. coli* along with an alarming increase of antimicrobial multi-resistance were observed in Europe from 2002 to 2009 (Gagliotti et al., 2011). In turn, of all the resistance traits that *S aureus* has acquired since the introduction of antimicrobial chemotherapy, meticillin resistance is clinically the most important, since a single genetic element confers resistance to the most commonly prescribed class of antimicrobials − the β-lactam antibiotics (Grundmann, Aires-de-Sousa, Boyce, & Tiemersma, 2006). The antimicrobial therapy used to treat infections caused by these microorganisms has therefore become problematic, emphasizing the need of a rational use along with a pharmaceutical investment in antibiotic research and development.

3.3.4. Fungi

Fungi are made up of a polyphyletic group of achlorophyllous, heterotrophic, eukaryotic and spore-bearing organisms (Sharma, 1988), surrounded by a cell wall mainly composed by chitin, glucans and glycoproteins (Bowman & Free, 2006). Most of fungi

belong to Eumykota (= Kingdom Fungi), commonly called *true fungi*, which are classified into four phyla: Chytridiomycota, Zygomycota, Ascomycota and Basidiomycota. The phylum Ascomycota (colloquially called ascomycetes) is by far the largest group of fungi. Ascomycetes could be found in a wide range of habitats and may grow either as yeasts, i.e., unicellular organisms multiplying by budding or fission, or as mycelia consisting of separate hyphae (molds). Ascomycetes present a wide range of lifestyles: while some of them are saprotrophs, others are necrotrophic or biotrophic parasites of both plants and animals, including humans (Bowman & Free, 2006).

Fungi have emerged as major causes of human disease, specially among the hospitalized patients with serious underlying disease and immunocompromised hosts (Pfaller & Diekema, 2007). In fact, the frequency of human invasive mycoses due to opportunistic fungal pathogens has increased significantly over the past two decades (Hajjeh et al., 2004; Pfaller & Diekema, 2004; Walsh et al., 2004). This increase in infections is associated with high morbidity and mortality (Gudlaugsson et al., 2003; Lin, Schranz, & Teutsch, 2001), being directly related to an increase in the number of patients who are at risk for the development of serious fungal infections, which includes individuals undergoing solid-organ transplantation (Pappas et al., 2010), blood and marrow transplantation (Marr & Bowden, 1999) and major surgery (Dean & Burchard, 1996); patients with AIDS (Armstrong-James, Meintjes, & Brown, 2014), neoplastic disease (Gold, 1984) and advanced age (Kauffman, 2001); patients receiving cytotoxic therapies, and very low birth weight infants. Besides the well-known opportunists *Candida albicans* and *Aspergillus fumigatus,* serious life-threatening infections are being reported with an ever increasing array of pathogens, including various dematiaceous molds such as *Alternaria* species (Walsh, 2003).

Candidiasis is a mycosis that can be caused by any of the pathogenic *Candida* species (Gow, van de Veerdonk, Brown, & Netea, 2011), which are the most common cause of fungal infections. Of them, *C. albicans* is the most prevalent (Gow et al., 2011), accounting for over half of all cases (Perlroth, Choi, & Spellberg, 2007; Pfaller & Diekema, 2007). Some fungal pathogens of humans, including *C. albicans*, may switch from the yeast to the filamentous state, a trait termed fungal dimorphism (Gow et al., 2011; Webster & Weber, 2007). *C. albicans* colonizes the skin, genital mucosa and/or intestinal mucosa of 30-70% of healthy individuals at any given moment. So, under normal circumstances, the fungus does not cause significant disease. However, in the absence of

proper immune recognition, the inability to control colonizing *C. albicans* on mucosal surfaces can lead to severe infection (Gow et al., 2011).

Aspergillosis is the name given to a wide variety of diseases caused by infection by fungi of the genus *Aspergillus*. Of them, allergic bronchopulmonary aspergillosis, pulmonary aspergilloma and invasive aspergillosis are the most common forms. These syndromes involve mycelial growth in the body, are difficult to detect (specially in early stages) and require aggressive therapeutic intervention. The genus *Aspergillus* comprises various human pathogens, however *Asp. fumigatus* stands out as the most prevalent airborne fungal pathogen in developing countries, causing severe and usually fatal invasive infections in immunocompromised hosts. *Asp. fumigatus* is a saprotroph widespread in nature, typically found in soil and decaying matter. It sporulates abundantly, with every conidial head producing thousands of easily dispersed small (2-3 μm) and slight conidia. These features turn them buoyant, keeping them airborne both indoors and outdoors. As a consequence, several thousands of conidia are daily inhaled by humans. Unlike what happens with immunocompromised patients, adverse effects rarely happen in immunocompetent individuals since the conidia are eliminated by innate immune mechanisms in a relatively efficient way (Latgé, 1999). However, this fungus is among one of the organisms recognized in fungal sensitization, a process operated by the competent immune system and that leads to respiratory allergies.

Dematiaceous fungi, i.e., pigmented filamentous fungi which contain melanin in their cell walls, are ubiquitous saprophytes in water, soil, and vegetation and may be plant pathogens or airborne spores (Georgiev, 2003). Furthermore, they can also infect humans, causing a wide range of diseases, such as phaeohyphomycosis, chromoblastomycosis, and eumycotic mycetoma (Brandt & Warnock, 2003). Among the members of *Dematiaceae*, *Alternaria* species are increasingly found as aetiologic agents of human disease due to the growing number of immunocompromised patients (Anjos et al., 2012). Despite *Alternaria infectoria* be a rare opportunistic agent of phaeohyphomycosis, several clinical cases have been reported, specially in renal transplant recipients (Halaby et al., 2001; Nulens et al., 2006; Segner, Jouret, Durant, Marot, & Kanaan, 2009) and immunocompromised hosts (Hipolito et al., 2009).

3.3.5. Antifungal treatment

Invasive fungal infections are a serious threat to human health and, although the current antifungal therapies have been significantly improved, the outcome is still far from satisfactory (Zhai & Lin, 2011).

On the one hand, the number of therapeutic options for the treatment of invasive fungal infections is quite limited when compared with those available to treat bacterial infections. In fact, two of the three classes of antifungal drugs (azoles and polyenes) that are currently used had been introduced in clinical practice by 1980, and the third class, the echinocandins, had been discovered more recently (Roemer & Krysan, 2014). Additionally, proper and early diagnosis is quite challenging, conditioning the future treatment. The choice of an appropriate antifungal therapy for invasive candidiasis depends upon specific clinical circumstances that includes, among others, the condition of the patient, relevant comorbidities, illness severity, histories of recent azole exposure and intolerance to antifungal agents (Pappas et al., 2009). Generally, amphotericin B–based preparations, the echinocandins antifungal agents and the azole antifungal agents play a role in treatment of invasive candidiasis, while therapy for mucosal infections is dominated by the latter (Pappas et al., 2004). In turn, the Food and Drug Administration (FDA)-approved antifungal compounds that have *in vitro*, *in vivo*, and clinical activity against *Aspergillus* species and are licensed for treatment of invasive aspergillosis are: the polyene amphotericin B deoxycolate (D-AMB) and its lipid formulations (AMB lipid complex [ABLC], L-AMB, and AMB colloidal dispersion [ABCD]); the azoles itraconazole, voriconazole and, posaconazole; and the echinocandins caspofungin (Walsh et al., 2008). Although there are no standardized therapies for infections caused by dematiaceous fungi, the azoles voriconazole, posaconazole and itraconazole, and, in some cases, AMB, have demonstrated the most consistent *in vitro* activity against this group of fungi (Chowdhary et al., 2014).

On the other hand, the development of resistance to current antifungals is a real and worrying burden, being reported either in *C. albicans* (Cannon et al., 2007) as in *Asp. fumigatus*. Azole resistance in *Asp. fumigatus* is an emerging problem (Chowdhary, Kathuria, Xu, & Meis, 2013; Howard et al., 2009; Snelders et al., 2008; van der Linden et al., 2015), causing resistant invasive and noninvasive aspergillosis and, therefore, compromising severely its clinical use. The increasing need for novel antifungal agents is therefore evident.

3.3.6. Antifungal drug targets

Because both fungi and mammals are eukaryotic organisms, the antifungal drugs have to exploit differences between their cells to kill only the fungi without damaging the mammalian host. Due to its specific composition, the fungal cell wall and the underlying plasma membrane are unique targets for the development of antifungal drugs (Tada, Latgé, & Aimanianda, 2013).

3.3.6.1. Antifungal drugs targeting the fungal cell membrane

Along with the glycerophospholipid bi-layer, sterols are the major lipid components of the eukaryotic cell membrane. In fungi, ergosterol is the main sterol, replacing the cholesterol found in the mammalian cell membrane (Tada et al., 2013). Ergosterol is one of the most important components in fungal membranes, being involved in several biological functions, such as membrane fluidity and asymmetry regulation, determinant to the membrane integrity (Ghannoum & Rice, 1999). Furthermore, it is also involved in the activity and distribution of integral proteins and control of the cellular cycle (Alcazar-Fuoli et al., 2008). Ergosterol is synthesized in the endoplasmic reticulum through arranged reactions involving more than 20-steps, being this pathway essential for fungal growth. Thus, ergosterol, as such in the membrane or its biosynthetic pathway, constitutes an attractive antifungal drug target. To date, four major classes of inhibitors targeting and/or interfering with ergosterol structure or its biosynthesis have been identified – morpholines, allylamines, polyenes and azoles (Tada et al., 2013).

Morphollines and allymalines interfere with ergosterol biosynthesis by acting on certain enzymes of the biosynthetic pathway (Tada et al., 2013).

Polyenes are amphiphilic molecules, being amphotericin B the most commonly used (Tada et al., 2013). AMB primarily acts by binding to ergosterol, leading to the formation of ion channels and fungal cell death. A second mechanism of action may involve oxidative damage on the cell through a cascade of oxidative reactions linked to lipoperoxidation of the plasma membrane (Walsh et al., 2008). However, this drug has several disadvantages (Tada et al., 2013). For example, AMB is toxic to humans because it also binds to cholesterol (although with less avidity than for ergosterol), resulting in cellular injury and end organ dysfunction (Walsh et al., 2008).

Azoles, especially the last generation of lipophilic triazoles, namely itraconazole, fluconazole, voriconazole and posaconazole, are the most used antifungals. Their main effect is to inhibit cytochrome P450 14-α-sterol demethylase and the subsequent C-22 sterol desaturase step in the ergosterol biosynthetic pathway. The lack of major toxicity for the patients constitutes an advantage of these drugs (Tada et al., 2013).

3.3.6.2. Agents interfering with the biosynthesis of the cell wall

The cell wall of fungi is a dynamic organelle that plays a role in numerous important processes (Bowman & Free, 2006; Latgé, 2007). It must provide the cell with sufficient mechanical strength to cope with variations in osmotic pressure imposed by the environment, but, at the same time, must retain adequate plasticity to allow for cell growth, cell division and the formation of a myriad of cell types during the life cycle of the fungus. Besides the maintenance of cell shape and integrity towards environmental stress, the wall allows the interaction between the fungal cell and its surroundings. Additionally, the cell wall mediates the adhesion of cells to one another and the substratum, and serves as a signalling centre to activate signal transduction pathways within the cell (Bowman & Free, 2006). Disruptions of cell wall structure could have a profound effect on the growth and morphology of the fungal cell, often rendering it susceptible to lysis and death (Bowman & Free, 2006; Latgé, 2007).

Fungal cells are characterized by the presence of a cell wall that accounts for almost 30% of its dry weight and for being mainly composed by different polysaccharides that are exclusive to the fungal kingdom (Tada et al., 2013). The skeletal fibrillar central core is made up of a β-(1,6)-branched β-(1,3)-glucan cross-linked to chitin, located proximate to the plasma membrane and attributes for the mechanical strength of the fungal cell. Amorphous polysaccharides are located in the entire cell wall, especially in the outer surface layer, along with glycoproteins (Bowman & Free, 2006; Tada et al., 2013).

Because of its vital biological role, unique biochemistry and structural organization, as well as the absence of most of its constitutive components in mammalian cells, the cell wall has long been considered an excellent target for the development of new antifungal agents (Latgé, 2007). In particular, the synthesis of chitin and β-(1,3)-glucan, due to their essentiality in the cell wall structure, have been the targets of past and ongoing search for new antifungal drugs (Tada et al., 2013).

a. β-(1,3)-Glucan synthase inhibitors

β-(1,3)-Glucan is the major polysaccharide component in the fungal cell wall, being synthesized by β-(1,3)-glucan synthase, a membrane-multiprotein associated complex. This enzyme has been clinically targeted by antifungals. In fact, three chemical families of antifungal drugs inhibiting β-(1,3)-glucan synthase activity (thus causing β-(1,3)-glucan defect within cell wall) have been identified: papulacandins, acidic terpenoids and echinocandins. However, currently, only the echinocandins, including micafungin, anidulafungin and caspofungin, are commercially available and applied into clinical practice. The major advantage of echinocandins is the absence of toxicity against mammalian cells, since their target (β-(1,3)-glucan synthase) does not exist in mammals. The mode of action of these non-competitive inhibitors of the glucan synthase remains unknown, and this lack of understanding is also appearing in the paradoxical effect, where high concentrations of drug do not kill better than low concentrations. Nevertheless, it has been found that echinocandins act on susceptible *Candida* and *Aspergillus* spp. On the one hand, they presented fungicidal activity against *Candida* spp. and are reported to be active against azole resistant *Candida* spp. On the other hand, and unlike other classes of antifungal drugs, echinocandins do not show clear growth end points against *Asp. fumigatus in vitro* but have been shown to be very effective in animal models of systemic fungal infections (Tada et al., 2013). Indeed, a clinical trial has demonstrated the echinocandins efficacy in the treatment of invasive aspergillosis in these models (Bowman & Free, 2006).

b. Chitin synthase inhibitors

Chitin synthases are enzymes that catalyze the formation of chitin, being delivered to areas of new growth by secretory vesicles called chitosomes (Munro, 2013). Fungi contain various chitin synthases that have been reported to perform distinct functions. Certain enzymes have specific roles at specific parts of the cell and have to be targeted to this sites to perform their function (Munro & Gow, 2001). While *C. albicans* has four chitin synthases (Chs1, Chs2, Chs3 and Chs8) (Munro & Gow, 2001; Munro, 2013), eight chitin synthase genes were identified in *A. infectoria* (*CHSA* to *CHSH*) (Fernandes et al., 2014) and in *Asp. fumigatus* (*CHSA*, *CHSB*, *CHSC*, *CHSD*, *CHSF*, *CHSG*, *CSMA* [earlier called CHSE] and *CSMB*) (Jiménez-Ortigosa et al., 2012). CsmA and CsmB are two chitin

synthases with a myosin-motor-like domain. Nikkomycins and polyoxins, the classical inhibitors of chitin synthesis, are substrate analogues of UDP-*N*-acetylglucosamine, the essential initial substrate for chitin biosynthesis (Tada et al., 2013). Polyoxins and nikkomycins could bind to the catalytic site of chitin synthases, which are not equally susceptible to the drugs. These agents are reported to be active *in vitro* but poorly effective *in vivo*. The low permeability, different susceptibility of fungal species and variable responses in animal models are the main problems for their successful use *in vivo* (Ruiz-Herrera & San-Blas, 2003; Tada et al., 2013).

4. Integrated multi-trophic aquaculture (IMTA)

The industry uses 7.5-8 million tonnes of wet seaweed annually, harvested either from naturally grown (wild) seaweed or from cultivated (marine agronomy, farmed) crops. The farming of seaweed has expanded rapidly as demand has outstripped the supply available from natural resources (FAO, 2004).

In marine aquaculture, integrated systems have emerged in the past decades as a response to the problems created by pollution caused by the excess of nutrients generated by intensive aquaculture. The increasing use of coastal areas around the world, along with the rapid growth and expansion of mariculture, led to a need for more sustainable and acceptable practices to consumers, as well as other users of the sea and coastal zones. Integrated multi-trophic aquaculture. Thus, integrated multi-trophic aquaculture (IMTA) arise as a concept of aquatic production that not only focus on the reduction of the negative environmental impacts caused by pollution, but also tries to reproduce natural systems and incorporate various productions in order to optimize resources utilization. The IMTA concept is quite flexible, comprising land-based or offshore mariculture systems, as well as fresh water aquaculture systems, and may include combinations of a number of different species (e.g. bivalve/fish and shrimp/seaweed/bivalve) (Pinto & Abreu, 2011).

In IMTA at the sea and in coastal areas, when there is an integration of different trophic levels of organisms into the same system, the wastes of one species are used by another one, as happens in natural ecosystems. Species fed with feed, such as fishes and shrimps, produce particulate organic matter (POM; food waste, feces) and excrete ammonium (NH_4^+) and carbon dioxide (CO_2). These subproducts are utilized by seaweeds that absorb and accumulate nitrogen (N) and phosphorous (P) from dissolved NH_4^+ and phosphate (PO_4^{3-}), respectively, as well as CO_2 (Figure 3). Filter feeders and scavenger

organisms utilize POM as feed. Besides, seaweed produce oxygen (O_2), which is utilized by the animals (Pinto & Abreu, 2011).

The outcome of the first workshop about IMTA in the United States of America (2010) is that this kind of systems has the potential to overcome some of the limitations that have prevented the growth of aquaculture, namely the public acceptance. This issue can be ameliorated since this practices have a low environmental impact and a diversification of products, which turns aquacultures more resistant to market price fluctuations and less affected by illnesses (Pinto & Abreu, 2011).

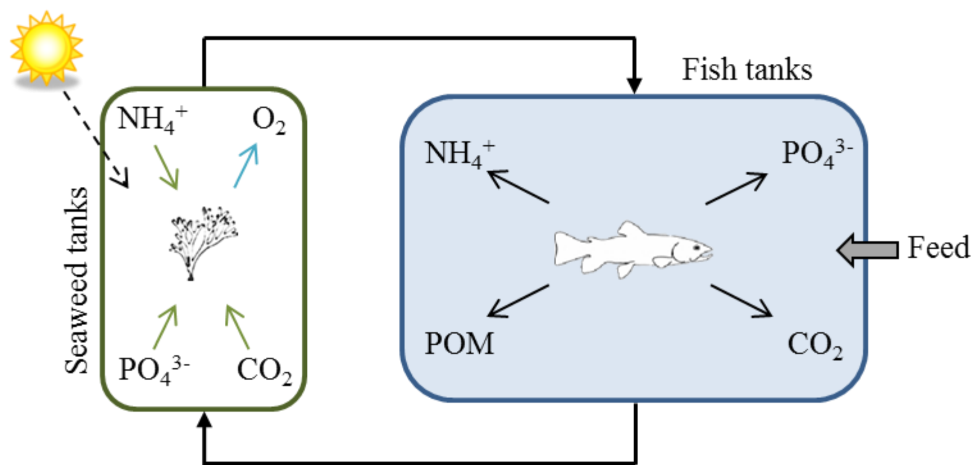


Figure 3. Schematic representation of a land-based integrated multi-trophic aquaculture (IMTA) system (adapted from Pinto & Abreu, 2011).

5. Aim and scope of the work

Osmundea pinnatifida is a red seaweed quite abundant throughout the year in the north and central coast of Portugal. This seaweed specie has the particularity of being edible, which indicate that, *a priori*, has no toxicity to humans. This feature facilitates the development and implementation of potential biotechnological applications, particularly in the area of human health. Recently, the nutritional value of this macroalgae have been the subject of a host of studies. However, its biological activities still need to be further exploited. Hence, the main aims of this study were to extract bioactive compounds from wild and IMTA-cultivated *O. pinnatifida* using organic solvents with different polarities – methanol (polar), dichloromethane (intermediate polarity) and *n*-Hexane (apolar) –, as well as to evaluate its biotechnological potential with regard to the antioxidant, antitumor and

antimicrobial (antibacterial and antifungal) activities. In order to evaluate the antioxidant capacity, DPPH and ORAC methods were used, along with the total phenols assay by FCR. In attempt to assess its cytotoxic potential, cell viability and cell proliferation studies were carried out, through MTT method, in a carcinoma model of a human hepatocellular cancer (HepG-2), a human breast adenocarcinoma model (MCF-7) and a model of human neuroblastoma (SH-SY5Y) cells. A standardized broth microdilution assay was used to determine the antibacterial potential against *E. coli* and *S. aureus*. In turn, to evaluate the antifungal activity, three organisms were tested: *C. albicans*, *A. infectoria* and *Asp. fumigatus*. The standardized disk diffusion and broth microdilution methods were used to evaluate the antifungal capacity against *C. albicans*. Radial growth inhibition and standardized broth microdilution assays were performed to assess the antifungal activity against *A. infectoria* and *Asp. fumigatus*. Lastly, light, confocal and fluorescence microscopy observation, along with chitin and β-glucan quantification were used to study the effect of *O. pinnatifida* extracts on the *A. infectoria* and *Asp. fumigatus* fungal cell wall.

MATERIALS AND METHODS

6. Seaweed collection and preparation

Osmundea pinnatifida specimens were collected in November 2013 at the rocky shore of Mondego Cape (40º 10’ 557” N, 8º 53’ 819”), Figueira da Foz, Portugal (Figure 4). After being removed from the substrate, the algae were firstly washed with current seawater. In the lab, a new wash was carried out with seawater to remove epiphytes and incrusting material, and then the biomass was centrifuged with a manual salad spinner, weighted and split into two portions. One of them was used to undergo small scale cultivation trials at ALGAplus facilities, located in Ria de Aveiro, Portugal. The other portion (wild biomass) was additionally washed with distilled water to remove salt, centrifuged, and hermetically stored at -20 ºC.

Figure 4. *Osmundea pinnatifida* collecting site: Mondego Cape (Figueira da Foz).

6.1. Seaweed cultivation trials

The seaweed farm ALGAplus is integrated with a seabream and seabass commercial aquaculture and uses the nutrient enriched water resultant from the fish production as its cultivation medium (IMTA concept). Two types of pilot axenic cultivation assays were carried out: (A) laboratory and (B) land-based IMTA trials (Figure 5). All biomass resultant from both types of assays was centrifuged and then frozen at -20 °C until further use.

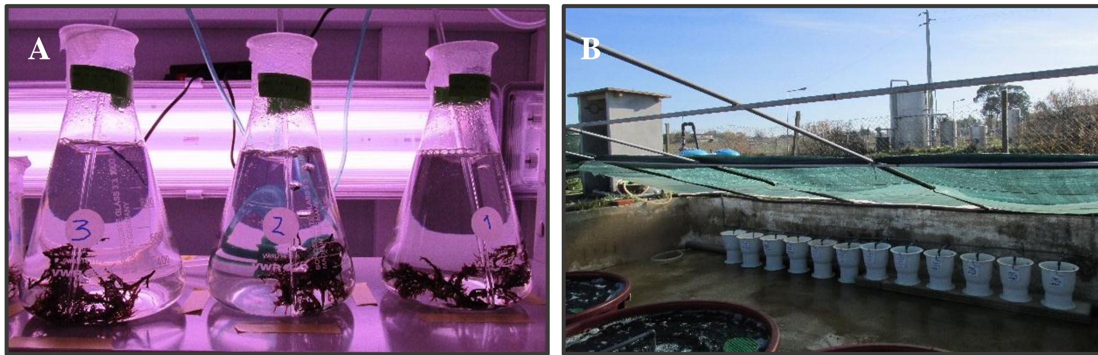


Figure 5. *Osmundea pinnatifida* cultivation trials: laboratory (A) and land-based IMTA (B) assays.

6.1.1. Laboratory trials

Two *in vitro* cultivation trials with temperature, photoperiod, irradiance and nutrients kept at values referred to as non-limiting for macroalgae development (15 ºC, alternating 16-h light and dark cycles, light intensity of 60 ± 10 μmol/m^2/s produced by fluorescent lamps [OSRAM L36W/77 Fluora, Germany]) evaluated the growth performance of *O. pinnatifida* (Figure 5A). Each laboratory trial was ran at least in triplicate ($n \geq 3$). Briefly, 4 g of biomass, previously washed with sterile salt water and centrifuged, were placed aseptically in each Erlenmeyer flask containing 1 L sterile salt water supplemented with culture medium. Flasks are coupled with an air bubbling system, responsible for maintaining the biomass in continuous circulation in the water column. Water and culture medium are changed twice a week, with 3/4 days of interval. Salinity, water temperature and pH are monitored with an HQ40d portable meter (Hach Company, USA) before and after carrying out the exchange. In the first laboratory trial (Lab TI), the biomass used was harvested in Mondego Cape. In addition to the procedures of preparation of samples described in the previous section, the biomass was further disinfected with a 10% betadine solution for 20 seconds before the assay begin. Nutrients are delivered by Guillard’s (f/2) marine water enrichment solution, which was provided by ALGAplus, and stocking density was maintained in each flask at 4g biomass/L in a weekly basis. In the second trial (Lab TII), *O. pinnatifida* specimens were collected in Mindelo beach, Vila do Conde, Portugal, at late January 2014 and were acclimated to the assay conditions for 3 months. In this case, each liter of filtered sterile seawater was enriched with 6 mL of von Stosch's enriched seawater medium (VSE) and with 1 mL germanium dioxide (GeO_2), which were provided by ALGAplus. The biomass remained in culture from the beginning to the end of the assay.

6.1.2. Land-based IMTA trials

Five week-long outdoor cultivation assays (Figure 5B) were conducted in two different periods, from November to December 2013 (*n*=4) and from May to June 2014 (*n*=3), in land-based IMTA conditions. Seaweed was grown by vegetative propagation in a batch-culture mode (in 15L fiber-glass tank), using the nutrient-enriched fish water (renewed every 3-4 days) and monitoring temperature, irradiance, salinity and pH. 150g of biomass were placed in each one of the 15L fiberglass tanks. Seaweed were maintained under continuous circulation in the water column through a bubbling air system. Irradiance measurement was carried out immediately below the water surface with a MQ-200 quantum sensor (Apogee Instruments, USA), which measure photosynthetically active radiation. The referred parameters were measured 3 times per day (11 a.m., 14 p.m. and 17 p.m.) from Monday through Friday. Stocking density in each tank was kept at 10g/L, by trimming the excess biomass, resulting from seaweed growth, on a weekly basis.

6.1.3. Growth parameters

The growth performance of *Osmundea pinnatifida*, both in laboratory and IMTA conditions, was assessed on a weekly basis through relative growth rate (RGR) and productivity.

The RGR of the seaweeds in flasks and tanks, expressed as percentage of growth per day (% day^{-1}), was calculated using the following equation:

$$\text{RGR (\% day}^{-1}) = \left(\frac{\ln(\text{Fw}) - \ln(\text{Iw})}{\text{t}}\right) \times 100,$$

where Fw is the final fresh weight (g) and Iw is the initial fresh weight (g) of seaweed; t corresponds to number of days in cultivation.

In turn, productivity of the tanks, expressed as mg dry weight per liter per week (mg dw L^{-1} wk^{-1}), was determined by the equation

$$\text{Productivity (mg dw L}^{-1}\text{ wk}^{-1}) = 0.164 \times \left(\frac{(\text{Fw} - \text{Iw})}{\text{V}}\right),$$

where 0.164 is the dry weight to fresh weight proportion (mean of 3 samples) and V is the volume of water in the tanks (L).

6.2. Preparation of seaweed extracts

All the biomass produced in the land-based IMTA assay conducted from November to December 2013 (cultivated sample), as well as wild biomass (wild sample) collected from Mondego Cape in November 2013 were used to obtain extracts.

Different *Osmundea pinnatifida* extracts were prepared according to the method of Barreto et al. (2012) with slight modifications.. Seaweed samples, previously frozen at -80 ºC (Thermo, Electron Corporation, USA) and freeze-dried (SCANVAC CoolSafe, Labogen, Sweeden), were separately ground to yield a powder, in which organic solvents of different polarities were added, namely methanol (> 99%), dichloromethane (> 99%) and *n*-hexane (99%). The powder was placed in a beaker, methanol was added in a 4:1 ratio (solvent:sample powder; mL/g), and extraction was carried out for 12 h at room temperature, under continuous stirring and protected from the light using aluminium foil. The content of the beaker was then filtered through Whatman™ filter paper, yielding the methanol extract (filtrate) and the sample powder (residue). The latter, after drying at room temperature, was placed in a beaker with dichloromethane, added in a 4:1 proportion (solvent: sample powder, mL/g) and extracted in the above-described conditions. It is therefore a sequential extraction procedure, wherein the sample used is the same from the beginning to the end of the process. The content of the beaker was then filtered, yielding the dichloromethane extract. In turn, methanol extract was placed in a separatory funnel and *n*-hexane was added in a 1:1 proportion (v/v), proceeding to a liquid-liquid extraction. Thus, methanol extract was partitioned, yielding the methanol and hexane fractions. The obtained extract and fractions were evaporated to dryness in vacuum, using a rotary evaporator (Laborota 4000, Heidolph), at 40 ºC. Additionally, they were subjected to a nitrogen flow, pointing downward on the sample surface, to ensure that all solvent was evaporated. At the end, the dry biomass of each extract/fraction were solubilized, or in dimethyl sulfoxide (DMSO; ≥99.9%, Sigma) – for antioxidant, cytotoxic and antiproliferative capacity assays – or in sterile ultrapure water – for antimicrobial assays - and kept at -20 ºC until further use.

7. Evaluation of antioxidant potential

As suggested by Huang et al. (2005), to comprehensively study different aspects of antioxidants, validated and specific assays are needed in addition to total phenols assay by FCR and ORAC assay. Thus, DPPH radical scavenging capacity assay was also carried out.

7.1. Total phenols assay

The concentration of polyphenols in *O. pinnatifida* extracts/fractions was determined with Folin-Ciocalteu method adapted to microscale (Zou, Chang, Gu, & Qian, 2011) with minor modifications. Gallic acid was used as a standard phenolic compound. Briefly, 2 µL of extract/fraction or standard gallic acid (Sigma-Aldrich) solutions (10, 30, 100, 300, and 1000 µg/mL), prepared with distilled water, were added to 158 µL of distilled water in a 96-well microplate, followed by 10 µL of Folin-Ciocalteu reagent (Sigma-Aldrich). The reaction mixture was pre-incubated for 2 min at room temperature and then 30 µL of 20% (weight/volume) sodium carbonate (Na_2CO_3; Panreac) was added and mixed. After one hour of reaction in the dark, the absorbance was measured at 755 nm (Synergy H1 Multi-Mode Microplate Reader, BioTek Instruments, USA) against blank solution (prepared by the same procedure described above, except that sample solution was replaced by 2 µL of water) and used to calculate the phenolic content. Total phenolic content (TPC) is expressed as mg gallic acid equivalents per gram of dry extract (mg GAE/g).

7.2. Evaluation of DPPH radical scavenging activity

DPPH radical scavenging activity was assessed by the DPPH decolourization assay, adapted to microscale (Herald, Gadgil, & Tilley, 2012) with slight modifications. DPPH radical (Sigma-Aldrich) was dissolved in absolute ethanol. Various concentrations of 2 µL of sample solution were added to 198 µL of the DPPH radical solution (0.1 mM). The mixture was vortexed for 1 min and allowed to stand at room temperature in the dark for 30 min, at which time the decrease in absorbance at 517 nm was measured in the microplate reader. The radical solution was freshly prepared each day. The synthetic antioxidant butylated hydroxytoluene (BHT; Sigma-Aldrich) was used as positive control.

The ability of test samples to scavenge DPPH radical was calculated using the following equation:

$$\text{DPPH radical scavenging activity (\% of control)} = \left[1 - \left(\frac{A_{smp} - A_{smp\ blk}}{A_{ct}}\right)\right] \times 100$$

where A_{ct} is the absorbance of the control (DPPH solution with DMSO), A_{smp} is the absorbance of the test sample (DPPH solution plus test sample), and $A_{smp\ blk}$ is the absorbance of the sample in ethanol (sample without DPPH solution). Results are expressed as mean values ± SD. EC_{50} values (µg/mL) were also determined for the extracts/fractions which scavenged over 50% of the DPPH radical.

7.3. Evaluation of oxygen radical absorbance capacity (ORAC)

ORAC-fluorescein assay was performed as described by Dávalos et al. (2004) as follows: the reaction was carried out in 75 mM phosphate buffer (pH 7.4), and the final reaction mixture was 200 µL. Sample (20 µL) and fluorescein (120 µL; 70 nM, final concentration; Sigma-Aldrich) were placed in the well of the 96-well microplate. The mixture was pre-incubated for 15 min at 37 °C. AAPH solution (60 µL; 12 mM, final concentration; Sigma-Aldrich) was added rapidly using a multichannel pipet. The microplate was immediately placed in the reader and the fluorescence ($\lambda_{excitation}$: 458 nm, $\lambda_{emission}$: 520 nm) recorded every minute for 240 min. The microplate was automatically shaken prior to each reading. A blank using phosphate buffer instead of the fluorescein and eight calibration solutions using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (1-8 µM, final concentration; Sigma-Aldrich) as antioxidant were also carried out in each assay. All the reaction mixtures were prepared in duplicate, and at least three independent assays were performed for each sample. Antioxidant curves (fluorescence versus time) were first normalized to the curve of the blank corresponding to the same assay by multiplying original data by the factor $\text{fluorescence}_{blank,\ t=0}/\text{fluorescence}_{sample,\ t=0}$. From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as:

$$\text{AUC} = 1 + \sum_{i=1}^{i=80} \frac{f_i}{f_0}$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time *i*. The net AUC corresponding to a sample was calculated by subtracting the AUC corresponding to the blank. Regression equations between net AUC and antioxidant concentration were calculated for all the samples. ORAC values were expressed as Trolox equivalents by using the standard curve calculated for each assay. Final results were in µmol of Trolox equivalents/g of dry extract (µmol TE/g).

8. Evaluation of antitumor potential

The evaluation of cytotoxic potential (cell viability and cell proliferation) of different *O. pinnatifida* extracts was performed in three human tumor cell lines: HepG-2 cells, MCF-7 cells and SH-SY5Y cells. The carcinoma model of a human hepatocellular cancer (HepG-2) was acquired in the American Type Culture Collection (ATCC) (ATCC HB-8065). The human breast adenocarcinoma model (MCF-7 cells) (ACC 115) and the model of human neuroblastoma (SH-SY5Y cells) (ACC 209) were, in turn, acquired in DMSZ bank. HepG-2 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich) supplemented with 10% of fetal bovine serum (FBS; SV30160.03, Hyclone) and 1% of antibiotic/antimycotic commercial solution (SV30079.01, Hyclone). MCF-7 cells were also cultured in RPMI-1640 medium, but supplemented with 1% of Minimum Essential Medium (MEM) non-essential amino acids solution (Sigma-Aldrich), 1mM of sodium pyruvate (Sigma-Aldrich), and 10 µg/mL of human insulin (Sigma-Aldrich). In turn, SH-SY5Y cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich) supplemented with 20% of FBS and 1% of antibiotic/antimycotic commercial solution. The culture medium was always renewed 12 hours after the seeding of the cells. Every two days, the culture medium was renewed in both cell lines. The cells were maintained in the following controlled conditions: 95% of humidity, 5% of CO_2 and constant temperature of 37ºC (Unitherm CO_2 Incubator).

For subculture, the cells used were dissociated with trypsin-EDTA (ethylenediaminetetraacetic acid; Sigma), split into a 1:3 (HepG-2) and 1:4 (MCF-7 and SH-SY5Y) ratio and subcultured into Petri dishes with 25 cm^2 growth area. Culture medium was replaced every 2 days until the cells reached the total confluence after 5-6 days of initial seeding. Cells were maintained in the conditions described above.

Cytotoxicity was evaluated after the cells reached total confluence and anti-proliferative activity after 24 hours of initial seeding. Cells were incubated with crude extracts, previously sterile filtered (0.2 µm, Whatman), during 24 hours at 1 mg/mL. For the samples with highest activity, dose–response assays were done (10 – 1000 µg/mL; 24 hours). Cisplatin (Sigma-Aldrich) was used as positive control. The effects were estimated by colorimetric assay based on the conversion of tetrazolium dye (MTT, 1.2mM) to a blue formazan product by living mitochondria (Yuan & Walsh, 2006). Results were expressed as IC_{50}, defined as the concentration causing a 50% reduction or inhibition of cell viability and cell proliferation, respectively.

9. Evaluation of antimicrobial activity

The *in vitro* antimicrobial activity of *Osmundea pinnatifida* extracts and fractions was screened against two bacteria and three fungal species.

9.1. Microorganisms

The study included a gram-positive (*Staphylococcus aureus*) and a gram-negative (*Escherichia coli*) bacterial clinical isolates, kindly provided by Centro Hospitalar e Universitário de Coimbra (Coimbra, Portugal). These strains were identified through standard microbiology methods. Gram staining, catalase test, oxidase test were carried out, as well as analytical profile index (API), using API® ID 32 STAPH and API® ID 32 GN test strips (Biomérieux, France), and antibiotic susceptibility tests using ATB G-5 test strips (Biomérieux, France) and antibiogram.

The fungal specimens comprised a yeast strain (*Candida albicans* YP0037), from Microbiology Pathogenic Yeast Collection, University of Coimbra (Portugal), and two clinical mold isolates (*Aspergillus fumigatus* CBS 500.90 and *Alternaria infectoria* CBS 137.90), obtained from CBS-KNAW Fungal Biodiversity Centre (Utrecht, Netherlands).

All microorganisms were stored in sterile 20% glycerol, at -80 °C, and subcultured in solid medium before each test to ensure optimal growth conditions and purity. *S. aureus* and *E. coli* were subcultured in Columbia agar (42.5 g/L) at 37 ºC. *C. albicans* was subcultured in yeast-extract-peptone-dextrose (YPD) agar (20 g/L glucose, 10 g/L peptone, 5 g/L yeast extract, 20 g/L agar) at 30 ºC. In turn, *Asp. fumigatus* and *A. infectoria* were subcultured in potato dextrose agar (PDA; Difco) (39 g/L) at 30 ºC, being the latter strain

incubated under alternating 8-h light enriched with UV (lamp F15W T8BLB; Grainger, USA) and 16-h dark periods.

9.2. Antibacterial activity

Minimum inhibitory concentrations (MICs) for the extracts in study were determined by broth microdilution method, according to procedures described in the reference document M07-A9 of Clinical and Laboratory Standards Institute (CLSI, 2012), with some modifications. This approved guideline addresses methods for dilution antimicrobial susceptibility testing for bacteria that grow aerobically. The inoculum was prepared through direct colony suspension. Briefly, isolated colonies of each bacteria were picked up from fresh overnight cultures, grown at 37 ºC in GS – gelose Columbia agar (Biomérieux, France), and suspended in ampoules containing 2 mL of 0.85% NaCl (Api, Biomérieux, France). Turbidity was adjusted to 0.5 McFarland using a densitometer, resulting in a suspension containing approximately 1 to 2×10^8 colony-forming units (CFU)/mL. The final inocula were obtained by 20-fold dilution in sterile 0.85% NaCl, resulting in a final concentration of 5×10^6 to 1×10^7 CFU/mL. Then, 100 µL of Mueller-Hinton Broth (MHB; Difco), 50 µL of different concentrations of *O. pinnatifida* extracts, and 10 µL of the bacterial inoculum were added into each well of a round-bottomed microtiter plate (Costar). Each microdilution tray include at least 4 growth control wells (broth + extract diluent (sterile ultrapure water) + microorganism) for each microorganism tested, 2 sterility wells (broth + extract + sterile 0.85% saline) for each tested extract concentration, and 2 control wells of MHB alone. The experiments were performed in duplicate (n=2) and repeated independently two times (N=2). The microdilution trays were incubated at 35±2°C for 16 to 20 hours in an ambient air incubator, without agitation, within 15 minutes of adding the inoculum. MIC was determined as the lowest extract concentration that completely inhibits the growth of microorganism in the microdilution wells, as detected by the unaided eye.

9.3. Antifungal activity

9.3.1. Antifungal activity of *O. pinnatifida* extracts against *C. albicans*

The antifungal activity of the extracts against *C. albicans* was assessed through two different approaches according to Clinical and Laboratory Standards Institute (CLSI) standard procedures: disk-based testing and broth microdilution testing.

A disk diffusion susceptibility test was performed based on the document M44-A of the CLSI guidelines (CLSI, 2004) with modifications. Briefly, 90-mm-diameter Petri dishes were used, with an agar depth of 4-5 mm. Testing was performed on YPD agar (10 g/L peptone, 5 g/L yeast extract, 20 g/L agar; (Panreac)) supplemented with 0.1% of glucose (Merck). The inoculum, a yeast suspension in sterile 0.85% saline density was adjusted to 0.5 McFarland standard (approximately 1 to 5×10^{6} CFU/mL) using a densitometer. Then, 100 µL were loaded and spread evenly on the dried surface of the YPD agar plate. Next, sterile 6-mm-diameter paper disks (Becton Dickinson) were impregnated with 5 mg/mL of the respective extracts and placed on the surface of the inoculated agar plates. Results were determined, after a 24 h-incubation period at 30 ºC, by measuring the diameter of the growth inhibition halos.

The broth microdilution method was performed as outlined in CLSI, M27-A2 document (CLSI, 2002), with minor changes. The medium used was yeast nitrogen base (YNB; Difco; 6.7 g/L), supplemented with 2% glucose. The inoculum was prepared as described above to 0.5 McFarland standard and diluted 2000-fold in YNB broth medium. 180 µL of this suspension were added to each well of microtiter plates, previously loaded with 20 µL of each extract, at the concentrations 10, 100, 250 and 500 µg/mL. Microdilution plates were incubated without agitation at 37 ºC for 46 to 50 hours. The amount of growth in the test wells was compared visually with the growth-control wells.

9.3.1.1. Effects of *O. pinnatifida* extracts on yeast-to-hyphal transition by *C. albicans*

The morphogenetic changes involved in the transition from yeast cells to hyphae in *C. albicans* are a crucial step for invasion (Gow et al., 2011). Inhibition of filamentation has a key role in the progression to active disease, and this process stands as a potential

target for the development of a novel class of therapeutic agents against established fungal infections (Saville et al., 2006).

A microtiter method was developed and optimized to evaluate the capacity of *O. pinnatifida* extracts to inhibit filamentation by *C. albicans*. RPMI 1640 with L-glutamine, (Sigma R6504) was used as broth medium. Yeast cell suspensions were prepared in sterile 0.85% saline to an optical density of 0.4 at 600 nm (Jenway 6300 spectrophotometer) and diluted 20-fold in RPMI 1640 broth medium. 160 µL of the resulting suspension were added to each well of a round-bottomed microtiter plate, previously loaded with 20 µL of each extract at a concentration of 250 µg/mL, and supplemented with 10% (20 µL) of FBS or sterile heat inactivated FBS (HI-FBS). After 3-h-incubation at 37 ºC, without agitation, microscope slides were prepared and immediately observed using an Eclipse E400 epi-fluorescence microscope (Nikon, Japan). Images were recorded with a DS-5M digital camera (Nikon, Japan).

9.3.2. Antifungal activity of *O. pinnatifida* extracts against *Asp. fumigatus* and *A. infectoria*

The studied organic extracts were screened for antifungal activity against *Asp. fumigatus* and *A. infectoria* using a radial growth inhibition assay and a microdilution assay.

For radial growth inhibition assay the extracts were added to PDA at 43 ºC to obtain a final concentration of 10 and 100 µg/mL. *A. infectoria* fragments were applied at a single point on the surface of petri dishes, and these plates were incubated during 8 days at 30ºC under 12 h-alternating light and dark cycle. Radial diameter of growth was measured and compared relative to control plates lacking extracts. *Asp. fumigatus* conidia suspensions were prepared in sterile 0.1% Tween 80 (Sigma). Spore suspension was diluted 100-fold in sterile distilled water. 5 µL of this final inoculum were placed in the center of each petri plate. The plates were incubated at 30 ºC for 8 days. Fungal growth was assessed by measuring the colony diameter. The inhibitory growth effect of each extract against *A. infectoria* and *Asp. fumigatus* was quantified in terms of percentage of inhibition of radial mycelial growth, as calculated by the formula:

$$\text{Growth inhibition (\%)} = \left(\frac{Dc - Dt}{Dc}\right) \times 100,$$

where Dc is the average of the diameter of colonies in control plates (mm) and Dt is the average of the diameter of colonies in treatment plates (mm).

Broth microdilution method was conducted according to procedures described in the CLSI M38-A document (2002) with modifications. The extracts were solubilized in sterile ultrapure water and 200 µL final volume added to the wells of a 96-well microplate in order to obtain, for each extract, a concentration of 10, 100, 250 and 500 µg/mL. Growth control wells, containing RPMI 1640 medium without extract (replaced by sterile ultrapure water) were included. The *Asp. fumigatus* and *A. infectoria* inocula were prepared using the same methodology, according to the CLSI M38-A standard. Briefly, 7-day-old cultures were covered with a sterile mixture of 0.85% saline with 1% Tween 20 (Sigma P5927). The suspension is prepared by gently scraping the colonies with an inoculation loop. The resulting mixture of conidia and hyphal fragments was withdrawn and transferred to a sterile tube. Heavy particles were allowed to settle for 3 to 5 minutes, in an ice bath, and the upper homogeneous suspension was transferred to a sterile tube and then mixed with a vortex mixer for 15 seconds. The conidial density in the suspensions was adjusted to an optical density from 0.09 to 0.11 (80 to 82% transmittance). The resulting suspensions were diluted 50-fold in RPMI 1640, which correspond to 2-fold of the density needed - approximately 0.4 to 5×10^4 CFU/mL. The test inocula was prepared in sufficient volume to directly inoculate each well with 100 µL of the corresponding diluted inoculum suspension. All microdilution trays were incubated at 30 ºC with orbital shaking (120 rpm) for 46 to 72 hours. Additionally, *A. infectoria* trays were incubated under alternating 8-h light enriched with UV and 16-h dark cycles. The amount of growth in the wells containing extracts is compared visually with that in the growth-control wells (no extracts added). Microdilution assay results were given by minimum effective concentration (MEC), corresponding to the lowest extract concentration at which the mold displayed microscopic morphological changes.

For microscopy, liquid *Asp. fumigatus* cultures were performed on sterile yeast malt extract (YME) with or without extract supplementation, in Erlenmeyer flasks. Each extract was separately added to YME to obtain two final test concentrations: 10 and 100 µg/mL. Inocula were prepared as described above in 0.85% saline with 0.1 % Tween 80. 30 µL of obtained spore suspension was inoculated into 20 mL YME medium. These suspensions were then incubated at 30 ºC, under constant orbital shaking at 120 revolutions per minute

(rpm), for 48 h. Microscope slides were prepared with the mycelia obtained, and covered with a coverslip. The preparations were observed by optical microscopy, using an Eclipse E400 epi-fluorescence microscope (Nikon, Japan). Images were recorded at different time periods with a DS-5M digital camera (Nikon, Japan).

9.4. Influence of *O. pinnatifida* extracts in fungal cell wall composition

Chitin and glucan are two essential components in the maintenance of fungal cell integrity and rigidity during growth and morphogenesis (Latgé, 2007). Once they are not present in mammals, they stand as potential targets for antifungal agents (Munro et al., 2003). Thus, the contents of chitin and glucan in the cell wall of the tested molds treated with *O. pinnatifida* extracts were evaluated. Chitin and beta-glucan contents were determined as described by Fernandes et al. (2014).

9.4.1. Determination of cell wall chitin content

The mycelia used in this assay were obtained from *Asp. fumigatus* and *A. infectoria* grown in YME liquid medium with or without extract. Fungal liquid cultures were performed in YME medium. The liquid cultures were incubated at 30 ºC, under constant orbital shaking at 120 rpm, for 72 h; for *A. infectoria* underneath 8 h-alternating light enriched with UV and dark cycle.

The cell wall chitin content was based on the measurement of the glucosamine released by acid hydrolysis of purified cell walls as described Fernandes et al. (2014). Lyophilized mycelia were weighted, resuspended in distilled water and disrupted by sonication. Samples were centrifuged and s pellets were extracted with SDS-Mer-OH buffer (50 mM Tris, 2% sodium dodecyl sulphate (SDS), 0.3 M β-mercaptoethanol and 1 mM ethylenediaminetetraacetic acid (EDTA); pH 8.0) at 100 ºC for 10 minutes. After centrifugation the supernatant was discarded and cell wall pellets were washed three times. Chitin contents were determined by measuring the glucosamine released by acid hydrolysis of purified cell walls, with 6 M HCl at 100 ºC for 17 hours. After evaporation at 65 ºC (thermoblock), the samples weredissolved in distilled water. Solution A (1.5 M Na_2CO_3 in 4% acetylacetone) was added to the sample The mixture was incubated at 100 ºC for 20 min. and then 96% ethanol was added to each tube, followed by solution B (0.8 g of β-dimethylaminobenzaldehyde in 15 mL of 37% concentrated HCl and 15 mL of absolute

ethanol). The tubes were incubated at room temperature for 1 hour. 0.22 mL of each reaction tube were transferred to a 96-well plate and absorbance was read at 520 nm on a plate reader (SpectraMAX Gemini XM, Molecular Devices, USA). A calibration curve was obtained using D-(+)-glucosamine hydrochloride (Merck). Chitin contents were reported as mg D-(+)-glucosamine hydrochloride equivalents (D-gluc HCl eq.)/mg mycelial dry weight (mdw), calculated by the formula:

$$\left[\frac{(\text{Abs}_{520}(\text{treatment})\text{-Abs}_{520}(\text{blank}))\text{-b}}{\text{m}}\right] / \text{mdw},$$

where b and m are the y-intercept and the slope of the calibration curve, respectively.

9.4.2. Determination of cell wall glucan content

Quantification of β-(1,3)-D-glucan levels in the cell walls of growing fungi was performed using the aniline blue assay as described by Fernandes et al. (2014). The fungal growth conditions, treatment and tissue harvesting were the same as those used for chitin quantification. After being weighted, the lyophilized mycelia were treated with 1 M NaOH, sonicated with a microprobe, and incubated at 52 ºC for 30 minutes. Each sample were transferred to a black/clear-bottom 96-well plate (Costar™), and aniline blue solution was added to each well. Aniline blue solution was prepared as previously (Fernandes et al., 2014). The plate was shaked briefly and then incubated, protected from light, at 52 ºC for 30 minutes and an additional 30 minutes at room temperature to allow reaction with the fluorochrome and decolorization of the aniline blue solution. Fluorescence was quantified immediately with a fluorescence plate reader at 405 nm excitation wavelength, 460 nm emission wavelength (SpectraMAX Gemini EM, Molecular Devices, USA), operated by SOFTMax PRO v5.0 software. Calibration curves were performed using different concentrations of curdlan (Sigma C7821). Glucan contents were determined by comparing the fluorescence of the sample with that of the curdlan solutions, being reported as µg curdlan equivalents (curdlan eq.)/mg mycelial dry weight (mdw), calculated as follows:

$$\left[\frac{(\text{Fluor.}_{\lambda\text{em:405 nm};\lambda\text{ex:460nm}}(\text{treatment}) - \text{Fluor.}_{\lambda\text{em:405 nm};\lambda\text{ex:460nm}}(\text{blank})) - \text{b}}{\text{m}}\right] / \text{mdw},$$

where b and m are the y-intercept and the slope of the calibration curve, respectively.

9.5. Influence of *O. pinnatifida* extracts in fungal morphology

Fungal morphology was studied by fluorescence microscopy using calcofluor white (CFW) stain. *Asp. fumigatus* liquid cultures were prepared and grown as described previously. In turn, A. *infectoria* inoculum was prepared as described for chitin and glucan quantification. After being inoculated in 1.5 mL microtubes containing YME with 0.5% agar supplemented with the respective extracts, it was grown overnight at 150 rpm under 8-h alternating UV light and dark cycles (Anjos et al., 2012; Fernandes et al., 2014). A solution of calcofluor white was prepared by mixing equal volumes of 10% KOH in glycerin and 0.1% CFW. For microscopic observation, fragments of the *Asp. fumigatus* aerial mycelium were placed in microscope slides and flooded with calcofluor white (CFW) solution. Digital images were acquired with a Zeiss LSM 510 Meta confocal microscope and managed on a Zeiss LSM Image Browser (version 4.2.0.121; Carl Zeiss Inc.).

10. Statistical analysis

All experiments were performed at least three times ($n \geq 3$), unless otherwise stated. Results are generally expressed as mean ± SD, mean ± SD, EC_{50}, IC_{50} or MEC. EC_{50} and IC_{50} values were calculated from non-linear regression analysis using the GraphPad Prism (v6.01) program with the equation $Y = 100/ (1+10^{(X-LogIC_{50})})$. Two-way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference (LSD) test was performed to test for differences between samples (wild and IMTA-cultivated) and extracts/fractions (dichloromethane, methanol and *n*-Hexane) using the software IBM SPSS v21.0. Significance of the differences was defined at the 5% level ($P<0.05$). The correlation between TPC, DPPH radical scavenging activity and ORAC was assessed by Pearson correlation test in GraphPad Prism software (v5.0). Principal components analysis (PCA) was also performed using CANOCO software (v4.5). One-way analysis of variance followed by Dunnet's *post hoc* test was performed to test for differences at the chitin and β-glucan contents of *A. infectoria* and *Asp. fumigatus* grown with and without extract supplementation. Significance of the differences was defined at the 5% level ($P<0.05$), at the 3% level ($P<0.03$), and at the 1% level ($P<0.01$).

RESULTS AND DISCUSSION

11. Cultivation trials

11.1. Laboratory trials

Osmundea pinnatifida registered positive growth values, i.e., was able to grow, throughout the entire course of both *in vitro* cultivation trials. The relative growth rate (RGR, % day^{-1}) and the productivity (mg dw L^{-1} day^{-1}) of the laboratory trials are displayed in Figure 6. Although *O. pinnatifida* cultivated in the Lab TI conditions gave higher RGR (Figure 6A), the Lab TII showed greater productivity (Figure 6B). In general, seaweed cultivated under the Lab TII conditions presented lower RGR and higher productivity than the cultivated under the Lab TI conditions. While both RGR and productivity values oscillated in the first pilot assay, in the second one there's a tendency to the increase of these parameters over experimental time. This feature can be due to the relatively long acclimatization period (3 months) that *O. pinnatifida* was submitted before the Trial II begin. Biomass cultivated under Trial II conditions displayed a substantially better morphological aspect. In the Trial I, despite biomass having been previously cleaned and disinfected, epiphytic seaweed (of 4 different genus) were often found, in large quantities, and removed. The appearance and growth of the latter may have been stimulated by f/2 medium, which figures among the major natural seawater enrichment media used for a broad spectrum of algae (Andersen, 2005).

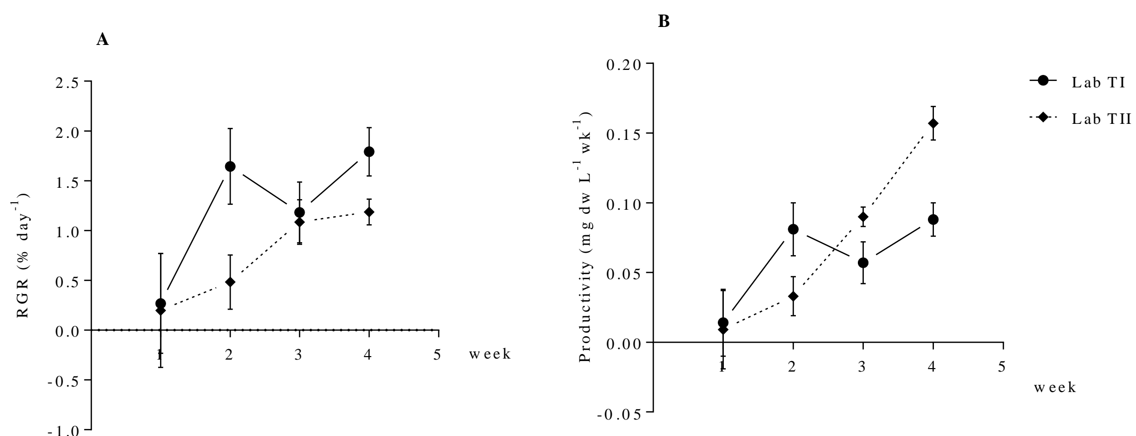


Figure 6. Relative growth rate (A; % day^{-1}) and productivity (B; mg dw L^{-1} wk^{-1}) of *O. pinnatifida* cultivated *in vitro* for 4 weeks in 1L flasks containing nutrient enriched water from a commercial fish aquaculture, supplemented with f/2 medium (Lab TI) and VSE medium + GeO_2 (Lab TII). Points correspond to the average of *n* = 3. Vertical lines correspond to SEM.

With respect to the outdoor, land-based IMTA trials, only the one carried out from November to December was valid. Although the verified higher production of biomass in the first two weeks in the second land-based IMTA trial (from May to June; data not shown), seaweed underwent a sharp decrease in growth, which was accompanied by loss of consistency. At the end of the third week, the seaweed was completely depigmented and extremely deteriorated. This could be due environmental factors, specially the extreme intensity of solar radiation. Among the realized cultivation trials, the first land-based IMTA trial (Nov-Dec) stood out for its tendency to the increase of RGR throughout the experimental period, as well as for the production of biomass with a high level of integrity.

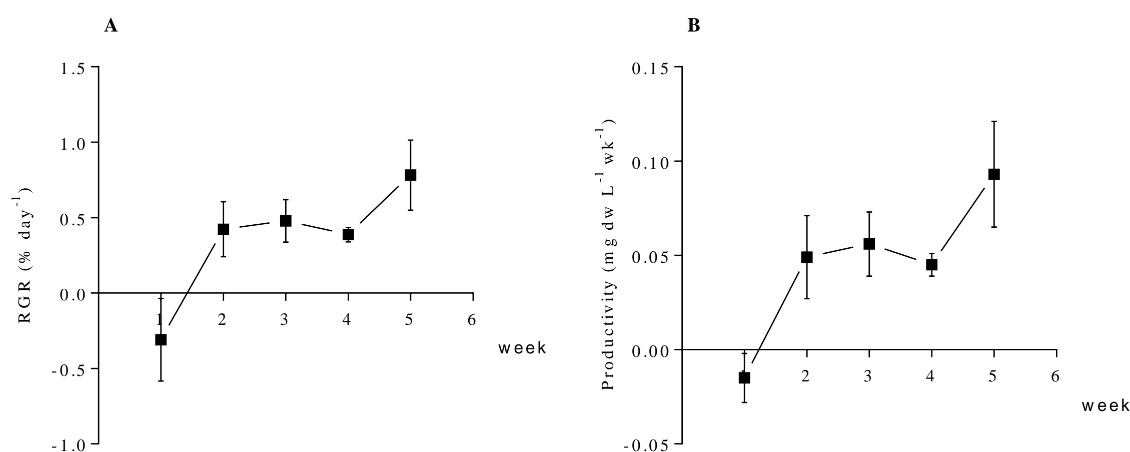


Figure 7. Relative growth rate (A; % day^{-1}) and productivity (B; mg dw L^{-1} wk^{-1}) of *Osmundea pinnatifida* cultivated in a land-based system for 5 weeks in 15L fiber glass tanks with nutrient enriched water from a commercial fish aquaculture (November-December 2013). Points correspond to the average of *n* = 4. Vertical lines correspond to SEM.

12. Extraction yield

Table 1 displays extraction yields using methanol, dichloromethane and *n*-Hexane as organic solvents to obtain extracts with different polarities from wild and IMTA-cultivated samples of *O. pinnatifida*. The highest yield was achieved by the extraction with methanol. The amount of extract obtained with the other solvents was very low, however the extraction with dichloromethane, on average, displayed a slightly higher yield than *n*-Hexane.

The isolation of compounds from natural sources frequently begins with the evaluation of their extracts properties. Solvent extractions, due to their ease of use, efficiency and wide applicability, are the most commonly used procedures to recover bioactive compounds from foodstuffs. The efficiency of extraction depends on several factors including solvent chemical properties (such as polarity), extraction time and temperature, sample-to-solvent ratio as well as on the chemical composition and physical characteristics of the samples (Bae, Jayaprakasha, Crosby, Jifon, & Patil, 2012; Dai & Mumper, 2010). Alves (2011) mentioned that the compounds typically isolated from the polar fraction may be esters, ethers, hydrocarbons, terpenoids, sterols, fatty acids, alkanes and coumarins; while some compounds such as alkaloids and flavonoids, among others, can be achieved by the extraction with solvents with intermediate polarity; polar solvents extract glycosides, tannins, some alkaloids, among others. As shown in Table 1, and in agreement with that obtained by Barreto et al. (2012), methanol (polarity index 5.1) was the most effective solvent to extract soluble compounds from *O. pinnatifida*. Similar yield values (2.85% to 5.01%) are given in methanol extracts from selected Indian red seaweeds (Ganesan, Kumar, & Bhaskar, 2008). Curiously, in our work, while the yield of extraction with dichloromethane and *n*-Hexane registered very little variation, the one obtained with methanol in wild sample was 2-fold higher than IMTA-cultivated. The chemical composition of seaweeds varies with individuals, species, habitats, maturity and environmental conditions (Ito & Hori, 1989). Wild and IMTA-cultivated samples grown in different environmental conditions (light, temperature, salinity) and were harvested in distinct seasons. As referred by Abreu et al. (2011), temperature and light are usually the most important environmental factors affecting growth and nutrient uptake of seaweeds. Furthermore, the observed yield variation may also be due to lack of ecological pressures, both biotic (e.g. predation, competition) and abiotic (e.g. nutrients) from the controlled environment in which the algae were grown. In turn, this may have led to a suppression of the production of certain polar chemical compounds in the IMTA-cultivated sample.

13. Antioxidant activity

As suggested by Huang et al. (2005), to comprehensively study different aspects of antioxidants, validated and specific assays, such as DPPH radical scavenging activity, are needed in addition to the total phenols assay using FCR and ORAC assay.

13.1. Total phenolic content

TPC values are presented in Table 1 and expressed as mg gallic acid equivalents per gram of dry extract (mg GAE/g). When TPC values for all three extracts are totalled, there is no statistically significant differences (*P*>0.05) between phenolic contents of wild and IMTA-cultivated samples (87.57 and 65.89 mg GAE/g, respectively). On the other hand, when analysing the effects at the extractant level, the extraction performed with dichloromethane had the highest value. The value of dichloromethane extract from wild sample (Dw) was 46.82 mg GAE/g while those of the remaining extracts were between 31.83 (methanol fraction) and 8.92 mg GAE/g (*n*-Hexane fraction). In the case of IMTA-cultivated sample, dichloromethane extract (Dc) registered a TPC value of 43.48 mg GAE/g while methanol (Mc) and *n*-Hexane (Hc) fractions presented 15.55 and 6.86 mg GAE/g, respectively. The amount of phenols extracted either with dichloromethane and *n*-Hexane solvents don't differ significantly (*P*>0.05) between wild and IMTA-cultivated samples. However, significant differences (*P*<0.05) were found in TPC among methanol fractions of these samples.

The total phenolic content evaluated through FCR has become a routine assay in studying phenolic antioxidants once it is a convenient, simple and reproducible assay (Huang, Ou, & Prior, 2005). Seaweed may contain phenolic compounds varying from simple (e.g. phenolic acids) to more complex substances (e.g. phlorotannins) in different quantities. The fact that phenolic compounds can be combined with other components (such as carbohydrates and proteins) means that there is no universal extraction method for removal of all phenolic compounds from plant matrices. Nevertheless, solvents such as methanol, ethanol, acetone and their combinations have been widely used for the extraction of phenolics (Dai & Mumper, 2010). Furthermore, it has been found that the phenolics extraction efficiency increase with increasing polarity of the extractant (Airanthi, Hosokawa, & Miyashita, 2011). Contrariwise, in the present study, phenolic compounds presented higher solubility in dichloromethane (polarity index, P', of 3.1) than in methanol (P' 5.1) solvent. In the case of the *O. pinnatifida* collected in Azores, the amount of phenolics extracted with methanol do not differ significantly from that extracted with dichloromethane (34.67 *versus* 33.26 mg GAE/g, respectively) (Barreto et al., 2012). As occurs with other chemical constituents, the composition of phenolic compounds both qualitatively and quantitatively might vary depending on the specie and many other variables such as habitat, season of harvesting, geographical distribution and

environmental conditions (salinity, light and temperature) (Ibañez, Herrero, Mendiola, & Castro-Puyana, 2012; Rodríguez-Bernaldo de Quirós, Lage-Yusty, & López-Hernández, 2010).

13.2. DPPH radical scavenging activity

Regarding antioxidant activity, the methanol fraction of wild sample (Mw) as well as *n*-Hexane fractions (both samples) showed the highest DPPH radical scavenging activity. No statistically significant differences (P>0.05) were found among these extracts. Their ability to scavenge DPPH radical in comparison to control were almost the same, ranging from 51.17 (Hc) to 55.23% (*n*-Hexane fraction of wild sample, Hw), being followed by dichloromethane extracts (42.03 and 44.82% for IMTA-cultivated and wild samples, respectively). The lowest DPPH radical scavenging activity (32.57) was observed in the Mc (Table 1). No significant correlation (P>0.05) was found between DPPH radical scavenging activity and TPC. Additionally, EC_{50} values (µg/mL) were determined for the samples which displayed over 50% reduction on the DPPH radical. All the extracts have been shown to scavenge the DPPH radical in a concentration-dependent manner (Figure 8). Mw, Hw and Hc scored, respectively, 911.1 (855.6-970.2), 1114 (873.1-1421) and 1346 (1149-1577) µg/mL. BHT was used as a positive reference (EC_{50}: 40.55 (27.39-60.05) µg/mL).

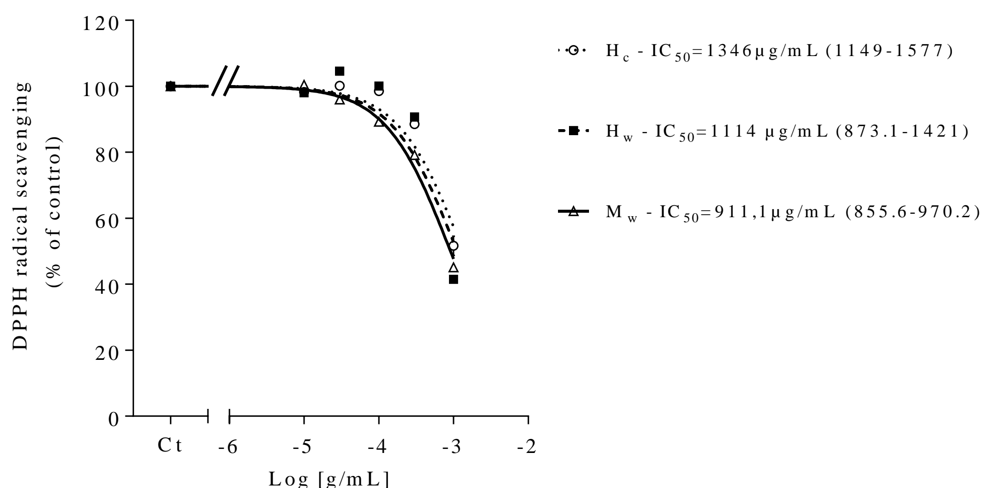


Figure 8. Dose-response curve (100 – 1000 µg/mL) of the fractions that scavenged (≥ 50%, 1000 µg/mL) the DPPH radical. Hc – *n*-Hexane fraction of the IMTA-cultivated seaweed; Hw – *n*-Hexane fraction of the wild seaweed; Mw – methanol fraction of the wild seaweed. Symbols are the mean ± SEM (*n*=8).

In the present study, despite of not statistically significative ($P>0.05$), TPC was negatively correlated ($r=-0.2288$) to their DPPH radical scavenging activity. This feature is in agreement with previous evaluation of antioxidant activities of 3 selected Indian red seaweeds – *Acanthophora spicifera*, *Euchema kappaphycus* and *Gracilaria edulis* – where DPPH radical scavenging activity increased with decreasing TPC (Ganesan et al., 2008). Thus, we presume that antioxidant compounds other than polyphenols are involved in the observed DPPH radical scavenging activity. Mw, Hw and Hc fractions are responsible for reducing the DPPH radical by more than 50% (52.85, 55.23 and 51.17, respectively). In the case of *n*-Hexane fractions, the radical scavenging activity could be related to less polar compounds, such as fatty acids, carotenoids (e.g. β-carotene, lutein), tocopherols, sterols and terpenoids, as referred by Burtin (2003). In order to measure the extracts' potency, the analysis of concentration-effect curves through the measurement of the extract concentration necessary to give 50% of the maximum response (EC_{50}) was made. The antioxidant activity of these 3 fractions exhibited dose dependency, increasing with increasing concentration of extract (data not shown). The determined EC_{50} values are pretty higher than butylated hydroxytoluene, BHT (EC_{50}: 40.55 µg/mL), used as positive control. These results are in agreement with that obtained by Barreto et al. (2012), revealing that *O. pinnatifida* extracts are weak DPPH radical scavengers.

13.3. Oxygen Radical Absorbance Capacity

Lastly, when ORAC values for all three extracts are totaled for each sample, there are no statistically significant differences ($P>0.05$) among them. The peroxyl radical scavenging activity of seaweed extracts ranged from 61.9 to 499.9 µmol of TE/ g of dry extract, depending on sample and extractant used (Table 1). Dichloromethane extracts showed higher scavenging activity against peroxyl radicals than methanol and n-Hexane fractions, being the mean difference statistically significant at the 0.05 level. The highest ORAC value was obtained for the Dc (499.9 µmol of TE/ g extract), followed by Dw (414 µmol of TE/ g extract) and Mw (271.32 µmol of TE/ g extract), whereas Hw (61.9 µmol of TE/ g extract) presented the lowest. There must note that ORAC values obtained with dichloromethane extraction don't differ significantly ($P>0.05$) among the wild and IMTA-cultivated samples. Interestingly, a significant strong correlation ($P<0.01$, $r=0.9499$, $r^2=0.9024$) was found between ORAC and TPC.

ORAC assay was selected to measure the peroxyl radical absorption capacity of seaweed extracts. This methodology is regarded to be more biologically relevant than DPPH and other similar protocols and has been demonstrated to be especially useful for food samples and crude plant extracts when multiple constituents co-exist and complex reaction mechanisms are involved (Wang et al., 2009). Furthermore, according to Zulueta et al. (2009), the ORAC method is the only one so far that combines the total inhibition time and the percentage of the free radical damage by the antioxidant into a single quantity, ensuring that, by the end of the process, all the antioxidants present in the sample have reacted with the radicals generated. Previous studies reported the presence of correlations between TPC and the ORAC of seaweed extracts (Tierney, Croft, & Hayes, 2010; Wang et al., 2009). In this study, ORAC values were positively, highly (r=0.9499; r^2=0.9024) and significantly (P<0.01) correlated with TPC values. This could indicate that phenolic compounds present may be mostly responsible for the verified peroxyl radical absorption capacity of *O. pinnatifida* extracts. In fact, among the myriad of bioactivities related to phenolic compounds, antioxidant activity seems to be the main one (Ibañez et al., 2012; Zhang et al., 2006). The multifunctional antioxidant activity of polyphenols is highly related to phenol rings which act as electron traps to scavenge peroxyl, superoxide anions and hydroxyl radicals (Sathya, Kanaga, Sankar, & Jeeva, 2013). However, due to the nature of the extracts, we cannot discard a synergistic or antagonistic interaction between phenolic and non-phenolic compounds that could be affecting the bioactivity.

Wojcikokowski et al. (2007) studied the *in vitro* antioxidant capacity of 55 medicinal plants through ORAC method using a sequential multi-solvent extraction process (ethyl-acetate, methanol and 50% aqueous methanol). Of them, only 6 species have registered higher total (sum of fractions) ORAC values than both wild (747.22 µmol of TE/ g extract) and IMTA-cultivated (771.59 µmol of TE/ g extract) *O. pinnatifida*. Even *Camelia sinensis* leaf and *Sylibum marianum* seed, which were included due to its known high antioxidant activity, showed lower total ORAC values (627.14 and 553.91, respectively) than both *O. pinnatifida* samples. Up to now, ORAC data on seaweeds is very limited. Indeed, to our knowledge, this is the first time that this methodology is applied for the screening of *Osmundea pinnatifida* antioxidant activity. Even so, the ORAC values obtained in this study for dichloromethane extracts (414 and 499.9 for wild and IMTA-cultivated sample, respectively) are higher than 70% acetone extracts of *Chondrus crispus*, *Palmaria palmata* (Rhodophyta), *Ulva lactuca* (Chlorophyta) (numerical data not shown) and *Laminaria digitata* (Phaeophyceae; 4 µmol of TE/ g extract); and comparable to those obtained for

Saccharina latissima and *Alaria esculenta* (Phaeophyceae) (Wang et al., 2009). As reported by Nogueira et al. (2014), amongst the red seaweeds, those belonging to the family Rhodomelaceae (order Ceramiales) are the most promising as potential producers of antioxidants; and this feature seems to be related to the ability to synthesize polyphenols and their derivatives, as bromophenols.

Table 1. Yield of extraction, total phenolic content (TPC) and radical scavenging activity of extracts from wild and IMTA-cultivated *Osmundea pinnatifida*

Sample	Solvent used for extraction	Yield (%)	TPC (mgGAE/g)	DPPH (%)	ORAC (µmol TE/g)
Wild	Methanol	4.191	31.83±3.71[a]	52.85±2.44[a]	271.32±59.15[a]
	Dichloromethane	0.330	46.82±5.14[b]	44.82±2.00[b]	414.00±67.07[b]
	n-Hexane	0.352	8.92±1.19[c]	55.23±4.48[a]	61.90±11,80[c]
IMTA	Methanol	2.036	15.55±3.30[c]	32.57±1.88[c]	134.17±17.53[c]
	Dichloromethane	0.466	43.48±6.53[b]	42.03±3.01[b]	499.90±48.95[b]
	n-Hexane	0.355	6.86±0.60[c]	51.17±5.19[a]	137.52±36.71[c]

Results are expressed as mean ± standard deviation (n≥3), except for the extraction yield. Yield=percentage of dry extract (g) to dry seaweed powder (g). DPPH=percentage of DPPH radical scavenged. Column wise values of different letters (a-c) indicate statistically significant differences ($P<0.05$)

13.4. Principal components analysis

Principal components analysis (PCA) was performed to have an overview of the differences and similarities between the 6 fractions (methanol, dichloromethane and *n*-Hexane) studied, obtained from seaweed into two distinct conditions (wild and IMTA-cultivated), and investigate the relationships between the different methods used to evaluate the antioxidant activity. The two first principal components (PC1 and PC2) explain 92.3% and 5.0% of the total variance of the data set, respectively (Figure 9). Through the analysis of the second principal component (PC2) (Figure 9), the vertical axis expressed an opposition between DPPH radical scavenging activity (left) and TPC (right). Moreover, TPC showed a negative correlation with DPPH radical scavenging activity. In fact, the fractions that showed high phenolic content presented low ability to scavenge

DPPH radical (for example, *n*-Hexane fraction of wild sample and methanol fraction of the IMTA-cultivated, Group II). On the other hand, dichloromethane fractions of both samples (Group I) showed high levels of TPC and weak DPPH radical scavenging activity, because it has located on the opposite side of PC2 (Figure 9). Additionally, through the PC2 analysis, it is also possible to observe that TPC has a positive correlation with ORAC (Figure 9). The first principal component (PC1), explain the variance between the samples in relation to the ability to neutralize peroxyl radicals (ORAC). Dichloromethane extracts showed highest ability to neutralize peroxyl radicals, being the same extracts where highest phenolic content was found (Figure 9).

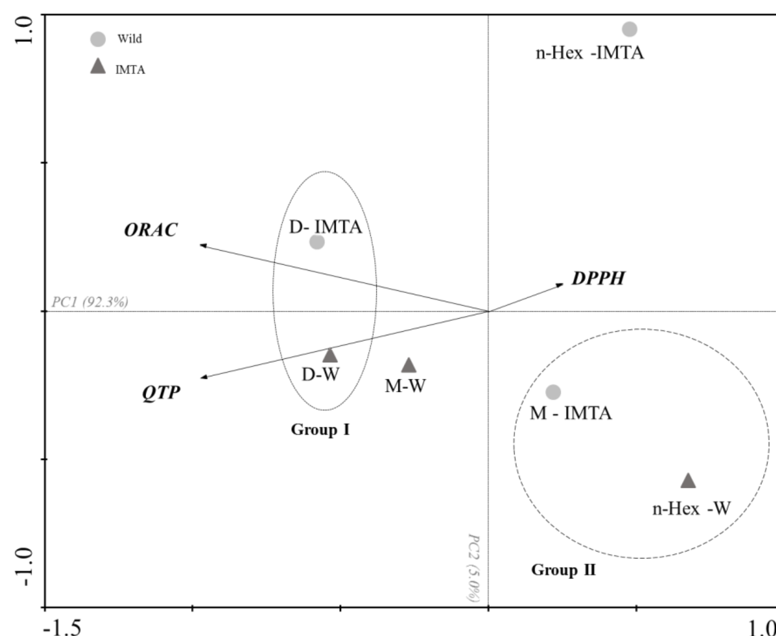


Figure 9. Principal component analysis (PCA) scatter plot of total phenolic content (TPC) and antioxidant activities (DPPH radical scavenging activity and ORAC) of dichloromethane extract (D), methanol (M) and n-hexane (n-Hex) fractions of Osmundea pinnatifida from Portuguese coast.

14. Antitumor activity

Cell viability and proliferation effects on HepG-2, MCF-7 and SH-SY5Y cell lines after exposure to extracts of *O. pinnatifida* (1000 µg/mL; 24 hours) were firstly quantified as percentage of control (Figure 10). As shown in Figure 10A, upon 24 h of incubation, all the tested extracts presented a very low level of cytotoxicity both in HepG-2 and MCF-7 cell lines. In fact, none of the extracts was able to reduce cell viability by over 20%.

However, except for the Hw, SH-SY5Y cells were more susceptible to the extracts than HepG-2 and MCF-7 cells. In fact, dichloromethane extracts (Dw and Dc) were responsible for reducing SH-SY5Y cell viability above 88%. There are no statistically significant differences at the 0.05 level between the effects caused by Dw and Dc in SH-SY5Y cells. The effects of these extracts were dose-dependent (Figure 11) and Dc presented a lower IC_{50} value than Dw (431.2 and 656.8 μg/mL, respectively).

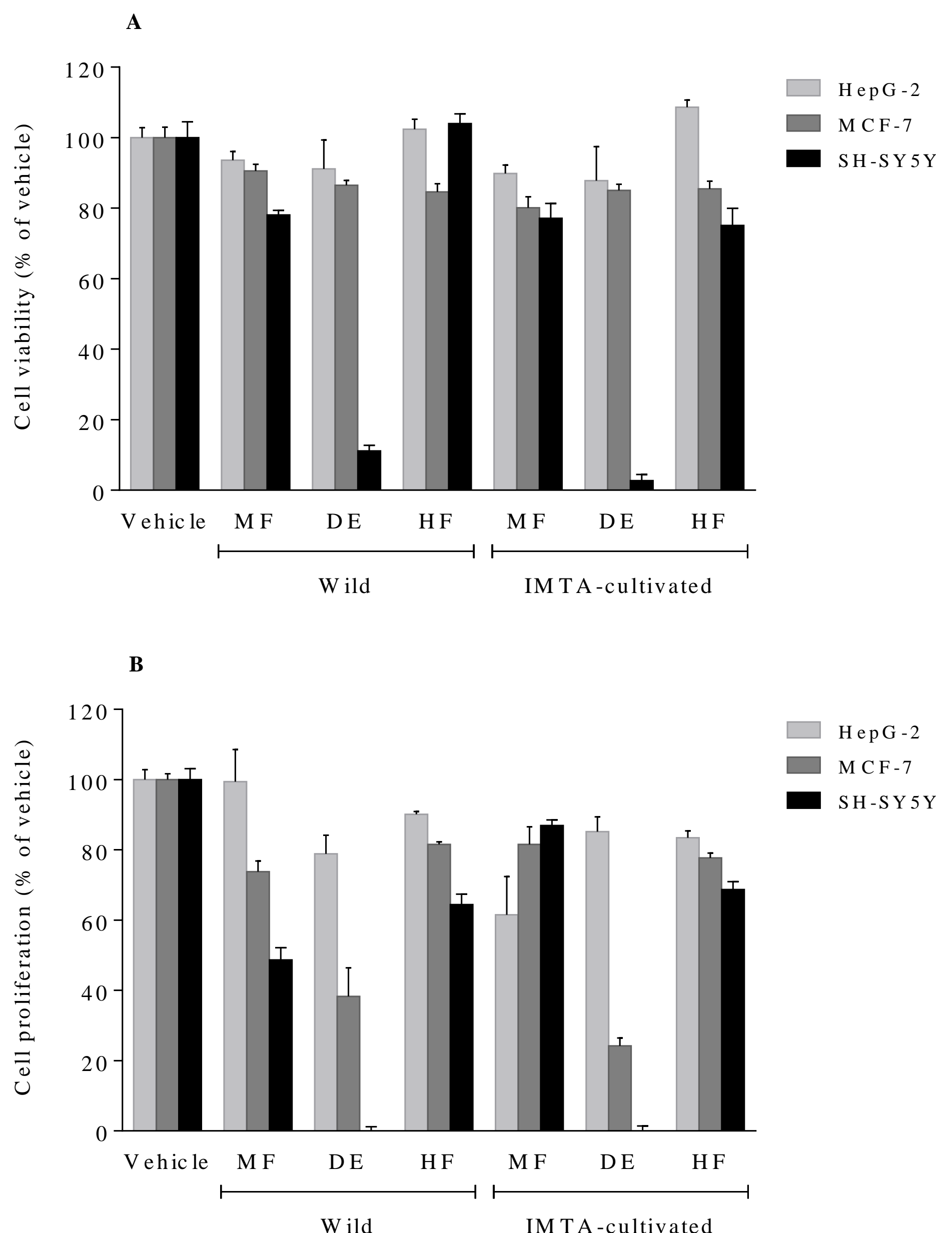


Figure 10. Effect of *Osmundea pinnatifida* extracts on viability (A) and proliferation (B) of HepG-2, MCF-7 and SH-SY5Y cells. Cells were treated with seaweed fractions (MF=methanol fraction, HF=*n*-Hexane fraction, DE=dichloromethane extract) at 1000 μg/mL for 24 hours.

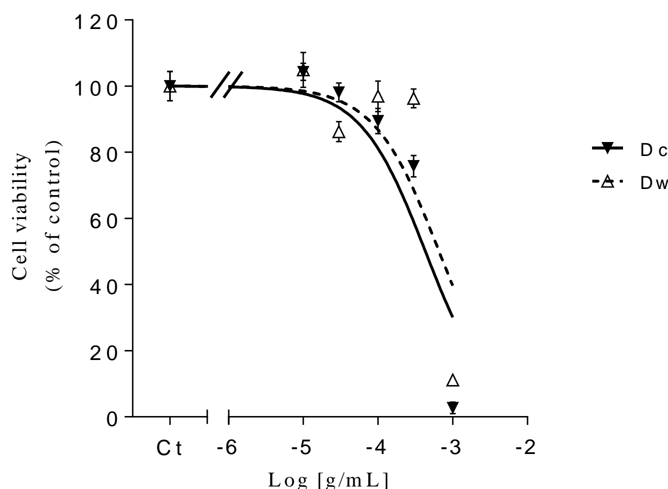


Figure 11. Dose-response curve (10 – 1000 μg/mL) of the fractions that reduced (≥ 50%, 1000 μg/mL) the cell viability of SH-SY5Y cells. Dc – dichloromethane extract of the IMTA-cultivated seaweed; Dw – dichloromethane extract of the wild seaweed. Symbols are the mean ± SEM (*n*=8).

On the other hand, effects on cell proliferation were also observed (Figure 10B). While the extracts inhibited from near 10% (Hw) to 38% (Mc) of the HepG-2 cell proliferation, in MCF-7 the percentage of inhibition ranged from 18 % (Hw and Mc) to 75% (Dc), whereas in SH-SY5Y it was between 13% (Mc) and 100% (Dw and Dc). Only dichloromethane extracts (Dw and Dc) have shown ability to reduce cell proliferation by more than 50% both in MCF-7 and SH-SY5Y cells. It is important to emphasize that these extracts also exhibited dose dependency in MCF-7 (Figure 12A) and SH-SY5Y (Figure 12B) cell proliferation. There are no statistically significant differences (P>0.05) between the effects caused by Dw and by Dc in MCF-7 and SH-SY5Ycells proliferation. As shown in Table 2, the Dc presented a lower IC_{50} value (923.6 μg/mL) than Dw (945.8 μg/mL) in MCF-7 cells. Contrariwise, Dw was more effective than Dc in SH-SY5Y cells (IC_{50}: 508.8 and 525.9 μg/mL, respectively).

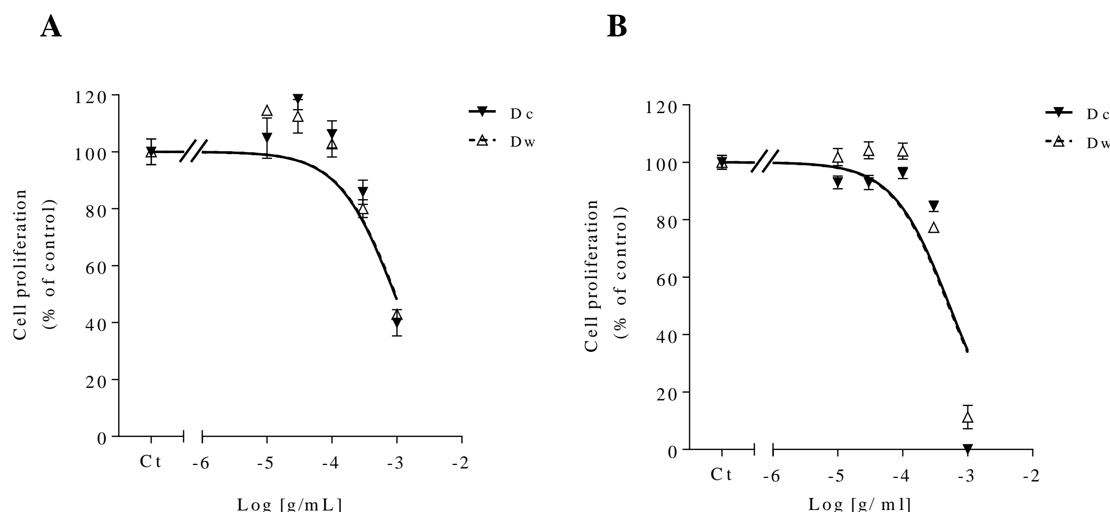


Figure 12. Dose-response curves (10 – 1000 µg/mL) of the fractions that reduced (≥ 50%, 1000 µg/mL) the MCF-7 (A) and the SH-SY5Y (B) cell proliferation. Dc – dichloromethane extract of the IMTA-cultivated seaweed; Dw – dichloromethane extract of the wild seaweed. Symbols are the mean ± SEM (*n*=8).

Currently, several research institutions have concentrated their efforts in the search for effective and safe novel drugs for the treatment of cancer. As reported by Abu-Dahab and Afifi (2007) and in agreement with the US NCI plant screening program, a pure compound is generally considered to have *in vitro* active cytotoxic effect if the IC_{50} value in carcinoma cells, following incubation between 48 to 72 h, is less than 4 µg/mL, while for crude extracts it is less than 20 µg/mL.

Despite of the fact that several *in vitro* tests did not differentiate between cytotoxic and cytostatic effects (Kroll, 2001), previous studies reported that seaweeds of Laurenciae tribe are a good source of metabolites that have displayed cytotoxic activity. Chamigren sesquiterpenoid metabolites isolated from algae of the genus *Laurencia* were found to possess cytotoxic activity, especially against colon tumor cell lines (Juagdan, Kalidindi, & Scheuer, 1997; Rashid, Gustafson, Cardellina, & Boyd, 1995) and NCI/ADR-RES cell line (Francisco & Erickson, 2001) which is believed to be derived from OVCAR-8 ovarian adenocarcinoma cells (Liscovitch & Ravid, 2007). In addition, a terpenoid isolated from *Laurencia viridis spec. nov.* has been demonstrated to exhibit cytotoxic activity in a panel of cancer cell lines (P-388, A-549, MEL 28, and HT 29; from DBA/2 mouse lymphoid neoplasm, human lung carcinoma, human colon carcinoma and human melanoma, respectively) (Norte, Fernández, Souto, Gavín, & García-Grávalos, 1997).

Table 2. Cytotoxic and antiproliferative effects induced on HepG-2, MCF-7 and SH-SY5Y cells by extracts from wild and IMTA-cultivated *Osmundea pinnatifida*

	Extract/ fraction	**HepG-2**		**MCF-7**		**SH-SY5Y**	
		Cytotox.	Antiprol.	Cytotox.	Antiprol.	Cytotox.	Antiprol.
Wild	Methanol	>1000	>1000	>1000	>1000	>1000	>1000
	Dichloromethane	>1000	>1000	>1000	945.8 (709.9-1260)	656.8 (431.1-1000)	508.8 (377.8-685.3)
	n-Hexane	>1000	>1000	>1000	>1000	>1000	>1000
IMTA	Methanol	>1000	>1000	>1000	>1000	>1000	>1000
	Dichloromethane	>1000	>1000	>1000	923.6 (663.4-1286)	431.2 (307-605.4)	525.9 (389.3-710.4)
	n-Hexane	>1000	>1000	>1000	>1000	>1000	>1000
	Cisplatin	116.45 (99.80-135.92)	14.80 (12.16-18.01)	136.5 (117.8-58.2)	22.65 (18.15-28.28)	14.12 (10.81-18.44)	7.45 (2.77-20.01)

Dose–response assays were carried out (10 – 1000 µg/mL; 24 hours; n≥3 for each concentration) and results are expressed as IC_{50} (µg/mL). 95% confidence intervals are presented within parenthesis. IC_{50} values were calculated from non-linear regression analysis using GraphPad Prism software with the equation Y = 100/ (1+10^(X-LogIC50)). Cisplatin was used as positive control.

In the present study, the tested extract and fractions have selective cytotoxic and antiproliferative effects, since, in general, SH-SY5Y cells were more susceptible than MCF-7 cells, being the latter more susceptible than HepG-2 cells (Figure 10). Additionally, a cytostatic effect has occurred in the MCF-7 cell line. The dichloromethane extracts of both samples did not induced cell mortality but kept them from proliferating in a 24h long *in vitro* colorimetric assay using MTT. However, the IC_{50} values obtained for this extracts (945.8 and 923.6 µg/mL for wild and IMTA-cultivated samples, respectively) in the MCF-7 antiproliferative activity do not fit in the NCI criteria for considering a crude extract as active. The cytotoxic and antiproliferative activity shown by both dichloromethane extracts (Dw and Dc) in SH-SY5Y cells do not fit in these criteria as well. It is necessary to evaluate the effects in a longer trial time, so to carry out fractionation of the dichloromethane crude extract in order to isolate and identify the compound responsible for the observed effect on MCF-7 and SH-SY5Y cell lines.

Nevertheless, previous studies also screened cytotoxic effects of *Osmundea* extracts in tumor cell lines. In agreement with our work, Barreto et al. (2012) also found that among methanol and *n*-Hexane fractions and dichloromethane extracts, the latter had strongest activity. Thus, *O. pinnatifida* dichloromethane extract also had selective and weak cytotoxicity, scoring an IC_{50} value of 129.3 µg/mL in HeLa (human cervix

carcinoma) cell line, whereas it was over than 200 μg/mL for Vero non tumor cell line. In addition, *O. hybrida* and *O. pinnatifida* extracts had no cytotoxicity towards L-6, another non tumor cell line.

15. Antimicrobial activity

15.1. Antibacterial activity

Solvent extracts of wild and IMTA-cultivated *O. pinnatifida* was investigated *in vitro* for their antibacterial activity against *Staphylococcus aureus* (Gram+) and *Escherichia coli* (Gram−). As shown in Figure 13, the microorganisms grew either in the control wells as in the treatment wells, which means that among the 6 extracts in study, none gave any inhibition against these clinical isolate strains at the tested concentrations (1562.5 – 31.25 μg/mL).

Until now, only a few studies aiming the screening of antibacterial capacity of seaweed extracts, through the broth microdilution, have been published (Kamei, Sueyoshi, Hayashi, Terada, & Nozaki, 2009; Kim, Kim, Jin, & Lee, 2013). Among them, the research carried out by Hellio et al. (2000) stands out as the most relevant for this work since they studied the antibacterial potential of *Laurencia pinnatifida*, a member of the *Laurencia* complex. They found out that ethanol and dichloromethane fractions exhibited, respectively, MICs of 96 and 24 μg/mL against *S. aureus*, and suggested that the latter extract is worthy for further investigation. However, in the particular case of *O. pinnatifida*, studies of this nature are even inexistent. Nevertheless, other methodologies have been applied for this purpose. Rizvi & Shameel (2005) studied the antibacterial activity of crude methanol extracts of fourteen seaweed species from Pakistan against four Gram+ and five Gram− bacteria through agar well diffusion technique. At the tested concentration (200 μg/100 μL DMSO), the *O. pinnatifida* methanol extract was unable to inhibit the growth of *S. aureus*. Rizvi (2010) used the same methodology to test the antibacterial activity of crude methanol extracts of twenty-six species of seaweeds (collected in Pakistan) against four Gram+ and seven Gram−. Once more, *O. pinnatifida* crude methanol extract (200 μg/100 μL DMSO) did not displayed antibacterial activity against *S. aureus*, and the same was verified against *E. coli*. Thus, results obtained in this

work corroborate those of the presented studies since none antibacterial effect was observed.

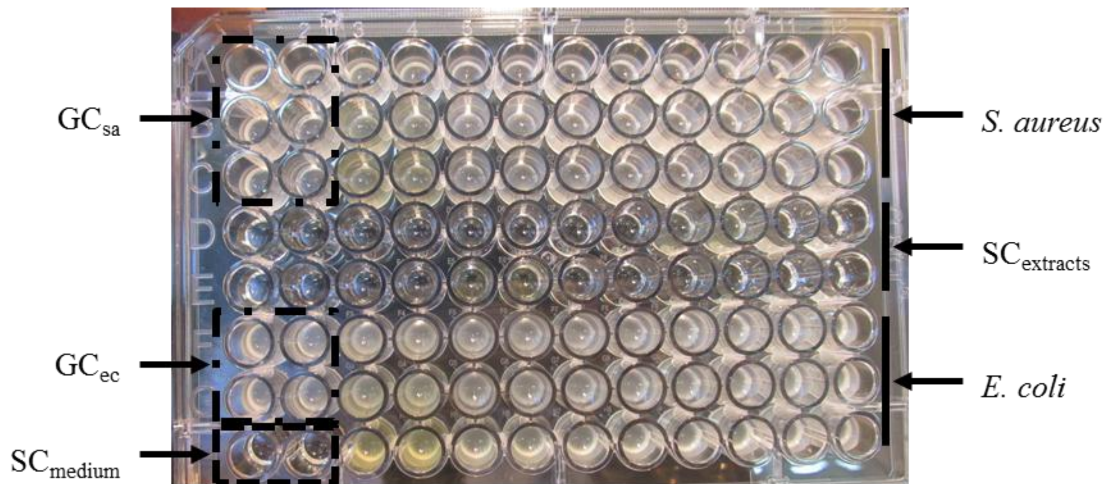


Figure 13. Microdilution assay performed to evaluate the antibacterial activity of *O. pinnatifida* extracts against *S. aureus* and *E. coli* (16-18h exposure, 35±2ºC). GC_{sa} – growth control wells of *S. aureus* (MHB + ultrapure water + microorganism); GC_{ec} – growth control wells of *E. coli* (MHB + ultrapure water + microorganism); SC_{medium} – sterility control wells of the culture medium (MHB alone); $SC_{extracts}$ – sterility control wells for each tested extract concentration (MHB + extract + 0.85% saline).

15.2. Antifungal activity

15.2.1. Antifungal capacity of *O. pinnatifida* extracts against *C. albicans*

The susceptibility of *C. albicans* to the *O. pinnatifida* extracts was evaluated through disk diffusion (Figure 14A) and broth microdilution (Figure 14B) methods. The outcome of both assays is in accordance, revealing that all the extracts were unable to prevent the growth of this yeast specie at the tested concentrations.

To date, studies in which the evaluation of *C. albicans* susceptibility to seaweed extracts was carried out using the standardized disk diffusion and broth microdilution methods are scarce. When concerning *O. pinnatifida* extracts, this is even the first time. Nevertheless, Rizvi & Shameel (2005), using a different methodology, found that *O. pinnatifida* crude methanol extract inhibited the *C. albicans* growth by 3.43% at 400 µg/mL. On the other hand, Hellio et al. (2000) evaluated, through a NCCLS standardized macrodilution method, the antifungal capacity of ethanol and dichloromethane fractions of

Laurencia pinnatifida (Laurencia complex) against *C. albicans*. The results were negative (did not inhibit the growth of the yeast) at the concentrations tested, from 4 µg/mL to 96 µg/mL. Despite the different methodologies and yeast strains used, as well as the distinct biogeography of the samples, the results obtained in the cited works are in agreement to that presented herein, suggesting that extracts of this nature doesn't have the potential to be used as a natural antifungal agent against *C. albicans*.

Furthermore, the capacity of the extracts in study to inhibit yeast-to-hypha transition in *C. albicans* was also tested, yielding negative results.

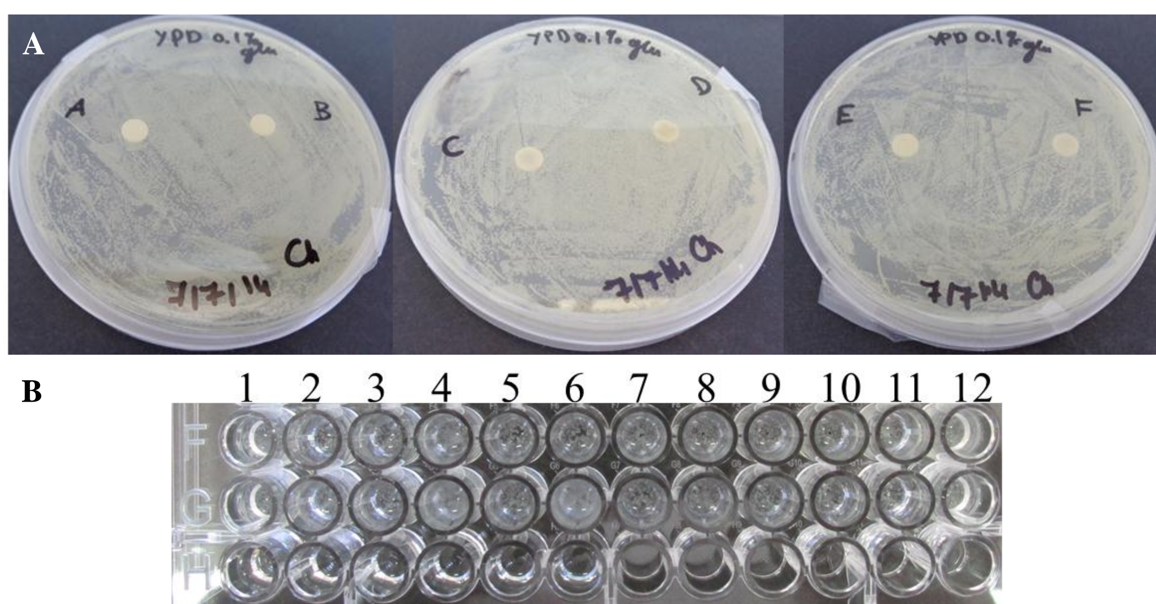


Figure 14. Susceptibility of *C. albicans* to *O. pinnatifida* extracts by disk diffusion (A) and broth microdilution (B) methods. In disk diffusion method (24 h exposure, 30 ºC), the disks were impregnated with extracts (A – Mw; B – Mc; C – Hc; D – Dw; E – Dc; F – Hw) at 5 g/L. In broth microdilution method (46-50h exposure, 37 ºC), F represents a row for the extract Hw (10 µg/mL: wells 1-3; 100 µg/mL: wells 4-6; 250 µg/mL: wells 7-9; 500 µg/mL: wells 10-12); the rows where the other extracts were tested are not shown; G is the row of growth control wells (suspension + sterile ultrapure water); H1-H6 – extract sterility control wells (an extract per well); H7-H12 – empty wells.

15.2.2. Antifungal capacity of *O. pinnatifida* extracts against *A. infectoria* and *Asp. fumigatus*

A radial growth inhibition assay was carried in PDA plates with or without extract supplementation. Results revealed that the great majority of *O. pinnatifida* extracts promoted the fungal growth of *A. infectoria*, as compared to the negative control (Table 3), being this feature observed regardless of the concentration tested (10 µg/mL and 100

μg/mL). A promoting fungal growth effect was also verified by Barreto et al. (2002), which found that *Osmundaria serrata* (family Rhodomelaceae) ethanol extract, at 31.25 μg/mL, stimulated *Colletotrichum gloeosporioides* growth, a common plant-pathogenic fungi that can also cause infection in humans (Cho et al., 2015). Nevertheless, Mw (10 μg/mL) and Hc (10 and 100 μg/mL) inhibited poorly the radial growth (3.7%, 1.79% and 3.7%, respectively) as compared with control, after incubation for 8 days. Although all treatments have been performed in duplicate, in some of them (namely 10 μg/mL Hc; 100 μg/mL Dw and 100 μg/mL Hw) a uniform radial growth was not observed, invalidating one of the tests.

Table 3. Effect of the different *O. pinnatifida* extracts, tested at 10 μg/mL and 100 μg/mL, on colony diameter (CD; mm) and on radial growth inhibition (RGI; %) of *A. infectoria*.

Extract	10 μg/mL		100 μg/mL	
	CD (mm)*	RGI (%)	CD (mm)*	RGI (%)
Mw	37.75 ± 4.60	3.70	42.75 ± 2.47	-9.06
Dw	39.50 ± 4.95	-0.77	42.50	-8.42
Hw	43.00 ± 5.29	-9.69	43.00	-9.69
Mc	40.33 ± 2.36	-2.89	41.00 ± 2.12	-4.59
Dc	40.10 ± 3.88	-2.30	40.00 ± 3.00	-2.04
Hc	38.50	1.79	37.75 ± 2.47	3.70

* Values refer to the average (n = 2) ± SD; incubation temperature: 30 ºC; incubation time: 8 days under alternating 8-h light enriched with UV and 16-h dark cycles. The control colony diameter value was 39.20 ± 3.70 mm.

On the other hand, all the tested extracts were found to be effective against *Asp. fumigatus*, reducing the radial growth as compared to control (Table 4). The extracts tested at 10 μg/mL show a percentage of radial growth inhibition that range from 2.94% to 9.28%, and from 7.84% to 19.83% for 100 μg/mL of extract (Table 4). The extracts tested at the highest concentration showed higher values of radial growth inhibition than those tested at the lowest concentration, which seems to indicate a dose-dependent effect. Among the extracts in study, the hexane fractions stand out from the remaining as the most effective against *Asp. fumigatus*. Curiously, the fraction obtained from IMTA-cultivated seaweed showed a stronger effect than the wild sample, inhibiting almost 20% of the mycelial growth as compared to control, after 4 days of exposure. Machado et al. (2014) also observed an increased antifungal potential of seaweed extracts after laboratory

cultivation, which could be associated with a change in concentration of chlorophyll-a and accessory pigments.

The radial growth inhibition assay was optimized for *Asp. fumigatus* testing given that, unlike *A. infectoria*, it is a fast grower mold that quickly covers up the plate with mycelium, making it impossible to quantify the effect of the extracts. The appropriate concentration of conidia was the one that allowed the measurement of *Asp. fumigatus* growth for, at least, 4 days. When radial growth inhibition assay is performed using a spore suspension as inocula instead of a mycelium agar square, the precision of the results of the experiment increase considerably, as show the low values of standard deviation obtained in *Asp. fumigatus* plates (Table 4). Curiously, higher variability was obtained for the fractions that gave higher radial growth inhibition, namely Hw and Hc at 100 µg/mL.

Table 4. Effect of the different *O. pinnatifida* extracts, tested at 10 µg/mL and 100 µg/mL, on colony diameter (CD; mm) and on radial growth inhibition (RGI; %) of *Asp. fumigatus*.

Extract	10 µg/mL		100 µg/mL	
	CD (mm)*	RGI (%)	CD (mm)*	RGI (%)
Mw	71.50 ± 1.32	6.54	70.50 ± 0.50	7.84
Dw	74.25 ± 1.77	2.94	70.17 ± 0.29	8.28
Hw	69.40 ± 1.56	9.28	65.30 ± 9.12	14.64
Mc	73.83 ± 0.76	3.49	70.50 ± 0.50	7.84
Dc	71.28 ± 0.86	6.83	71.50 ± 0.50	6.54
Hc	70.83 ± 0.76	7.41	61.33 ± 10.73	19.83

* Values refer to the average (n = 2) ± SD; incubation temperature: 30 ºC; incubation time: 4 days. The control presented a colony diameter of 76.50 ± 0 mm.

Regarding broth microdilution assays, the extracts in study did not inhibit the growth in none of the yeast specie at the tested concentrations. However, microscopy observations revealed changes in the production of conidia by *A. infectoria* when mycelium was incubated for 72 h with Hw and Dw (Figure 15). Thus, results are given in MEC instead of MIC. *A. infectoria* treated with 20 µg/mL Hw presented normal conidiation (Figure 15B). Yet, conidia has no longer been observed from 30 µg/mL (Figure 15C & D), which corresponds to MEC. In the other hand, Dw was only able to cause the same effect at a slightly higher concentration − 100 µg/mL (Figure 15F). This features contrasted with the control, which presented a high number of conidia in a single preparation (Figure 15A). Lastly, preliminary results indicated that Hc could also have an inhibitory effect on *A.*

infectoria. Still, this effect faded with successive dilutions used for the determination of the MEC.

Despite the sporulation of *A. infectoria* in submerged cultures has not been described yet, it has occurred in this assay. A progressive slight decrease in the RPMI 1640 medium volume in the wells was observed along the experiment due to evaporation. This may have led to an increase in the interface area, allowing a higher aeration of the wells and, consequently, stimulating the conidiation.

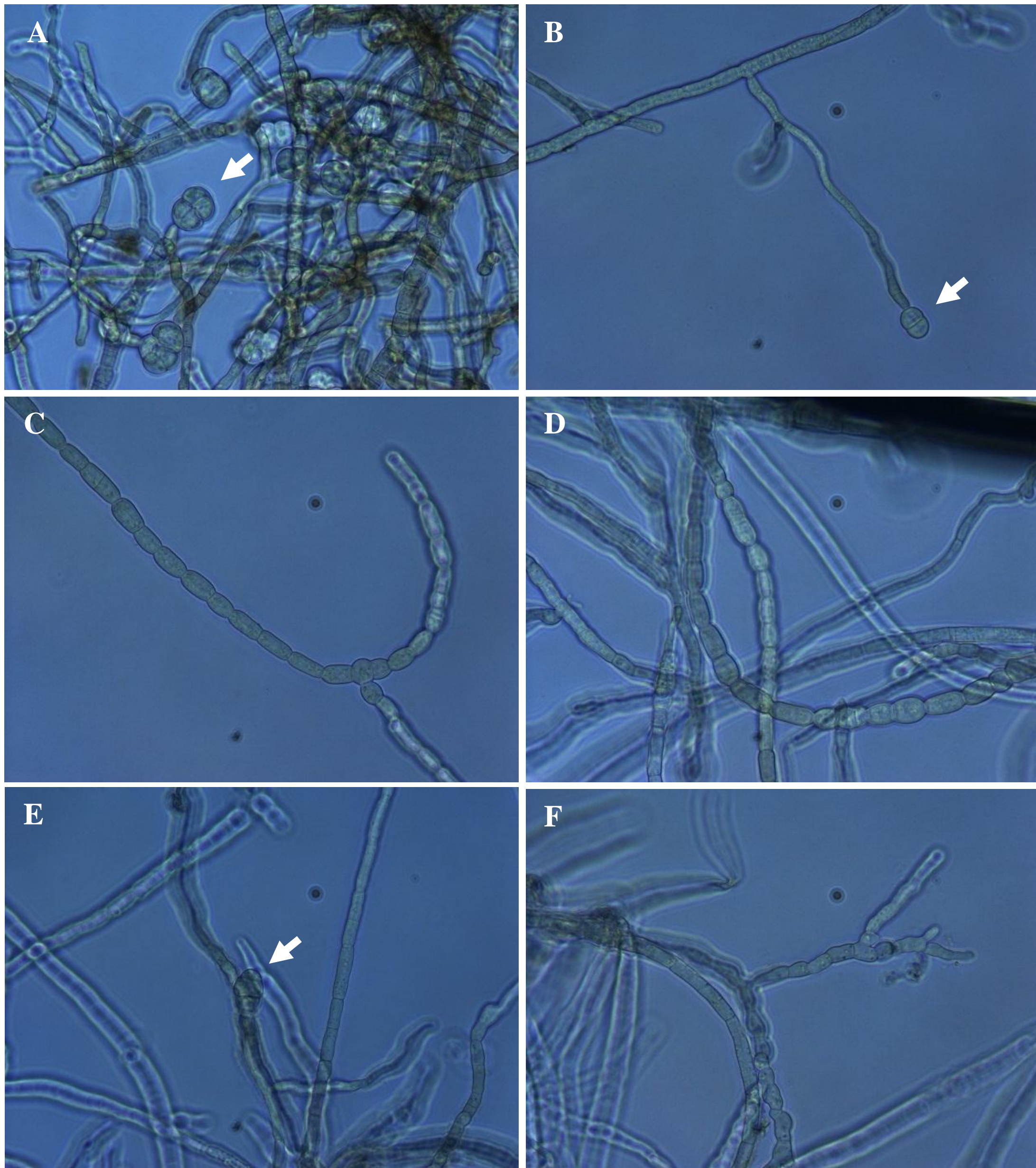
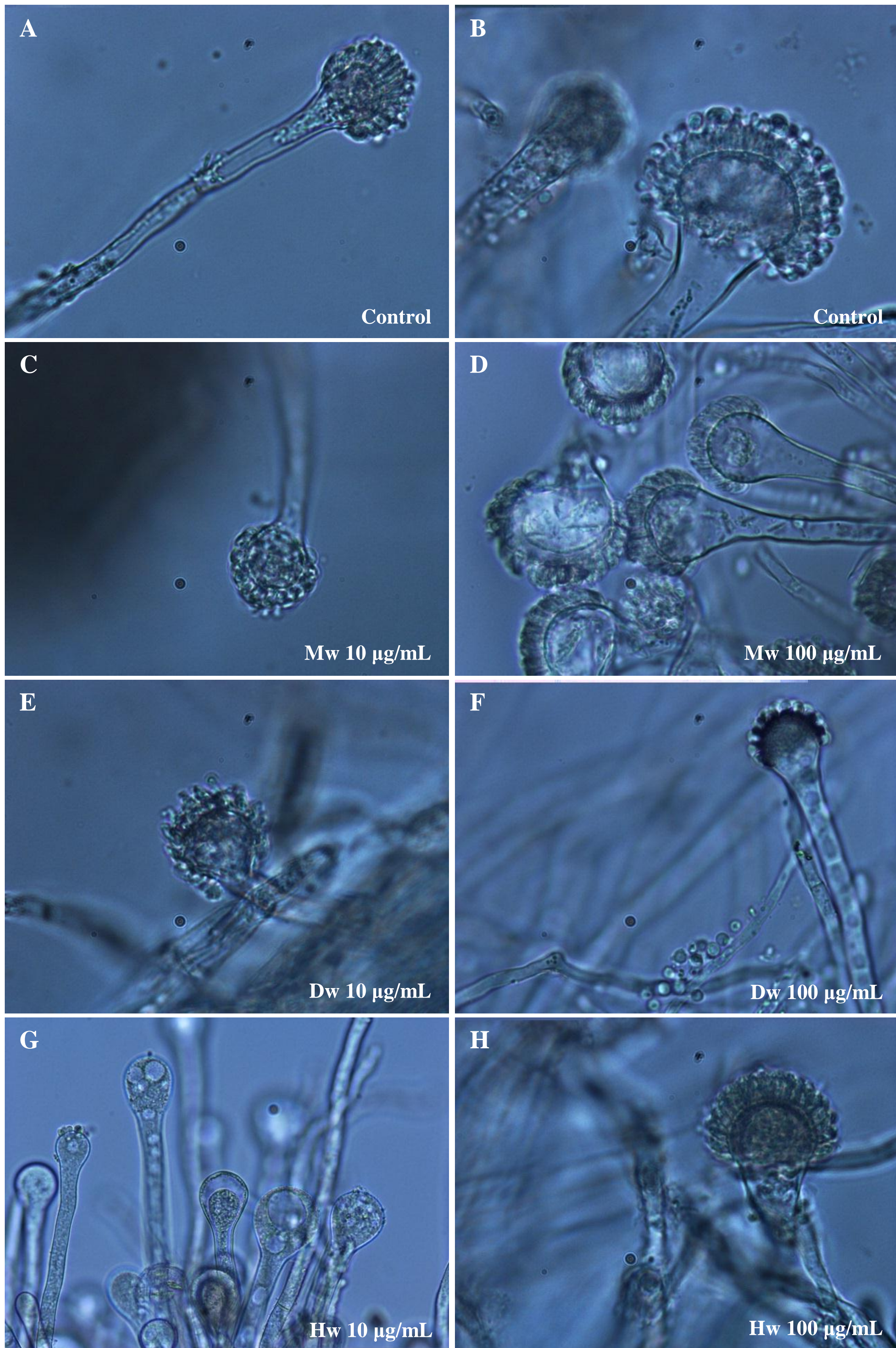


Figure 15. Effect of *O. pinnatifida* extracts on the sporulation of *A. infectoria* grown in RPMI 1640 medium for 72 h (30 ºC, 120 rpm, under alternating 8-h light enriched with UV and 16-h dark cycles). An extract-free control (A) and treatments with 20 µg/mL Hw (B), 30 µg/mL Hw (C) and 40 µg/mL Hw (D); and with 90 µg/mL Dw (E) and 100 µg/mL Dw (F) are displayed. Images were captured with a DS-5M-L1 digital camera (Nikon, Japan), coupled to an Eclipse E400 epi-fluorescence microscope (Nikon, Japan) at 400× total magnification. Arrows indicate presence of conidia.

Liquid *Asp. fumigatus* cultures in YME medium were carried out to study the effects of the extracts that previously showed radial growth inhibition on the morphology of the aerial mycelia (Figure 16). When liquid cultures are grown as shake cultures, the submerged mycelium grows homogeneously due to the constantly moving of liquid nutrient (Booth, 1971). The aeration is also promoted, leading to the development of an aerial mycelium ring above the medium surface, which was used to microscopic observation. The typical morphology of *Asp. fumigatus* conidial heads is shown in control figures, being characterized by the presence of green echinulate conidia (2.5 to 3 μm in diameter) produced in chains basipetally from greenish phialides. This chains of conidia are borne directly on a broadly clavate vesicle (20 to 30 μm in diameter) located at the end of the conidiophore (Latgé, 1999; Mahon, Lehman, & Manuselis Jr, 2014). As shown in Figure 16, methanol fractions (Mw and Mc) at 10 μg/mL and 100 μg/mL, dichloromethane extracts (Dw and Dc) at 10 μg/mL, and hexane fractions (Hw and Hc) at 100 μg/mL didn't changed the morphology of the conidial heads. In turn, dichloromethane extracts at 100 μg/mL led to a reduction in the number of phialides and conidia (Figure 16F & L). 10 μg/mL Hw caused a vacuolization in vesicles and a suppression in the formation of both phialides and conidia (Figure 16G). The impairment in phialide formation was also described in *Asp. fumigatus* Δ*csmA*, Δ*csmB* and Δ*csmA*/Δ*csmB* mutant strains, in which the conidiation defects are phenotypically very similar, implying a role for these enzymes in conidiophore wall biosynthesis (Mellado, Aufauvre-Brown, Gow, & Holden, 1996; Jimeńez-Ortigosa et al., 2012). As previously mentioned, the hexane extracts were the most effective in the reduction of colony radial growth rate. Hc, specially at 10 μg/mL, also appears to have some effect on the morphology of conidial heads, however this is not completely clear due to its relatively early development stage.


A
Control
B
Control
C
Mw 10 µg/mL
D
Mw 100 µg/mL
E
Dw 10 µg/mL
F
Dw 100 µg/mL
G
Hw 10 µg/mL
H
Hw 100 µg/mL

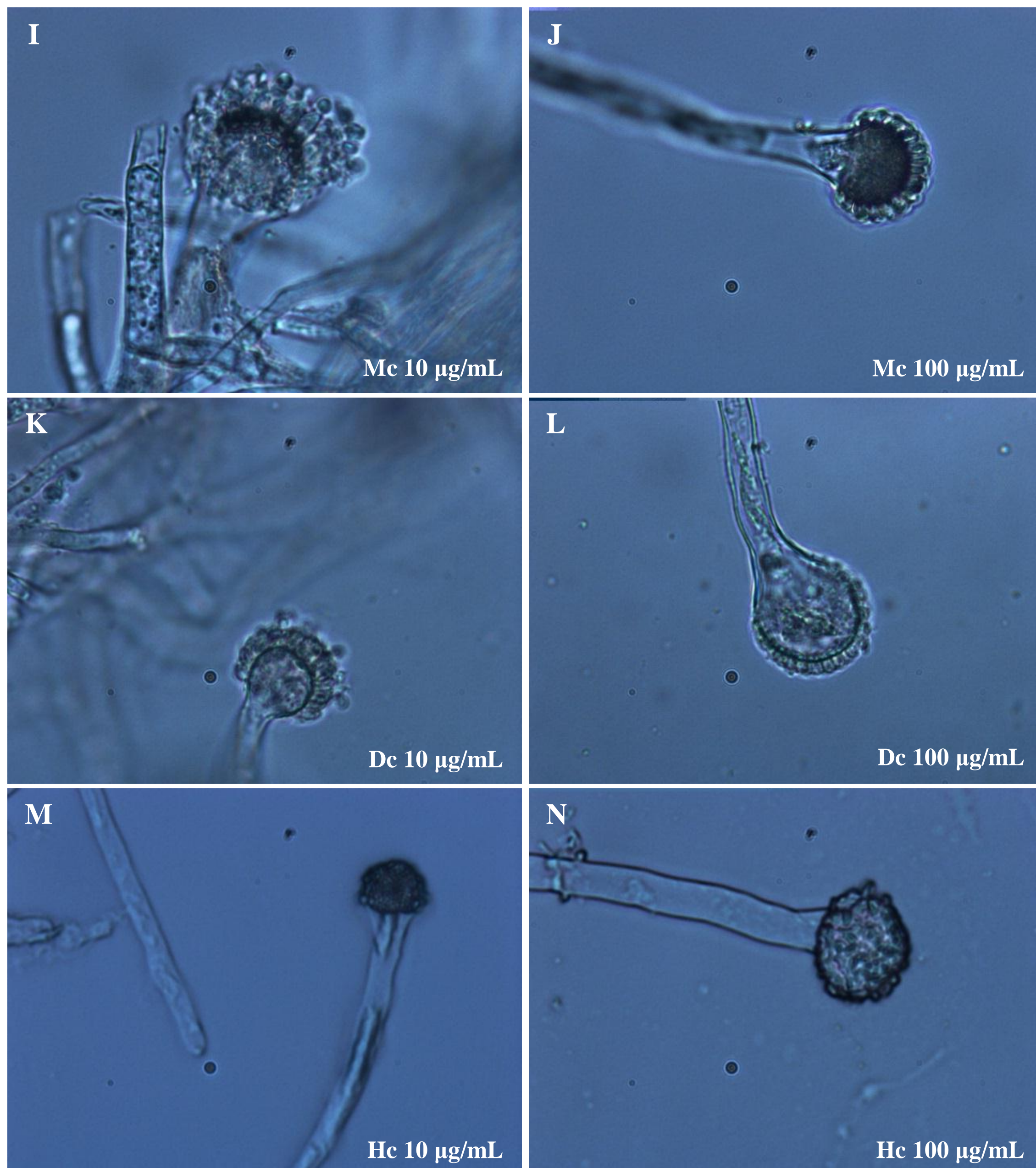


Figure 16. Conidial heads morphology of *Asp. fumigatus* upon exposure to *O. pinnatifida* extracts. Images show *Asp. fumigatus* grown in YME (48 h; 30ºC; 120 rpm) without (A & B – control) or with extract supplementation. C – 10 µg/mL Mw; D – 100 µg/mL Mw; E – 10 µg/mL Dw; F – 100 µg/mL Dw; G – 10 µg/mL Hw and H – 100 µg/mL Hw (previous page). I – 10 µg/mL Mc; J – 100 µg/mL Mc; K – 10 µg/mL Dc; L – 100 µg/mL Dc; M – 10 µg/mL Hc; N – 100 µg/mL Hc (current page). Images were captured with a DS-5M-L1 digital camera (Nikon, Japan), coupled to an Eclipse E400 epi-fluorescence microscope (Nikon, Japan) at 1000× total magnification, except for Hw and Dc at 10 µg/mL (400×).

15.2.3. Influence of *O. pinnatifida* extracts in fungal cell wall composition

Cell wall chitin and β-glucan contents of mycelia of *A. infectoria* and *Asp. fumigatus* were quantified after exposure to *O. pinnatifida* extracts.

As shown in Figure 17, while chitin levels remained almost unchanged, a decrease in β-glucan content of *A. infectoria* occurred upon treatment with 30 µg/mL Hw for 3 days. This observation is in agreement with Fernandes and co-workers (2014), which reported the same effect with the same *A. infectoria* strain, grown in the same conditions, but treated with 1 µg/mL caspofungin, a β-1,3-glucan synthase inhibitor. They also found that this strain did not activate the salvage mechanism in the presence of caspofungin. Here, despite the slight (but insignificant) increase in chitin content upon exposure to 30 µg/mL Hw, it can be stated that *A. infectoria* did not activate a salvage mechanism as well. Curiously, this fraction corresponds to the one that inhibited the sporulation of *A. infectoria* in RPMI 1640 medium, as previously described.

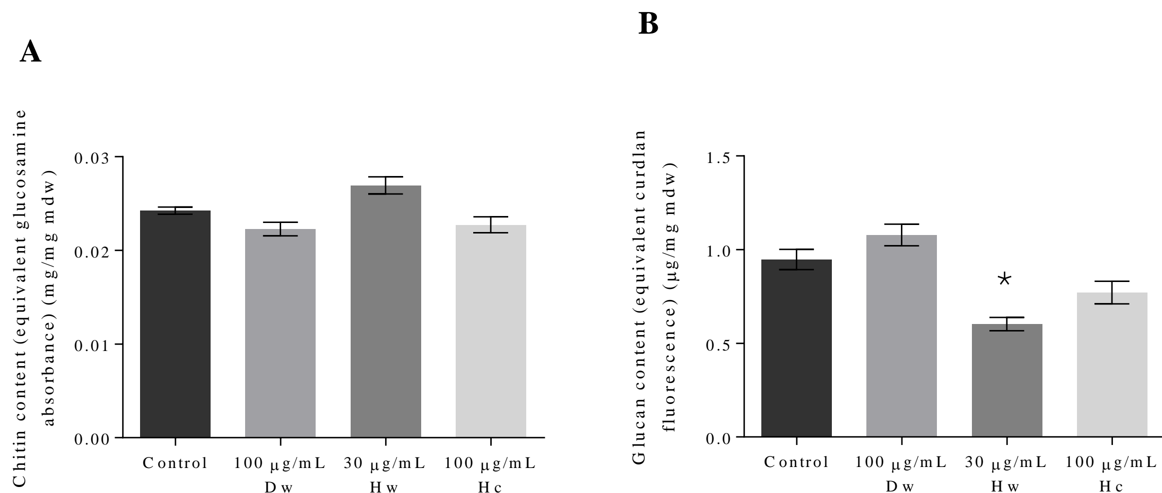


Figure 17. Effects of *O. pinnatifida* extracts on cell wall components of *A. infectoria*. Chitin (A) and β-glucan (B) contents are presented. The strain was grown in liquid cultures (72 h; 30 ºC; under alternating 8-h light enriched with UV and 16-h dark cycles; 120 rpm) on YME without (control) or with extract supplementation (100 µg/mL Dw; 30 µg/mL Hw; 100 µg/mL Hc). Results are the average ± SEM of two independent experiments performed in duplicate. * $P < 0.01$ (Dunnet's post-hoc test).

Treatment with 100 µg/mL Dw and 100 µg/mL Dc led to a significant decrease in the cell wall chitin content of *Asp. fumigatus*, although this was more marked in Dw than in Dc (Figure 18A). *Asp. fumigatus* treated with 10 µg/mL Hw also presented a slight but

statistically insignificant decrease in chitin content. Verwer et al. (2012) found that inhibition of chitin synthesis in *Asp. fumigatus* ATCC 204305 by 1 μg/mL and 64 μg/mL nikkomycin Z, a chitin synthesis inhibitor, resulted in increased synthesis of β-glucan. Here, the mold was somehow unable to compensate for the altered ratio of chitin with β-glucan synthesis when treated with 100 μg/mL Dw, 100 μg/mL Dc, 10 μg/mL Hw and 10 μg/mL Hc. Thus, the referred extracts did not affect the cell wall β-glucan but the chitin contents in *Asp. fumigatus*.

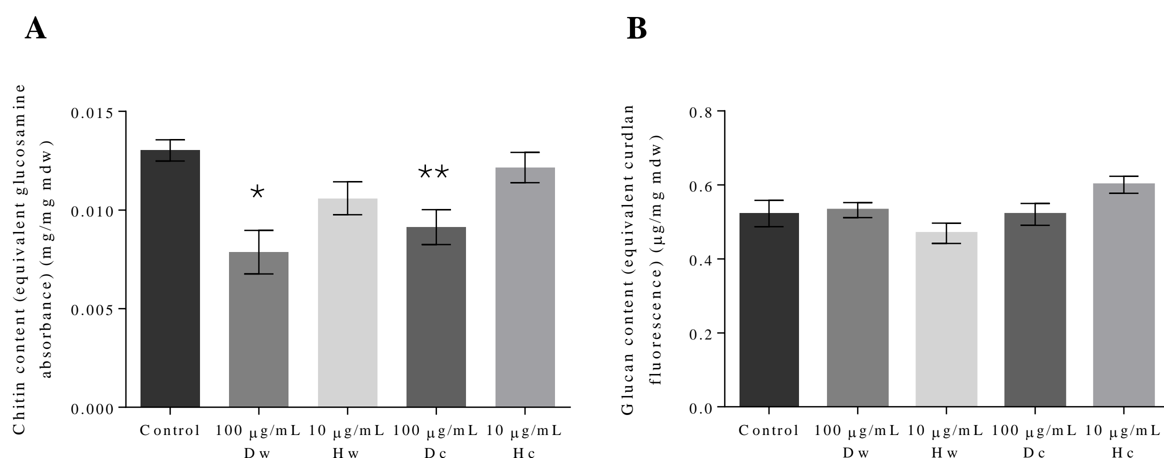


Figure 18. Effects of *O. pinnatifida* extracts on cell wall components of *Asp. fumigatus*. Chitin (A) and β-glucan (B) contents are displayed. The strain was grown in liquid cultures (48 h; 30 ºC; 120 rpm) on YME without (control) or with extract supplementation (100 μg/mL Dw; 10 μg/mL Hw; 100 μg/mL Dc; 10 μg/mL Hc). Results are the average ± SEM of two independent experiments performed in duplicate. * $P < 0.01$; ** $P < 0.03$; (Dunnet's post-hoc test).

15.2.4. Influence of *O. pinnatifida* extracts in fungal morphology

The accumulation of chitin in the cell wall and septa of *A. infectoria* and *Asp. fumigatus* was visualized by CFW fluorescence. As shown in Figure 19, confocal microscopy revealed that chitin is distributed uniformly along the hyphal trunk of *A. infectoria* under all tested conditions. Chitin has also accumulated uniformly along the hyphal trunks and conidial heads in *Asp. fumigatus* under the conditions tested, except for 100 μg/mL Dw (Figure 20B), which led to an apparent decrease in chitin levels when compared to the other treatments, including control. Morphological changes were observed in conidial heads upon exposure to 100 μg/mL Dw and 10 μg/mL extract Hw, confirming

preliminary observations by optical microscopy. When exposed to this extracts, conidial heads exhibited a lack of conidia (Figure 20B & D). In the particular case of *Asp. fumigatus* treated with 10 µg/mL Hw, pore-like structures are still observed at the vesicles' surface, which could correspond to the anchor sites of phialides (Figure 20D).

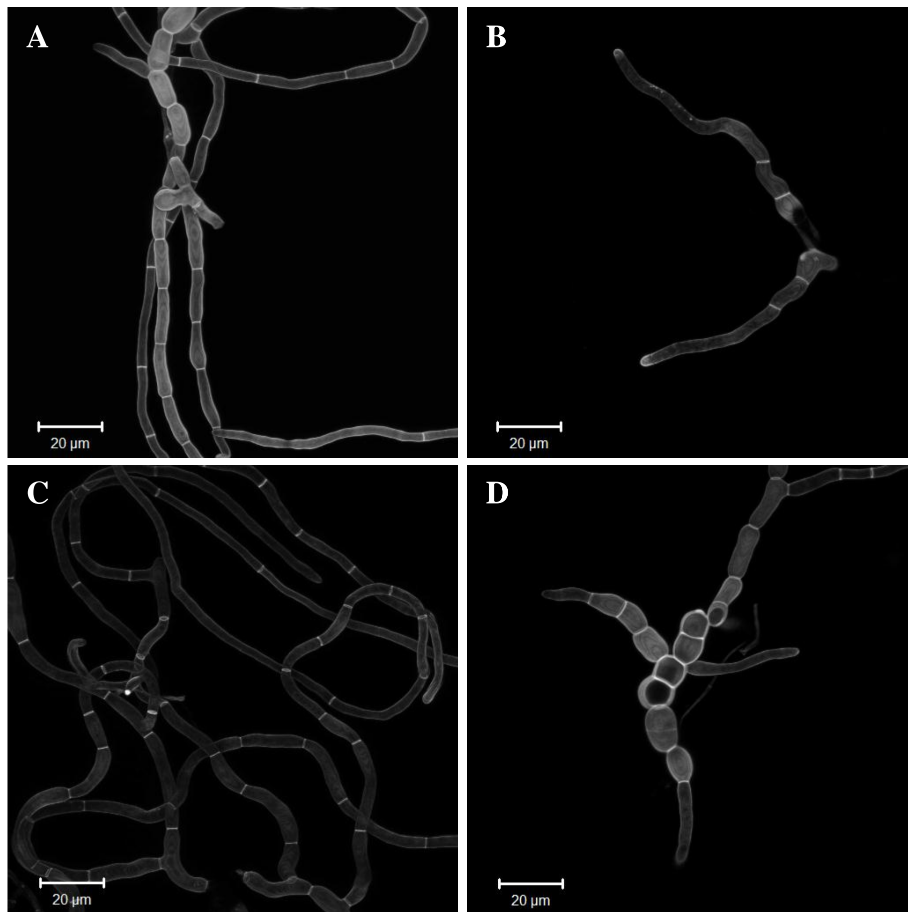


Figure 19. Chitin distribution upon exposure to *O. pinnatifida* extracts. Images show a three-dimensional projection obtained by confocal imaging of chitin stained with CFW in *A. infectoria* grown in YME with 0.5% agar (overnight; 30 ºC; 150 rpm; under alternating 8-h UV light and dark cycles) without (A) or with extract supplementation (B – 100 µg/mL Dw; C – 30 µg/mL Hw; D – 100 µg/mL Hc). For each condition, maximum-intensity projections of z-stack images were obtained with FIJI software. Cell imaging was performed with a Zeiss LSM 510 Meta confocal microscope with a Plan-Apochromat 63× (numerical aperture 1.4) oil objective.

O. pinnatifida extracts had no inhibitory impact in the radial growth of *A. infectoria* in PDA plates, but 30 µg/mL Hw inhibited its' sporulation in RPMI medium. This extract, at the same concentration, was the single one that induced a decrease in β-glucan content of *A. infectoria* grown in YME. However, the morphology of the mold remained

unchanged after 72h exposure. This means that this extract affected *A. infectoria* sporulation, but not the germination process. On the other hand, all the studied extracts inhibited the radial growth of *Asp. fumigatus*. Hexane fractions at 100 µg/mL were the most effective, suggesting the involvement of apolar compounds of the seaweed. Curiously, the fraction obtained from IMTA-cultivated sample presented higher radial growth inhibitory activity (almost 20%) than the wild one. Furthermore, liquid cultures in YME supplemented with 100 µg/mL Dw and 30 µg/mL Hw showed abnormal conidial heads, completely devoid of conidia. The observed effects in *Asp. fumigatus* upon exposure to 100 µg/mL Dw seems to be committed with the verified reduction in chitin levels. Lastly, the prominent phenotype caused by 30 µg/mL Hw exposure appears to be unrelated to chitin and β-glucan contents, but with other factors.

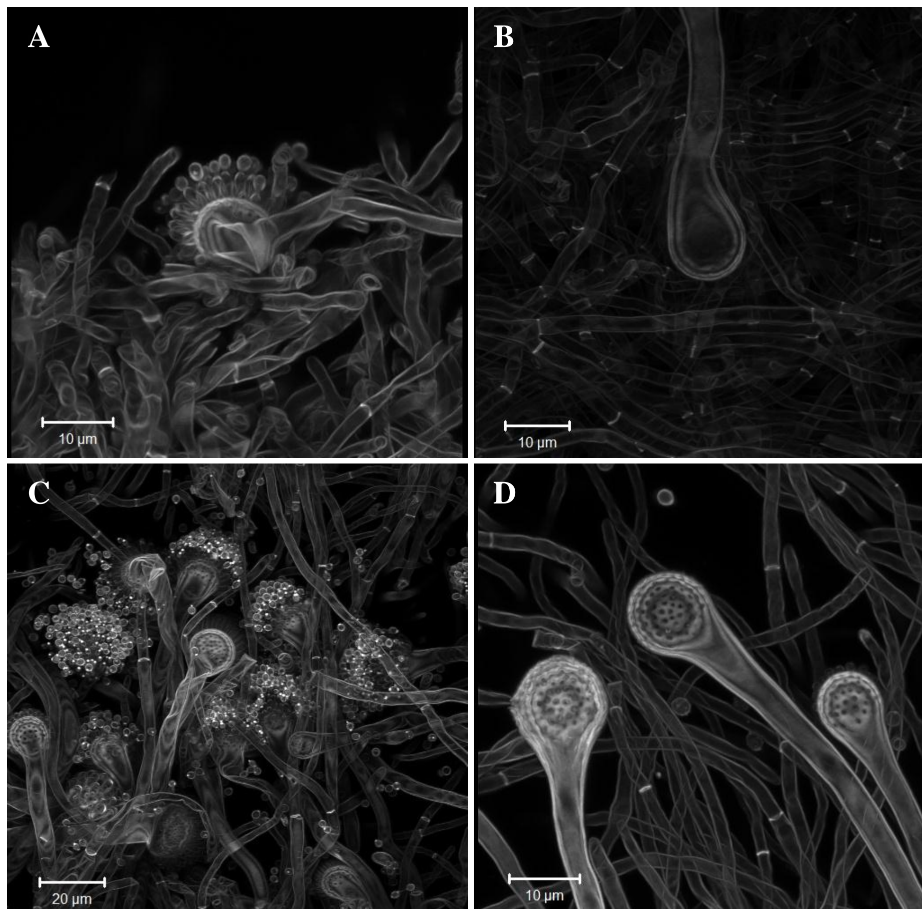


Figure 20. Changes in morphology of the conidial heads and chitin distribution upon exposure to *O. pinnatifida* extracts. Images show a three-dimensional projection obtained by confocal imaging of chitin stained with CFW in *Asp. fumigatus* grown in YME (48 h; 30ºC; 120 rpm) without (A) or with extract supplementation (B – 100 µg/mL Dw; C – 100 µg/mL Dc; D – 10 µg/mL extract Hw). For each condition, maximum-intensity projections of z-stack images were obtained with FIJI software. Cell imaging was performed with with a Zeiss LSM 510 Meta confocal microscope with a Plan-Apochromat 63× (numerical aperture 1.4) oil objective.

CONCLUSIONS AND FUTURE PERSPECTIVES

16. Conclusion and future perspectives

The work carried out under the subject of this dissertation allowed reaching the following conclusions:

Among the cultivation trials of *Osmundea pinnatifida*, the land-based IMTA trial carried out in the winter stand out as the most suitable, since it was the only one in which the morphological integrity of algae was maintained.

Methanol proved to be the most effective organic solvent used for the extraction of compounds from wild and IMTA-cultivated *O. pinnatifida*.

Dichloromethane extracts of both wild (Dw) and IMTA-cultivated seaweed (Dc) were the best source of antioxidants, encompassing higher total phenols content and ORAC values. Phenolic compounds may be responsible for the high peroxyl radical absorption capacity observed.

With respect to antitumor activity, it was found that the cells show different susceptibilities to the action of the extracts. All the tested extracts had no marked effects in HepG-2 cells, in terms of both cell proliferation and cell viability. Both dichloromethane extracts induced a cytostatic effect in MCF-7 cells. Dichloromethane extracts (Dw and Dc) were also effective against SH-SY5Y cell line, inhibited the cell proliferation and compromised the cell viability.

No antibacterial activity was detected against *E. coli* and *S. aureus* under the conditions tested, in any of the studied extracts.

The dichloromethane extract and the hexane fraction of wild *O. pinnatifida*, besides the verified radial growth inhibitory activity, completely inhibited *Asp. fumigatus* sporulation. Inhibition of sporulation may be important in fungal growth control since spores consists in the main dispersal agents of fungi, allowing them to be reproduced, and are linked to respiratory and other health issues, specially in immunocompromised patients. Despite the effect caused by Dw seems to be related with a reduction in chitin content, the same cannot be said for Hw, whose chitin and glucan remained almost unchanged.

These preliminary results are important, since they reveal that *Osmundea pinnatifida* has a great biotechnological potential, opening a door to further investigation.

It should be emphasized that seaweed farming is a recent and emergent practice, requiring substantial improvements at the level of cultivation conditions and

methodologies used. With regard to the *O. pinnatifida* cultivation, it will be interesting to extend the experimental period in the cold season, as well as to submit it to stress conditions in order to verify its response at the chemical composition level, which can be associated with an increase in the production of biologically active secondary metabolites.

All the observed effects throughout this study may be due to a single compound, or to synergistic or antagonistic effects among the various compounds that are present in the extracts. Thus, the isolation and purification of these compounds proves to be of utmost importance, since these procedures will help to uncover if there is any compound in the extract that has a single and effective biological activity. Posteriorly, these compounds may be tested in more complex models to understand their applicability, such as co-cultures for the study of antitumor capacity, prior to *in* vivo testing.

Regarding antifungal activity, further studies, such as the identification of the chitin and β-glucan synthases affected by these extracts, are needed to elucidate the mechanisms that led to the verified effects on *Asp. fumigatus*. Additionally, the isolation and identification of the bioactive components of the extract responsible for the observed phenotypes in the conidial heads are crucial for biotechnological applications, either as antifungal agents from natural sources or for the remediation of fungal contamination in residential and occupational buildings.

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