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Extracts of the seaweed *Bifurcaria bifurcata* display antifungal activity against human dermatophyte fungi

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Abstract *Bifurcaria bifurcata* is a seaweed of the Order Fucales (Ochrophyta, Phaeophyceae) that can be found all year round along the Portuguese Atlantic shore. Although it is considered edible in some countries, its biotechnological potential has not yet been assessed in detail and here we report its antimicrobial potential against human dermatophyte fungi. Three samples were harvested from Baleal Island (Peniche, Portugal) harvested in winter (BbPe); from Aguda Beach (Arcozelo, Vila Nova de Gaia, Portugal), harvested in autumn (BbAg) and a sample obtained by culturing the Aguda sample under laboratory conditions (BbLC). A broth macrodilution assay was applied to determine the MIC (minimum inhibitory concentration) and MLC (minimum lethal concentration) of the *B*. *bifurcata* extracts. Results show that the BbPe MeOH extracts were the most effective and had higher antifungal activity against all the tested dermatophyte strains than the BbLC and BaAg extracts [*Epidermophyton floccosum* FF9 (100–200 μg/mL MIC; 200 μg/mL MLC), *Microsporum canis* FF1 (400 μg/mL MIC; ≥400 μg/mL MLC), *Trichophyton mentagrophytes* FF7 (100 μg/mL MIC; >800 μg/mL MLC), *M*. *gypseum* CECT 2908 (800 μg/mL MIC; ≥800 μg/mL MLC), *T*. *mentagrophytes* var. *interdigitale* CECT 2958 (800 μg/mL MIC; ≥800 μg/mL MLC), *T*. *rubrum* CECT 2794 (200 μg/mL MIC; ≥400 μg/mL MLC)]. In fact, only *E*. *floccosum* FF9 was sensitive to BbAg and BbLC MeOH extracts. To our knowledge, this is the first report of antifungal activity of *B*. *bifurcata* against human dermatophyte fungi.

Keyword: seaweed; *Bifurcaria bifurcata*; bioactivity; antifungal; dermatophytes; minimum inhibitory concentration; minimum lethal concentration

1 INTRODUCTION

Nowadays, seaweeds are a blooming subject in the field of marine biotechnology, be it for direct consumption as food or as ingredients in food, agriculture, pharmaceutical or cosmetic industries. They can have significant effects as biostimulants for plant growth (Cardoso et al., 2014), as cosmetic ingredients and their bioproducts, mainly polysaccharides, have been shown to have a range of bioactivities (Cardoso et al., 2014; Carvalho and Pereira, 2015). New drug discovery is a very active field of research in seaweed biotechnology with evidence for antimicrobial, antitumoral, anti-inflammatory and immunomodulation effects (Cardoso et al., 2014; Carvalho and Pereira, 2015). There has been work on antibacterial and antiviral effects of seaweed extracts, but in comparison, there is much less published regarding antifungal (specifically human fungal pathogens) and anti-protozoal activities. A variety of seaweed extracts show interesting results

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as antifungal agents. For example, *Turbinaria conoids*, *Padina gymnospora*, *Sargassum tenerrimum* (Phaeophyceae) revealed antifungal activities against *Candida albicans*, *Penicillium* sp., *Aspergillus flavus*, *Aspergillus tetreus*, *Candida glabrata*, and *Cryptococcus neoformans* (Manivannan et al., 2011). Extracts from *Codium decorticatum*, *Caulerpa scalpelliformis* (Chlorophyta), *Turbinaria conoides*, *Sargassum swartzii* (formerly *Sargassum wightii*) (Phaeophyceae) and *Acanthophora spicifera* (Rhodophyta) have also been reported as an antifungal (Lavanya and Veerappan, 2012).

Bifurcaria bifurcata is a macroalga from the Order Fucales (Ochrophyta, Phaeophyceae) that can be found all along the European Atlantic shores (Pereira, 2015, 2018a). It is perennial, fixed in the rocky substrate that, depending on local hydrodynamics, can be found all year around from the mid-littoral zone. Research has focused on the biochemical composition on *B. bifurcata* with reports of pigments (e.g. carotenoids, chlorophylls *e* xanthophyll), fatty acids, polar compounds (e.g. phlorotannins), as well as sterols and diterpenes (Hellio et al., 2001; Maréchal et al., 2004; Abboud et al., 2009; Gallé et al., 2013). The potential economic value of this seaweed is unknown, although there is work pointing to biological activity, such as anti-proliferative, antioxidant, antitumor, antifouling, heavy metal bioremediation and even industrial use for hydroseeding (Pereira, 2015). Although not consumed in most countries where it is native, this alga is considered edible (Pereira, 2016) which means that it has generally regarded as safe to handle and use for food and product development.

The main of this work is to explore the antifungal potential of *B. bifurcata* against human dermatophyte fungi.

2 MATERIAL AND METHOD

2.1 Sampling location, sample acclimatization, and preparation

Samples were harvested from two different geographical locations in Portugal. The first was at North Baleal Beach, from Baleal Island (Peniche, Portugal), in winter (BbPe). It is a mixed substrate location with rocky zones (calcareous rocks) and sand zones, very exposed to hydrodynamics of sea waves (Pereira, 2018b). However, the exact spot of the harvest was protected against waves and showed a local low gradient slope. The second harvest location was at Aguda Beach (Arcozelo, Vila Nova de Gaia, Portugal), in autumn, samples termed BbAg. This place consists of a large rocky shore area, with low gradient slope but subjected to strong hydrodynamics of sea waves (Pereira, 2018c).

All harvested samples were washed with seawater on site and were transported in insulated sealed ice-boxes for temperature stress reduction until arrival to the laboratory where they were cleansed of sand, mollusks, crustaceans, and other seaweeds before freezing (-22°C) and subsequent freeze-drying.

Part of the sample harvested in Aguda Beach was also submitted for aquaculture at the company AlgaPlus (Ílhavo, Portugal—www.algaplus.pt) using a batch culture system in laboratory conditions with controlled light intensity, temperature, and photoperiod and using enriched water from a fish aquaculture grown for 4–5 weeks. After culture, this material was freeze-dried to produce the *B. bifurcata* laboratory cultured sample (BbLC).

2.2 Extraction procedure

2.2.1 Sample preparation for extraction

After freeze-drying, the samples of *B. bifurcata* (BbPe, BbAg, and BbLC) were pulverized to a diameter <0.5 mm.

2.2.2 Extraction method

The extraction method for this work was designed to extract the widest variety of compounds with different polarities from the *B. bifurcata* biomass. The method (Fig.1) used a sequential extraction with organic solvents, hexane then methanol (MeOH) in the proportion of 1:20 (m/v) and at room temperature, for the extraction of apolar and polar compounds, respectively. Following the organic solvents, water (H_2O) in the proportion of 1:100 (m/v) at 80–100°C was used to extract the remaining polar compounds and polysaccharides. Polysaccharides in this extract were precipitated by ethanol (EtOH) addition and recovered by filtration. Four distinct extracts: hexane extract, MeOH extract, polysaccharides extract and final H_2O extract (after polysaccharides recovery) were obtained.

The MeOH and H_2O extracts were then concentrated at low pressure by rotary evaporation then freeze-dried. Hexane extracts were stored as liquids and the polysaccharides were stored in EtOH (99%) for 24 h at 4°C before vacuum filtration, using G3 Büchner funnels, and finally freeze-dried (Fig.1).

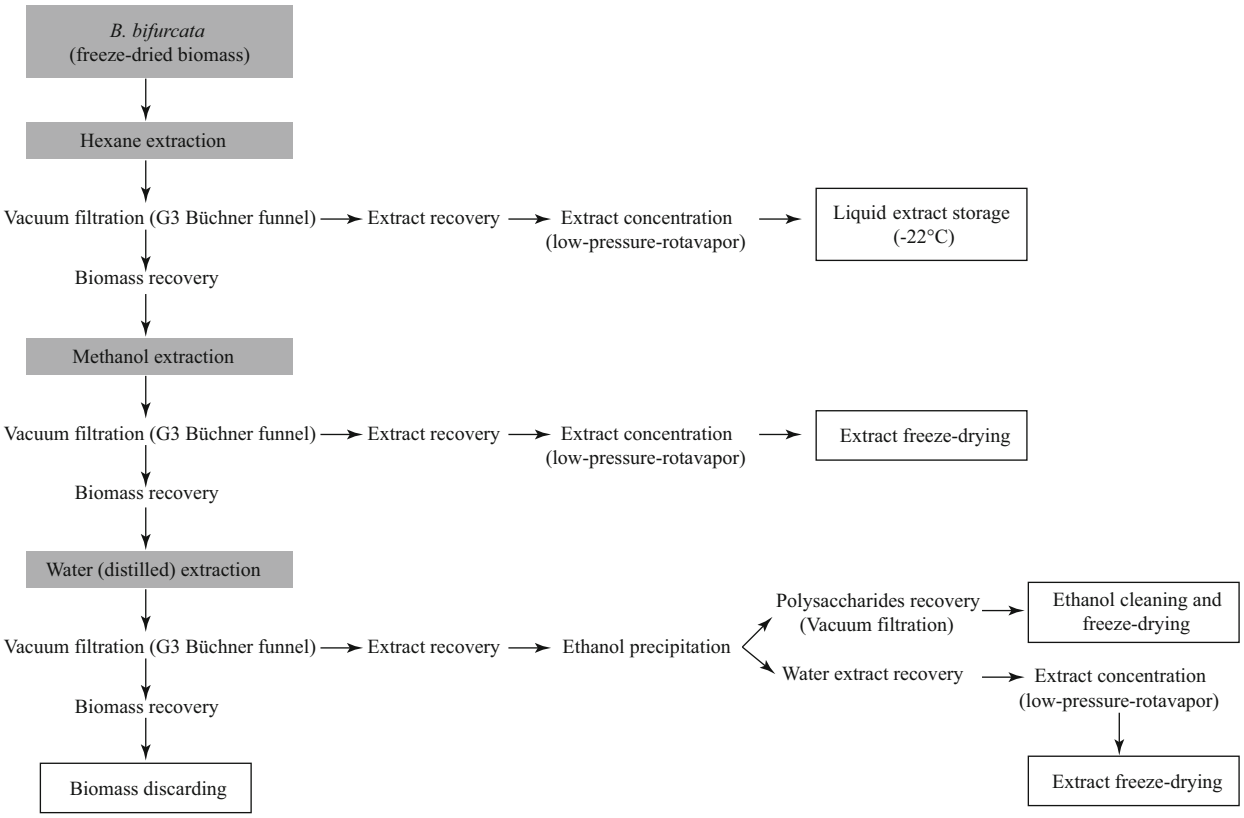

Fig.1 Experiment design of the sequenced extraction procedure

2.3 Antifungal assay

For evaluation of antifungal activity, a broth macrodilution assay was developed, according to M38-A2 (CLSI, 2008), for the determination of the minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) of the extracts obtained from the 3 different *B. bifurcata* (BbPe, BbAg, and BbLC) samples.

For this assay, the Roswell Park Memorial Institute (RPMI 1640, Biochrom) medium was used, at the concentration of 1.04 g/L with an addition of the 3-(n-morpholino) propanesulfonic acid (MOPS) buffer at a final concentration of 0.165 mol/L. All the components were dissolved in distilled water and the pH was adjusted to pH 7 before filtering the medium for sterilization.

2.3.1 Fungal strains

The dermatophyte fungi used in this work were: three strains of clinical dermatophyte isolated from nails and skin (*Epidermophyton floccosum* FF9, *Microsporum canis* FF1 and *Trichophyton mentagrophytes* FF7) and four dermatophyte type strains from the Colección Espanõla de Cultivos Tipo (*Microsporum gypseum* CECT 2908, *Trichophyton mentagrophytes* var. *interdigitale* CECT 2958, *Thrichophyton rubrum* CECT 2794, *Trichophyton verrucosum* CECT 2992). These isolates stored in a Sabouraud broth with glycerol at -70°C. Before the assays, the fungi were inoculated in Sabouraud dextrose agar (SDA) or in potato dextrose agar (PDA) to ensure the optimal growth, quality, and purity of the cultures.

2.3.2 Seaweed extracts preparation for the assay

Due to the difficulty of solubilizing seaweed hexane extracts in a way that would comply with the antifungal assay, these were not used for this work. Seaweed MeOH extracts were re-dissolved in 40% aqueous EtOH solution and dried H_2O and polysaccharides extracts were re-dissolved in sterilized distilled H_2O. Subsequently, these extracts were homogenized by vortex and ultrasonic treatment.

2.3.3 Inoculum preparation

Fungi were cultivated in tubes for 7 days at 30°C in SDA medium and suspensions of spores and mycelia were obtained by adding physiological saline solution and vortexing with glass pearls (3-mm diameter). The resulting mixture of conidia and hyphal fragments

Table 1 Antifungal activity (MIC and MLC) of *B. bifurcata* MeOH extracts against human dermatophyte strains (*n*=3, tests performed in duplicate)

Strain	BbPe		BbAg		BbLC		Fluconazole*	
	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC
Epidermophyton floccosum FF9	100–200	200	800	800	400	400	16	16
Microsporum canis FF1	400	≥400	800	>800	400	800	128	128
Trichophyton mentagrophytes FF7	100	>800	800	>800	800	≥800	16–32	32–64
Microsporum gypseum CECT 2908	800	≥800	>800	>800	800	>800	128	>128
Trichophyton mentagrophytes var. *interdigitale* CECT 2958	800	≥800	>800	>800	>800	>800	128	≥128
Thrichophyton rubrum CECT 2794	200	≥400	800	>800	800	≥800	16	64
Trichophyton verrucosum CECT 2992	> 800	> 800	>800	>800	>800	>800	128	>128

MIC (minimum inhibitory concentration) and MLC (minimum lethal concentration) were determined by the macrodilution method and are expressed in μg/mL dry weight (m/v). BbPe: wild-type sample from Peniche; BbAg: wild-type sample from Praia da Aguda; BbLC: sample from laboratory culture. *: fluconazole was the control antifungal antibiotic.

were transferred to a sterile tube and adjusted to the desired concentration by hemocytometer counting.

2.3.4 Antifungal test

Serial dilutions of the extract (0.8, 0.4, 0.2, 0.1, 0.05 mg/mL) were prepared in distilled water. In each test tube, 100 μL of the diluted extract and 900 μL of inoculated RPMI media containing $(1–2)\times10^4$ cells/mL was added. The tubes were incubated aerobically at 30°C for 7 days and MICs were determined from negative growth tubes. To evaluate MLCs, aliquots (20 μL) of broth were taken from each negative tube and cultured in SDA plates. Plates were then incubated for 7 days at 30°C. In addition, a reference antifungal compound, fluconazole (Pfizer) was used as a standard control to assess the sensitivity of tested microorganisms. For each strain tested, the growing conditions and the sterility of the medium were checked in two control tubes. All experiments were performed in duplicate for a total of three independent assays.

3 RESULT AND DISCUSSION

Before testing against the seven dermatophyte strains, the extracts were screened for antifungal potential using the same methodology but applied to only *Epidermophyton floccosum* FF9, *Microsporum canis* FF1 and *Trichophyton mentagrophytes* FF7 (data not shown). Only MeOH extracts of all *B. bifurcata* samples (BbPe, BbAg, BbLC) demonstrated antifungal activity so only those were tested against all seven strains and the polysaccharides and H_2O extracts were not tested.

MIC and MLC data was gathered for each extract and the respective dermatophyte strain (Table 1).

Of the tested seaweed samples, BbPe was the most active against dermatophyte strains with BpAg being the less active (Table 1). Significant inhibitory and lethal activities were verified for the *Epidermophyton floccosum* FF9. Moreover, BbPe presented the highest inhibitory capacity against *Trichophyton mentagrophytes* FF7 (100 μg/mL MIC) and *Trichophyton rubrum* CECT 2794 (200 μg/mL MIC) (Table 1).

Fluconazole shows much lower MIC and MLC values but in terms of fungi resistance it can be observed the same pattern with BbPe where *E. floccosum* FF9, *T. mentagrophytes* FF7, and *T. rubrum* CECT 2794 are the less resistant and *Microsporum gypseum* CECT 2908, *Trichophyton mentagrophytes* var. *interdigitale* CECT 2958 and *T. verrucosum* CECT 2992 the most resistant strains (Table 1).

To our knowledge, this is the first study to demonstrate the antifungal capacity of *B. bifurcata* against human dermatophyte fungi. The fact that only the MeOH extracts showed antifungal activity is quite in line with other published work where polar fractions or extracts reveal antifungal activity (Hellio et al. (2001), Manivannan et al. (2011) and Lavanya and Veerappan (2012). As described by Oumaskour et al. (2012), dichloromethane/MeOH extracts of *Laminaria ochroleuca* (Phaeophyceae) had activity against *Cryptococcus neoformans*, and MeOH extracts of *Colpomenia sinuosa* and *Saccorhiza polyschides* (formerly *Saccorhiza bulbosa*) (Phaeophyceae) had activity towards *Candida tropicalis*. The greater effectiveness of polar extracts was reported with ethyl acetate and MeOH extracts of *Cystoseira sedoides*, *Cystoseira crinita* and *Cystoseira compressa* against 5 yeast species (*Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida*

dubliniensis, and *Candida kefyr*) (Mhadhebi et al., 2012).

Antifungal activity against human dermatophyte fungi strains was found in MeOH extracts produced from *Ulva rigida* (Chlorophyta) and *Gelidium microdon* (Rhodophyta) (Silva et al., 2013) but these seaweed extracts were apparently less active than *B. bifurcata*, though direct comparisons are difficult as the maximum concentration used was 256 μg/mL and the fungal strains used were not the same. Nevertheless, *B. bifurcata* (BbPe) had higher antifungal activity than *U. rigida* and *G. microdon* extracts against *T. mentagrophytes* FF7 and *E. floccosum* FF9 (Silva et al., 2013). Lopes et al. (2012) used eleven brown algae (Ochrophyta-Phaeophyceae) to obtain phlorotannin-enriched extracts, but their lowest MIC and MLC values were 3.9 and 7.8 mg/mL respectively from *Cystoseira nodicaulis* extracts, substantially higher to our best results.

Although the antifungal activity from the *B. bifurcata* extracts was not greater than Fluconazole, the samples used were crude MeOH extracts and purification of the active components may be relevant, and some studies suggest the possible origin of the group of metabolites that cause the antifungal activity. Twelve purified extracts of acyclic diterpenes and complex mixtures of non-acetylated diterpenes from *B. bifurcata* from four different geographical locations (Quiberon, France; Port Sail, France; Oualidia, Morocco; and Black Head, Ireland), were tested for antifouling capacity (Hellio et al., 2001) and they revealed antifungal effects against marine fungi cultures (*Corollospora maritima*, *Lulworthia* sp. and *Dendryphiella salina*) with MIC values of 8 μg/mL (Hellio et al., 2001). Although this antifungal activity was not against dermatophytes, this suggests that the antifungal activity in our extracts may be associated with diterpenes.

In an interesting work, Gallé et al. (2013) selected twenty seaweed species to evaluate bioactivity against *Trypanosoma brucei rhodesiense* trypomastigotes and reported impressive results from hydro-alcoholic and ethyl acetate extracts from *B. bifurcata*. This might suggest that the antifungal effect of BbPe might arise from a general cytotoxic effect, not specific for fungi but for other microorganisms as well. It is notable that after fractionation, the most active extract contained the diterpene eleganolone. However, when this fraction was tested alone, the activity was decreased compared to the whole extracts, which suggests a synergistic effect between different *B. bifurcata* metabolites (Maréchal et al., 2004; Gallé et al., 2013).

Fractionation of the BbPe MeOH extract could determine which fraction or fractions, alone or mixed, were responsible for the antifungal activity. Use of metabolomics strategies with liquid chromatography coupled to mass spectrometry (LC-MS) could elucidate the metabolites present in these tested fractions that might be responsible for the antifungal effect. This approach could also explain the differences between the three different *B. bifurcata* samples in this work (BbPe, BbAg, and BbLC).

Another subject where a metabolomics approach would help is in determining if the seasonal and geographical variation, as reported in by Hellio et al., (2001) and Maréchal et al., (2004), might influence the different antifungal activities registered from the *B. bifurcata* extracts of this work (BbPe, BbAg, and BbLC). Seaweed metabolism changes as the local climate changes during the year. The sunny winters common in Portugal encourage biomass production in perennial seaweed species, depending on wave hydrodynamics. Indeed, *Bifurcaria bifurcata* is perennial and adult specimens can be easily found during winter if the location is somehow protected against the strong waves.

However, looking for effects due to seasonal variation among our bioactivity results is hampered as we can only compare BbPe (harvested in January) with BbAg (harvested October) and these samples were obtained from different locations. Therefore, we cannot confirm if the higher antifungal activity of winter-harvested BbPe (January) over autumn-harvested BbAg (October) was due to location or season. Indeed, a future perspective would be a geographical and seasonality study with the collection of samples at different times of a year and from different places to compare activity results and extrapolate seasonal influence among the optimal period of a year and optimal climatic factors for harvesting. This information could direct future harvesting or culturing protocols for maximizing antifungal components from *B. bifurcata*. The fact that the bioactivity of the cultured sample (BbLC) was inferior to the wild-type sample BbPe might suggest that encouragement of vegetative growth in culture does not encourage the production of the metabolites responsible for the antifungal activity.

In summary, *B. bifurcata* MeOH extracts demonstrated antifungal capacity against human dermatophyte fungi and appear to be the most

effective seaweed in published literature. It would be interesting to extend the screen for antifungal activity to other important fungal species. The effectiveness of the crude extracts suggests the potential for pharmaceutical studies to purify and identify candidate antifungal components. Previous work strongly suggests that the compounds responsible for this activity might be diterpenes. This work indicates that bioactivity might be seasonally or geographically influenced, and the next steps could be to apply a metabolomics approach using a seasonal and geographical sensitive sampling method. This way, characterization and identification of the responsible metabolites for antifungal effect will be achieved together with the knowledge on the optimal growth conditions for culturing this seaweed should the antifungal properties justify exploitation.

4 CONCLUSION

For the first time, we report the antifungal capacity of *B. bifurcata* against human pathogen dermatophyte fungi strains, by evaluating the Minimum Inhibitory Concentrations (MICs) and Minimum Lethal Concentrations (MLCs). Such bioactivity needs to be more explored to discover the active compounds and to understand the effect of external factors such as climate and local geography on this bioactivity. A metabolomics approach together with a strategic seasonal and geographical harvest of samples would be a promising strategy to discover which metabolites are responsible for the activity. Also, testing these extracts against a wider range of fungi, perhaps those which cause problems on crops and/or food would be of major interest.

5 DATA AVAIBILITY STATEMENT

The data that support the findings of this study are available in the repository "Estudo Geral" from the University of Coimbra, with the identifier http://hdl.handle.net/10316/24928 (in Portuguese).

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