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Original Article

Antibacterial, antifungal and cytotoxic activities exhibited by endophytic fungi from the Brazilian marine red alga *Bostrychia tenella* (Ceramiales)

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ABSTRACT

Marine environment is one of the most important sources regarding natural products research. Besides, marine microorganisms have been denominated as a talented natural source for discovery of new leads. Although the association of macroalgae and fungi has been described regarding ecological issues, there is a lack of studies about marine seaweed endophytic fungi. In this context, the goal of this study was to evaluate cytotoxic, antifungal and antibacterial activities of endophytic fungi isolated from the Brazilian marine seaweed *Bostrychia tenella* (J.V. Lamouroux) J. Agardh (Ceramiales, Rhodophyta). Forty-five endophytic microorganism strains were isolated from *B. tenella*. Crude extracts and organic fractions of ten selected strains were obtained after growth in rice medium. Samples were evaluated for cytotoxicity, antifungal and antibacterial assays. *Penicillium* strains showed positive results in a diversity of assays, and other five strains were active in at least one test. In addition, cytochalasin D was isolated from *Xylaria* sp. This alga is composed of a microbiological potential, since its endophytic strains exhibited remarkable biological properties. Moreover, cytochalasin D isolation has confirmed chemical potential of marine endophytic strains. This is the first study in which cultured fungi isolates from the Brazilian macroalga *B. tenella* were evaluated concerning biological properties. Results corroborated that this species could be a pharmaceutical source from marine environment. Furthermore, *Acremonium implicatum* is being firstly described as marine endophyte and *Xylaria* sp., *Trichoderma atroviride* and *Nigrospora oryzae* as marine seaweed endophytes. Thus, this work reports the first study relating detailed isolation, cultivation and biological evaluation (cytotoxic, antifungal and antibacterial) of endophytes *Penicillium decaturense* and *P. waksmanii* from the Brazilian marine red alga *B. tenella*. We are also reporting the isolation of cytochalasin D, a known antitumor and antibiotic compound, from *Xylaria* sp. strain. Despite widespread prevalence in terrestrial and marine habitats, this present work describes the first occurrence of cytochalasin D as a metabolite from marine seaweed endophyte.

Introduction

Marine environment is currently pointed as one of the most important sources regarding natural products research field, since organisms from oceans have been exhibited remarkable biological, biochemical and biosynthetic potential (Gerwick and Moore, 2012; Mayer et al., 2010, 2011; Molinski et al., 2009; Newman and Cragg, 2014). Biodiversity is very expressive concerning high taxonomic levels: from 76 phyla described for *Eukariota*, about sixty can be found in marine areas, meanwhile forty for terrestrial or freshwater environments (Blunt et al., 2013). Marine natural products research have often been mentioned as fundamental for discovery

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of new chemical structures, mainly for featuring unusual mechanisms of action (Molinski et al., 2009). In a recent data, up to 2009, 2840 marine species have been investigated resulting on the isolation of 20,057 metabolites, which were published in 7795 articles. Despite exciting numbers, considering the total of 250,000 recognized marine species, it is estimated that only 1% of them have already been studied (Blunt et al., 2013), which means there are an amazing roll of unexplored living beings comprising chemical and biological treasures.

Similarly, microbial natural products represent an extensive area for new therapeutic compounds search (Berdy, 2012; Cragg and Newman, 2013; Demain, 2014; Vederas and Li, 2009; Walsh and Fischbach, 2010). Relevant reviews emphasized microbial metabolites as targets for discovery and development of new drugs, mostly anticancer and antibiotics (Berdy, 2012; Butler et al., 2013; Demain, 2014), antifungals, antiparasitics, among others (Amedei and D'Elios, 2012). Microorganisms are very versatile and found everywhere, even in inhospitable habitats, in all ecosystems around the globe. It is preconized that less than 1% of all bacteria species and less than 5% of all fungi species are described, suggesting at least 10 million microbial species are unknown, remaining hidden in nature (Berdy, 2012). Besides, based on genetic researches, 90% of biosynthetic skill of microorganisms keeps unattainable, what ratifies the significance of microbial natural products research for drug discovery and, even for complete biodiversity knowledge and ecological relationships understanding (Walsh and Fischbach, 2010).

Additionally, assembling these concepts and overlapping remarkable uncharted fields, marine microorganisms have been denominated as a talented natural source for discovery of new leads, for showing notable biosynthetic ability as producers of functional metabolites (Blunt et al., 2013; Cragg and Newman, 2013; Fenical and Jensen, 2006; Gerwick and Fenner, 2013; Gerwick and Moore, 2012). Predicted numbers suggest approximately 3.7×10^{30} microorganisms fighting for survival in oceans and seas, most owning extraordinary undiscovered biochemistry (Fenical and Jensen, 2006; Li and Vederas, 2009). In this context, there are few evidences proving that invertebrate marine hosts and microorganisms establish an intense symbiotic relationship, since several prospections indicated that active substances produced by sponges, tunicate, soft corals etc., are actually microbial products (Dorrestein et al., 2008; Gerwick and Fenner, 2013; Gerwick and Moore, 2012; Glaser and Mayer, 2009).

Twenty approved marine drugs and/or in clinical or preclinical trials were organized accordingly field collected source and biosynthesis pathways origin source (Gerwick and Moore, 2012). In this work, authors have strongly proponed that heterotrophic bacteria and cyanobacteria are the real ocean biologic treasures, being genetically responsible for 80% of these previously mentioned marine leads. Moreover, with the advance of genetic techniques that will allow isolation and expression of biosynthetic clusters, microorganisms and respective marine invertebrate hosts will represent a new frontier for natural products drug discovery (Cragg and Newman, 2013).

Concerning ecological issues, it is reasonable to exploit the specific role of endophytic microorganisms in marine life style. Some authors have been described endophytes as any microbe that spends part of their life cycle in plant host health tissues, in a balance relationship that can alternate between latent pathogenesis and mutualistic symbiosis (Kusari et al., 2013). However, the widely spread definition explains that an endophytic microorganism is a fungus or bacteria that colonizes plants inner tissues offering no apparent impact to hosts (Gutierrez et al., 2012; Zhao et al., 2011). Despite slight differences, there is a common agreement about an evident genic intercommunication between hosts and endophytes, strongly proved by shared metabolic abilities (Gutierrez et al., 2012; Kusari et al., 2013; Zhao et al., 2011). They present chemical diversity, since are producers of alkaloids, terpenoids, flavonoids, steroids, besides owners of biological richness (Guo et al., 2008). Recent works have reported the isolation of antitumor compounds from endophytic fungi of plants, thus arousing the interest of scientific community. Paclitaxel, camptotecin, podophyllotoxin, vinblastine and vincristine were detected in endophytes obtained from original producer plant (Cragg and Newman, 2013; Kusari et al., 2013; Zhao et al., 2011).

The association of macroalgae and fungi has been already described regarding ecological issues; however, there is a lack of studies about marine seaweed endophytic fungi, especially from tropical/subtropical regions (Jones et al., 2008). Recently, about eighty unknown and/or bioactive metabolites isolated from endophytic fungi associated to marine macroalgae (Chlorophyta, Phaeophyta e Rhodophyta) were reviewed (Leandrini de Oliveira et al., 2012), indicating an impressive chemical and biological diversity from these poorly explored source of natural compounds.

According to the issues reported above, we found it could be very interesting to work in a new research frontier, by exploring endophytic microorganism from marine red alga *Bostrychia tenella* (J.V. Lamouroux) J. Agardh (Rhodomelaceae, Ceramiales). Aiming to develop this purpose, endophytic fungi were isolated and cultivated under laboratories conditions, and their crude extracts (CE) were evaluated in cytotoxic, antifungal and antibacterial assays. *B. tenella* belongs to Rhodomelaceae family, the most important in Rhodophyta phyla, and pointed as halogenated metabolites producers, many of which present unusual carbonic frame and bioactivity (Suzuki and Vairappan, 2005). In a previous study of our research group involving *B. tenella*, we reported active volatile apolar fractions against the parasites *Trypanosoma cruzi* and *Leishmania amazonensis*, as well against *Cladosporium cladosporioides* and *C. sphaerospermum* fungi (de Felicio et al., 2010).

It has been reported a screening for evaluating promissory sources of novel and biologically active metabolites, comparing endophytic from aquatic plants and algae associated fungi, including *B. tenella*. However, both unknown metabolites and strains microorganism isolation were not attributed clearly for *B. tenella*. Furthermore, the applied methodology was not satisfactory for classifying the strains as endophytic (Schulz et al., 2008). Thus, this is the first work relating detailed isolation, cultivation and biological evaluation (cytotoxic, antifungal and antibacterial) of endophytes from the Brazilian marine red alga *B. tenella*.

In order to validate chemical potential of marine seaweed endophytes, in this work, we are also reporting the isolation of cytochalasin D (**1**), a known antitumor and antibiotic compound, from *Xylaria* sp. strain. Despite widespread prevalence in terrestrial and marine habitats, this present work describes the first occurrence of cytochalasin D as a metabolite from marine seaweed endophyte.

Materials and methods

Isolation of endophytic microorganisms associated to B. tenella

Algae collection and superficial sterilization

Bostrychia tenella (J.V. Lamouroux) J. Agardh specimens were collected in rocky shores located at Praia Dura, Ubatuba city, São Paulo state, Brazil (23°20′07″S, 45°10′27″W), in September 2008. Voucher specimens were deposited in the herbarium of Instituto de Botânica (Herbarium SP), with the accession number SP 371456.

The general procedures adopted for isolation of the microorganisms were proposed based in previously described methodologies (Erbert et al., 2012; Proksch et al., 2010). The algal material was cleaned in seawater, stored in flasks with sterilized seawater supplemented with chloramphenicol (200 mg l^{-1}), and maintained in

low temperature. Aseptically, the algal material was fragmented with scalpel and separated in three parts, in which each part was submitted to different superficial sterilization methods (SSM): (i) 15 s in ethanol 70%; (ii) 5 s in ethanol 70% and 5 s in sodium hypochlorite (NaOCl); (iii) 5 s in ethanol 70% and 10 s in NaOCl. After each SSM, thallus fragments were rinsed in three sequential sterilized seawater recipients and inoculated in Petri dishes (13–15 fragments/plate) containing semi-solid medium: potato-dextrose-agar (PDA) or algae-dextrose-agar (ADA), supplemented with chloramphenicol (200 mgL^{-1}). For efficacy control of algal thalli sterilization, drops of the last wash seawater were plated in other medium Petri dishes (PDA and ADA) as well as sterilized thallus were touched in medium plates control before inoculation. Growth and control plates were incubated at 30 °C, and periodically observed.

Endophytic strains isolation and preservation

Microorganism colonies were growing around sterilized thalli fragments in semi-solid medium plates. For strains isolation, mycelial discs were taken from growing plates and inoculated in PDA plates. Once isolated, strains preservation were performed using sterile penicillin tubes containing PDA medium and mineral oil.

Crude extracts and fractions obtained from B. tenella *endophytes*

From the total number purified endophytic strains, ten of them (BT01–BT10) were selected to growth in rice solid medium. The selection criteria were relied based on visual and morphological differences, in order to choose distinguished strains from each other, taking into account coloration and macroscopic morphoanatomical structures.

Each selected strain was growth in PDA plates for 15–20 days, at 30 °C. In sequence, ten discs of agar containing mycelium were added in two Erlenmeyers flaks (500 ml) containing 90 g of sterilized rice. Flasks were maintained at room temperature (at 25 ± 2 °C), for 28 days. For organic extraction, rice cultured was submitted to maceration in methanol for 24 h twice. It was made a control culture containing only rice solid medium. The CE were concentrated under reduced pressure and, in sequence, they were dissolved in MeOH:H_2O (9:1) and submitted to liquid–liquid partition, yielding *n*-hexane (HF), ethyl acetate (EF) and hydroalcoholic (MF) fractions. Forty-four samples (CE and organic fractions) of ten strains and one culture control were reached and submitted to biological assays.

Endophytes identification

Strain BT04 was identified under responsibility of Dr[a]. Lara D. Sette, microbiology specialist researcher in Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas (CPQBA), Universidade de Campinas (Unicamp, Campinas, SP, Brazil). Genomic DNA was extracted according to the procedure described in the literature (de Hoog et al., 2003). Using PCR, D1/D2 (DNAr 28S) and ITS1-5.8S-ITS2 regions were amplified using the respective primers: NL-1m and NL-4m; ITS-1 and ITS-4. The purified fragments sequencing were obtained in a MegaBACE 100 (GE Healthcare) automatic sequencer, using NL-1m, NL-2m, NL-3m and NI-4m for D1/D2 region, and ITS-1 and ITS-4 for ITS region. Phylogenetic analysis was carried out in a partial sequence comparing of D1/D2 and ITS regions and assembled in a *contig* (unique sequence combining different fragments), with the organisms sequences contained in Genbank and CBS database. The sequences were aligned using CLUSTAL X program (Thompson et al., 1994) and phylogenetic analysis was conducted using Mega v3.0 program (Kumar et al., 2004). The evolutionary distance matrix was calculated according to Kimura (1980) model and the genetic tree was proposed from evolutionary distances made by Neighbor-Joining method (Saitou and Nei, 1987), using bootstrap values calculated from 1000 resampling, with the support of software included in MEGA 3.0 program.

Other nine strains (BT01–BT03; BT05–BT10) were identified following the Sanger method (Sanger et al., 1977) by Genotyping Biotechnologia Ltd. (Botucatu, SP, Brazil). The microorganisms were submitted to semi-solid growth in PDA, their DNA was extracted and amplified by PCR for ITS region in Bio-Rad My Cycler Thermal Cycler thermocycler. Sequentially, the reaction was purified in agarose gel 1% and the sequencing was performed using three different colonies and BigDye® Terminator v3.1 Cycle Sequencing kit. Capillary electrophoresis automatic sequencing was carried out using ABI 3730 Genetic Analyzer (Applied Biosystems) and alignment of produced electropherograms against respective sequencing (.ab1) of reference gene ITS deposit in GenBank.

Cytotoxicity activity (MTT assay)

The cytotoxicity activity was evaluated against HL-60 (human leukemia), HCT-8 (human colon carcinoma) and SF-295 (glioblastoma) cell lines acquired from National Cancer Institute, USA. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 μg ml^{-1} streptomycin and 100 U ml^{-1} penicillin, and incubation at 37 °C with 5% CO_2 atmosphere. For experiments, the cells were added in 96-well plates (1.0×10^5 cells/well for adherent cells or 0.5×10^5 cells/well for suspended cells in 100 μl of medium) and the samples (100 μg ml^{-1} final concentration) were added to each well (final volume of 200 μl) and incubated for 72 h. Control groups received the same amount of sterile DMSO. Tumor cell growth was quantified by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium (MTT) to a purple formazan product. Sequentially, plates were centrifuged and the medium was replaced by 150 μl of fresh medium containing 0.5 mg ml^{-1} of MTT and incubated for 3 h. The formazan salt was dissolved in 150 μl DMSO and the absorbance was measured using a multiplate reader (Multimode Detector DTX 880, Beckman Coulter) (Berridge and Tan, 1993; Mosmann, 1983). Samples effect was quantified as the percentage of control absorbance of reduced dye at 595 nm. Doxorubicin was used as positive control.

Data for the MTT were displayed as growth inhibition percentage in 100 μg ml^{-1} final concentration. For selected samples, it was also measured the IC_{50} values and their respective confidence intervals were obtained by nonlinear regression using the GRAPHPAD program v5.0 (Intuitive Software for Science, San Diego, USA).

Antifungal activity (bioautography assay)

The phytopathogenic microorganisms evaluated in the antifungal assays *C. sphaerospermum* (Penzig) SPC 491 and *C. cladosporioides* (Fresen) de Vries SPC 140 were cultured at the Instituto de Botânica, São Paulo, SP, Brazil. Samples were dissolved in polarity-compatible solvents and applied to pre-coated TLC plates. For each sample arrays (CE, HF, EF and MF), the TLC plates were developed with organic solvent mixtures previously established, as the mobile phase. After eluting, the plates were dried for complete solvents removal. Chromatogram plates were sprayed with a spore suspension of *C. sphaerospermum* or *C. cladosporioides* in a glucose and salt solution and incubated for 72 h in a dark moistened chamber at 25 °C. A clear inhibition zone appeared against a dark background, demonstrating favorable sample activity. Nystatin was used as the positive control (Homans and Fuchs, 1970).

Antibacterial activity (minimum inhibitory concentration assay)

Minimum inhibitory concentration (MIC) values were determined by the microdilution broth method in 96-well microplates [National Committee for Clinical Laboratory Standards, NCCLS (2003)]. Standard strains from the American Type Culture Collection of the following microorganisms were evaluated: *Staphylococcus aureus* Rosenbach 1884 (ATCC 6538) and *Klebsiella pneumoniae* (Schroeter 1886) Trevisan 1887 (ATCC 13883). Samples were dissolved in DMSO (1 mg ml^{-1}), followed by dilution in Mueller Hinton broth. Concentrations ranging from 400 to 25 μg ml^{-1} were achieved. The final DMSO content was 5% (v/v), and this solution was used as negative control. The inoculums were adjusted for each organism, to yield a cell concentration of 5×10^5 colony forming units (CFU) ml^{-1}, according to McFarland scale (0.5). One inoculated well was included, to allow control of the adequacy of the broth for organism growth. One non-inoculated well, free of antimicrobial agent, was also included, to ensure medium sterility. Penicillin G and Streptomycin commercial standards were used as positive controls. The microplates (96-wells) were incubated at 37 °C for 24 h. Subsequently, resazurin (30 μl) in aqueous solution (0.02%) was added to the microplates, to indicate microorganism viability.

*BT05 strain (*Xylaria *sp.) fermentation and extraction*

BT05 strain (identified as *Xylaria* sp.) was grown on PDA for 15–20 days at 30 °C. Ten plugs of mycelia agar were inoculated into 20 Erlenmeyer flasks (500 ml) containing autoclaved rice and sterilized seawater (90 g:90 ml) for 28 days at room temperature (25 ± 2 °C). The mycelia mass was extracted with dichloromethane and methanol (2:1, 200 ml each flask, twice), overnight, filtered under vacuum, and phases were separated. Organic phase (CH_2Cl_2) was concentrated under reduced pressure, yielding 21 g of crude extract.

Cytochalasin D purification and characterization

For chromatography separation, 20 g of crude extract was fractionated by silica gel VLC using a stepwise gradient solvent system of increasing polarity from 100% *n*-hexane to 100% MeOH, to yield nine fractions (A–I). The fraction eluting with 100% EtOAc (fraction G) was purified using RP HPLC [Supelcosil TM LC-18, 250 mm × 10 mm, 5 μm, gradient elution: 5–80% MeCN/H_2O (0.05% formic acid) in 40 min]. A major compound was collected in t_R = 24 min (32 mg, a 0.16% yield from crude extract).

Identification of the cytochalasin D (**1**) was carried out using spectroscopy techniques, including 1D and 2D nuclear magnetic resonance (NMR) (^{1}H, ^{13}C, DEPT, COSY, HMQC and HMBC). NMR spectra were obtained in Bruker spectrometer DRX-500 (Bruker Optics, Billerica, MA, USA) using CD_3OD (Sigma–Aldrich) as a solvent and the compound was identified by comparing their physical properties with the literature data (Xu et al., 2001). High-resolution electrospray ionization mass spectra (HRESIMS) were obtained on an UltrO-TOF instrument (Bruker-Daltonics, Billerica, MA, USA) equipped with an ESI ion source. The sample was dissolved in methanol/water 4:1 (v/v) and infused into the ESI source at a flow rate of 5 μl min^{-1}, using a Harvard Apparatus model 1746 (Holliston, MA, USA) syringe pump.

Results

Endophytic microorganism isolation associated to Bostrychia tenella

Forty-five endophytic strains were isolated from *B. tenella* through three SSM (SSM 1, 2 and 3) and by using two semi-solid medium PDA and ADA as carbon source for primary endophyte development (Fig. 1). From SSM 1, 2 and 3 were obtained, respectively, 11 (24%), 17 (38%) and 17 (38%) strains. From semi-solid medium PDA and ADA were obtained, respectively, 26 (58%) and 19 (42%). Microbial growth was not observed on the control plates.

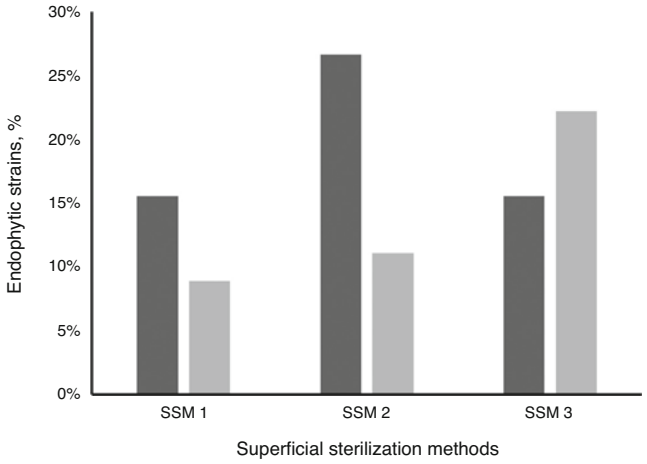


Fig. 1. Distribution of isolated strains from *Bostrychia tenella* according to superficial sterilization methods (black bar: PDA medium; gray bar: ADA medium).

Table 1
Identification of endophytic strains associated to *Bostrychia tenella* specimens.

Strains code	Identification	Similarity (%)
BT01	*Trichoderma atroviride*	99
BT02	*Nigrospora oryzae*	99
BT03	*Phomopsis* sp.	99
BT04	*Penicillium decaturense*	100
BT05	*Xylaria* sp.	99
BT06	*Penicillium waksmanii*	99
BT07	Xylariaceae sp.	99
BT08	*Eurotium* sp.	100
BT09	*Acremonium* sp.	99
BT10	*Acremonium implicatum*	99

Endophytes identification

Strains identification is presented in Table 1.

Biological activities

CE, *n*-hexane, ethyl acetate and methanol-water fractions of ten endophytic microorganisms, besides respective culture controls, were submitted to three assays: cytotoxic activity using SF-295 (glioblastoma), HL-60 (leukemia) and HCT-8 (human colon carcinoma) cell lines; antifungal activity against *C. cladosporioides* and *C. sphaerospermum* phytopathogenic fungi; and antibacterial activity against *S. aureus* and *K. pneumoniae* human pathogens. The culture control (pure rice) samples were inactive in all biological evaluations, indicating rice solid medium have not interfered in results.

The overall biological activities evaluations data are presented in Table 2. Eight samples showed a cellular growth inhibition more than 50% in at least one cancer cell line, in an assay that evaluated samples in a concentration of 100 μg ml^{-1}. Three samples (HF06, EF04 and EF06) have exhibited efficient cell growth inhibition (80–100%) in all the tumor cell lines. Considering this potential, IC_{50} (sample concentration that promotes 50% of cell deaths) were obtained (Table 3). Fraction HF06 was the most cytotoxic sample, since its IC_{50} values obtained ranged in 0–1 μg ml^{-1}, comparable to doxorubicin, especially for SF-295 tumor cell line.

Table 2
Biological activities of extracts and organic fractions from endophytic fungi (associated to *Bostrychia tenella*).

Samples	Cytotoxicity			Antifungal						Antibacterial			
	SF-295	HCT-8	HL-60	*C. cladosporioides*			*C. sphaerospermum*			*S. aureus*		*K. pneumoniae*	
				R	R_f	*P*	R	R_f	*P*	R	MIC	R	MIC
CE01	3.74 ± 4.86	8.23 ± 6.02	11.20 ± 1.22	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
CE02	13.21± 7.70	12.91 ± 3.72	16.67 ± 0.27	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
CE03	18.36 ± 6.38	21.43 ± 1.19	19.37 ± 4.21	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
CE04	22.09 ± 8.46	20.69 ± 6.84	16.87 ± 0.82	+	0.87	2	+	0.84	2	–	>400	–	>400
CE05	32.10 ± 8.74	67.39 ± 46.11	22.83 ± 4.09	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
CE06	55.40 ± 11.03	92.32 ± 10.86	23.26 ± 20.32	+	0.86	1	+	0.83	1	–	>400	–	>400
CE07	23.04 ± 5.66	19.36 ± 4.80	24.06 ± 4.97	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
CE08	35.64 ± 2.60	44.10 ± 2.30	24.27 ± 2.12	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
CE09	16.88 ± 0.74	−13.43 ± 5.07	9.02 ± 1.00	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
CE10	22.19 ± 1.56	14.46 ± 1.54	6.16 ± 1.33	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
CECC	−2.69 ± 1.46	−1.13 ± 7.51	4.81 ± 0.61	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
HF01	1.53 ± 10.34	−2.02 ± 1.34	−13.02 ± 6.66	–	[b]	[b]	+	0.32	1	–	>400	–	>400
HF02	5.46 ± 3.82	−13.80 ± 4.16	−12.49 ± 5.50	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
HF03	2.37 ± 8.46	39.00 ± 5.21	38.97 ± 4.49	–	[b]	[b]	–	[b]	[b]	+	400	–	>400
HF04	1.78 ± 4.86	−8.02 ± 4.91	−31.86 ± 4.62	+	0.2	2	+	0.21	2	+	200	–	>400
HF05	7.18 ± 1.80	−3.71 ± 1.49	−16.39 ± 16.31	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
HF06	93.05 ± 0.11	97.78 ± 0.00	92.23 ± 2.60	+	Trail	3	+	Trail	3	+	≤25	+	400
HF07	−9.65 ± 2.84	5.18 ± 7.81	−10.57 ± 0.75	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
HF08	−3.91 ± 0.14	−7.70 ± 2.83	−9.18 ± 1.09	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
HF09	14.44 ± 2.22	2.02 ± 4.83	2.84 ± 0.82	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
HF10	8.94 ± 0.28	−3.76 ± 0.22	−9.23 ± 0.88	–	[b]	[b]	–	[b]	[b]	+	100	–	>400
HFCC	2.81 ± 7.01	8.76 ± 2.90	15.86 ± 11.62	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
EF01	7.18 ± 2.36	17.85 ± 0.15	82.17 ± 0.07	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
EF02	−6.32 ± 1.04	7.81 ± 3.35	4.76 ± 2.17	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
EF03	13.75 ± 4.30	28.32 ± 1.12	15.95 ± 4.42	–	[b]	[b]	+	0.84/0.76	2/1	–	>400	–	>400
EF04	89.30 ± 6.38	87.90 ± 2.83	86.88 ± 1.97	+	0.83/0.21	2/3	+	0.89/0.22/0.12	3/1/1	+	400	–	>400
EF05	58.74 ± 0.21	68.03 ± 1.49	29.17 ± 0.41	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
EF06	79.38 ± 1.06	88.46 ± 0.65	84.93 ± 1.86	+	0.85	1	–	[b]	[b]	+	400	–	>400
EF07	17.72 ± 0.49	19.43 ± 2.38	5.81 ± 1.22	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
EF08	50.84 ± 2.08	59.61 ± 0.00	30.37 ± 0.48	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
EF09	−4.31 ± 0.42	−18.43 ± 2.23	−2.74 ± 11.42	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
EF10	−14.07 ± 6.59	−6.39 ± 2.31	−5.57 ± 3.19	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
EFCC	6.14 ± 8.67	−1.50 ± 1.49	−2.21 ± 14.20	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
MF01	11.69 ± 0.69	4.55 ± 2.60	1.35 ± 2.79	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
MF02	11.30 ± 1.53	6.55 ± 2.75	1.35 ± 2.11	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
MF03	3.05 ± 1.53	−5.97 ± 9.15	8.89 ± 8.29	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
MF04	−14.17 ± 1.73	3.29 ± 5.88	7.88 ± 3.06	+	0.92	1	+	0.92	1	–	>400	–	>400
MF05	4.97 ± 0.76	5.29 ± 0.67	11.20 ± 1.36	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
MF06	11.89 ± 2.91	7.02 ± 0.30	6.73 ± 3.60	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
MF07	14.68 ± 0.76	12.38 ± 3.12	12.01 ± 1.02	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
MF08	12.42 ± 3.12	5.39 ± 0.97	3.41 ± 5.30	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
MF09	12.82 ± 2.29	4.71 ± 2.83	3.56 ± 3.74	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
MF10	10.71 ± 1.80	6.13 ± 0.37	3.99 ± 2.31	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
MFCC	8.11 ± 5.07	9.49 ± 3.05	0.14 ± 2.04	–	[b]	[b]	–	[b]	[b]	–	>400	–	>400
Doxorubicin	88.13 ± 1.74	93.09 ± 0.37	87.41 ± 0.31	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]
Nistatin	[a]	[a]	[a]	+	0	3		0	3	[a]	[a]	[a]	[a]
DMSO 5%	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]	–	>400	–	>400
Penicillin G	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]	+	0.0028	[a]	[a]
Streptomycin	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]	+	0.7375

R, results; +, active; –, inactive; R_f, retention factor; *P*, potency; grade: 1 – weak, 2 – medium, 3 – strong.
CE means "crude extract", HF means "*n*-hexane fraction", EF means "ethyl-acetate fraction", MF means "methanolic fraction", CC means "control" (rice medium), numbers in front of CE, HF, EF and MF means each strain (01–10).
[a] Not assayed.
[b] Not obtained.

Nine evaluated samples displayed fungal growth inhibition in at least one *Cladosporium* evaluated strain. HF06 and EF04 samples provided strong inhibition halos (3, in a scale from 0 to 3) in both phytopathogenic fungi. HF01, EF03 and EF06 were active in only one fungi species, instigating the possibility of secondary metabolites be acting selectively. Moreover, EF03 and EF04 presented more than one inhibition spot, suggesting the presence of two or more active substances in each sample.

Table 3
IC_{50} values for selected samples.

Samples	Cytotoxicity		
	SF-295	HCT-8	HL-60
HF06	0.87	0.43	0.56
EF04	20.93	6.63	3.78
EF06	14.57	4.38	11.73
Doxorubicin	0.23	0.04	0.02

For antibacterial assay, five samples were active against *S. aureus*, highlighting HF06 sample for showing remarkable MIC value against *S. aureus* ($\leq 25\ \mu g\ ml^{-1}$) and for being active against *K. pneumoniae* ($400\ \mu g\ ml^{-1}$). Gram-positive and Gram-negative bacteria feature antibiotic action mechanisms in distinguished ways; therefore, HF06 should be supposedly composed by metabolites comprising different antibiotic actions or even by a nonspecific bacteria killer substance.

In addition, Figs. 2 and 3 were displayed to bring out other key views about this biological screening. In Fig. 2, active samples were

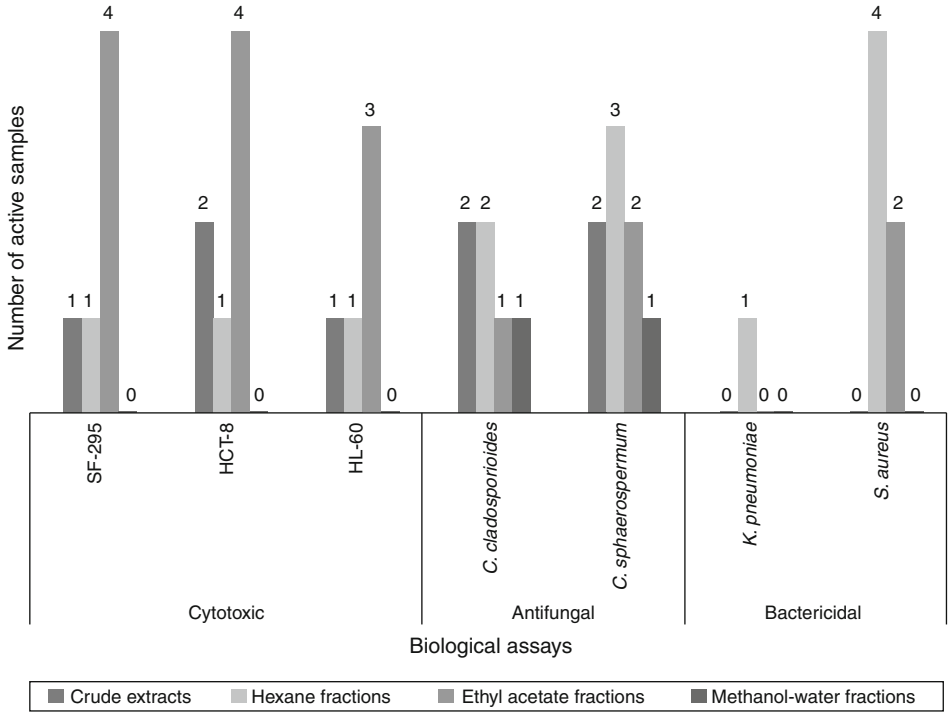


Fig. 2. Biological activities of *Bostrychia tenella* associated endophytes: distribution by evaluated assays.

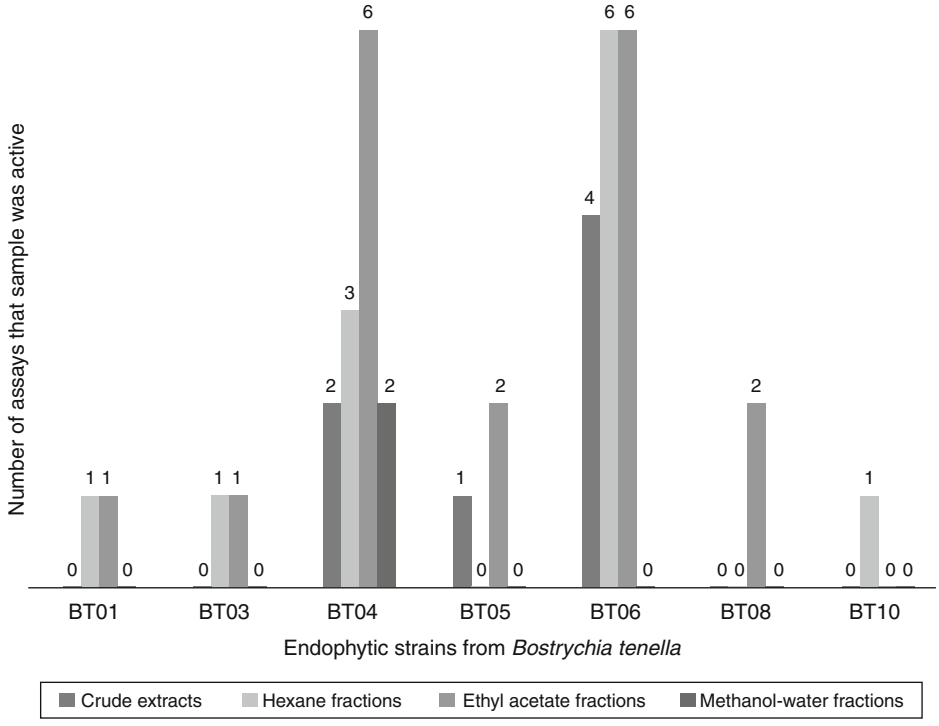


Fig. 3. Biological activities of *Bostrychia tenella* associated endophytes: distribution by endophytic strain (BT02, BT07 and BT09 have not showed any activity in evaluated assays).

classified according biological assay – including count by cancer cell line or fungi/bacteria strain evaluated. Concerning cytotoxicity, EFs were the most active samples (11 of 18, or 61%), whereas HFs (5 of 7, or 71%) in antibacterial activity, and for antifungal, active samples were balance arranged among crude and organic fractions. In Fig. 3, comparative biological potential of individual endophytic strains can be observed. Three strains (BT04, BT05 and BT06) have exhibited impressive results, showing activity almost all assays; meanwhile other three strains (BT02, BT07, BT09) were not active in none of them.

Cytochalasin D characterization

According to HRESIMS data (see in sequence), isolated pure crystals corresponded to a compound formula $C_{30}H_{37}NO_6$. Searching in Dictionary of Natural Products (http://dnp.chemnetbase.com), and

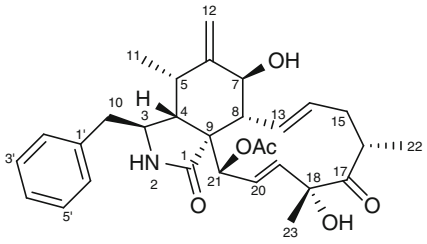
Table 4
Nuclear magnetic resonance of hydrogen-1 and carbon-13 for cytochalasin D (**1**) and comparison with literature data.

Structure position	^{13}C[a]	^{13}C[b]	Structure position	^{1}H[a]	^{1}H[b]
1	173.64	176.77			
3	53.53	55.06	3 a	3.22, m, 4.5, 4.0 1H	3.28, ddd, 8.2; 5.3; 2.6, 1H
4	46.96	47.93	4	2.84, m, 5.0, 2.0, 1H	2.88, m, 1H
5	49.96	49.77	5 b	2.14, t, 5.0, 1H	2.17, dd, 5.2; 2.6, 1H
6	147.46	151.04			
7	69.80	72.47	7 a	3.80, d, 10.5, 1H	3.75, d, 10.1, 1H
8	32.63	33.56	8 b	2.83, m, 10.5, 5.0, 2.0, 1H	2.58–2.64, m, 1H
9	53.24	55.40			
10	45.28	45.05	10 a	2.65, dd, 13.5, 9.5, 1H	2.70, dd, 13.2; 8.3, 1H
			10 b	2.83, m, 13.5, 10.0, 2.5, 1H	2.89, m, 1H
11	13.63	13.63	11	0.95, d, 7.0, 3H	0.55, d, 6.7, 3H
12	114.50	113.63	12	5.09, s, 5.29, s 2H	4.97, s, 1H; 5.2, s, 1H
13	134.11	134.50	13	5.35, m, 15.5, 10.0, 5.0, 1H	5.26–5.31, m, 1H
14	130.59	129.02	14	5.65, dd, 15.5, 10.0, 1H	5.23–5.26, m, 1H
15	37.70	39.51	15 a	2.02, dd, 13.5, 5.0, 1H	1.99, dd, 12.8; 4.8, 1H
			15 b	2.51, dd, 13.5, 11.0, 1H	2.32–2.42, m, 1H
16	42.29	43.54	16 b	2.73, m, 11.0, 4.5, 1H	2.85, m, 1H
17	210.23	211.83			
18	77.66	79.46			
19	127.08	131.94	19	5.15, dd, 16.0, 2.5, 1H	5.55, dd, 15.9; 9.7, 1H
20	132.26	133.57	20	6.11, dd, 16.0, 2.5, 1H	5.98, dd, 15.9; 2.5, 1H
21	77.26	78.35	21 a	5.63, t, 2.5, 1H	5.42, t, 2.3, 1H
22	19.39	19.87	22	1.20, d, 7.0, 3H	1.14, d, 7.0, 3H
23	24.16	24.75	23	1.51, s, 3H	1.48, s, 3H
1′	137.21	138.52			
2′,6′	129.06	131.10			7.28–7.32 (m, 2H)
3′,5′	128.92	129.79	Ph-H	7.25, m, 15.0, 7.0, 1.5, 5H	7.17–7.24 (m, 3H)
4′	127.57	128.02			
$O(CO)CH_3$	169.69	171.92			
$OCO(CH_3)$	20.84	20.79			
OAc				2.26, s, 3H	2.29, s, 3H
NH				5.53, brs, 1H	–

[a] NMR data obtained in our study (Bruker DRX 500 (11.7 T), sample in CD_3OD.
[b] NMR data from cytochalasin D (Xu et al., 2001).

through comparison of our NMR data with previous reported, the purified substance structure was established as cytochalasin D (**1**) (Table 4).

$[M+H]^+$ *m/z* 508.2644 (calc. $C_{30}H_{38}NO_6^+$ 508.2694, error 9.84 ppm), $[M+Na]^+$ *m/z* 530.2470 (calc. $C_{30}H_{37}NNaO_6^+$ 530.2508, error 7.16 ppm), $[M+K]^+$ *m/z* 546.2212 (calc. $C_{30}H_{37}NKO_6^+$ 546.2247, error 6.4 ppm).



1

Discussion

Brazil hosts huge biodiversity, distributed across several biomes and ecosystems, containing 10–20% of the world's known living species, many of which have not been described (Valli et al., 2012). Our continental country is gifted by 8000 km of tropical and subtropical marine coastline, bathed by Atlantic Ocean. Despite rising scientific efforts, support of Brazilian researchers concerning investigation of chemistry and biological properties of marine organisms calls for a quantitative increasing (Blunt et al., 2014). Brazilian seaweeds have unique characteristics that can be exploited to create the basis for new products and processes, such as novel pharmaceuticals (Pereira and Costa-Lotufo, 2012). However, associated microbiota remains poorly explored: from two hundred eighty natural products isolated from Brazilian microorganisms were reviewed and only 6% were sourced from marine macroalgae endophytic fungi (Ioca et al., 2014). From our group contribution, we reported antibacterial compounds from endophytic fungus *Phomopsis longicola* isolated from the tropical red seaweed *Bostrychia radicans* (Montagne) Montagne (Erbert et al., 2012). Herein, biological and chemistry data of endophytes from *B. tenella* species has come to subsidize the development of marine natural products research in Brazil.

Concerning the applied microbiological isolation, the milder superficial sterilization method (SSM 1) determined the lower isolated strains number (11 or 24%), while the other two SSMs (SSM 2 and 3) showed greater numbers of isolates (17 or 38%). Regarding favorable semi-solid medium for development of endophytic microorganisms from sterilized algae thalli, PDA medium demonstrated to be more efficient than ADA medium, since promoted growth of 58% of isolates. Potato-dextrose culture media is known by being nutritional rich, especially for comparing with an adapted growth medium (ADA) – although marine red algae being agar and sulfated polysaccharides producers (Kim et al., 2011). In addition, non-growth in control plates confirmed isolates as endophytes, and validated superficial sterilization methodology applied.

The literature is considerably poor in reports about microbial isolation details from algae species, since the major reports do not mention the sterilization time. The methodology proposed in this work was adapted from (Proksch et al., 2010), where the authors have described in details the fungi isolation from marine species. However, they have not made any distinction regarding different organic matrix, since they pointed out a sterilization method using ethanol 70% in period of 60–120 s. As *B. tenella* has delicate thallus, we decided to reduce the time to 15 s and also to use NaOCl as sterilization agent. Schulz et al. (2008) used NaOCl 1–3% solutions with success for sterilizing plant issues, after an optimization process.

However, they did not specify clearly the use of NaOCl in the algae species.

B. tenella endophytes showed cytotoxic, antifungal and antibacterial activities in a range of intensity grades. Both *Penicillium decaturense* and *P. waksmanii* showed outstanding results in biological screening, being active in almost all assays. We can suggest the production of different compounds with distinguished properties or versatility, by acting in evaluated tests. Frequently, non-specific mechanism action is more related to toxicity to cell, even health ones. Nevertheless, multipurpose metabolites are valuable to understand target/metabolite interactions, and can be inspirational for synthetic modifications aiming fewer adverse effects. On the other hand, five strains showed activity in 1–2 assays/targets, suggesting specific actions, adding promising value concerning further bioguided studies.

The biological potential of marine algae endophytic microorganisms has been reported along last years. Eighty unknown metabolites from marine seaweed endophytic fungi were reviewed, some of them described as cytotoxic, antifungal and antibacterial properties (Leandrini de Oliveira et al., 2012). Moreover, some reviews have listed ranges of endophytes metabolites with biological properties: 73 cytotoxic, 84 antifungal and 50 antibacterial compounds – including human pathogenic microbial (Gunatilaka, 2006; Pupo et al., 2009; Strobel et al., 2004; Tan and Zou, 2001).

From our ten selected strains, we obtained at least six genera. *Penicillium*, *Acremonium*, *Trichoderma*, *Phomopsis*, *Eurotium* and *Xylaria* have already been found from marine environment and/or as endophytic fungi. However, some details about biodiversity, ecology, chemical and biological potential must be explored aiming to understand the significance of our results.

Most active strains were *P. decaturense* and *P. waksmanii*, for displaying positive results in all evaluated assays. Crude and all organic fractions of both strains showed remarkably moderate/strong antifungal properties. *n*-Hexane and ethyl acetate fractions of both displayed activity for *S. aureus*, highlighting *n*-hexane fraction of *P. waksmanii* that was also active against Gram-negative *K. pneumoniae*. Crude, *n*-hexane and ethyl acetate fractions of *P. waksmanii* and ethyl acetate fraction of *P. decaturense* were cytotoxic to three tumor evaluated cell lines. For *P. waksmanii n*-hexane fraction, the IC_{50} value was ranged 0–1 $\mu g\,ml^{-1}$ for all three-tumor cell lines, an impressive result for non-purified sample.

Penicillium is a cosmopolitan genera and has been investigated for many reasons, mainly because production of micotoxins, antibiotics, bioactive and pharmaceutical applied compounds. Biosynthetic arsenal of *Penicillium* includes azaphilones, intriguing structural alkaloids, diketopiperazines, benzodiasepins, quinolones, quinazolines, poliketides and cyclic peptides (Finefield et al., 2012; Kozlovskii et al., 2013; Li, 2010; Schultze et al., 2010).

P. decaturense was first reported as source of insecticide alkaloids (Zhang et al., 2003). Recently, marine sponge derived *P. decaturense* was reported showing biotransformation and biodegradation properties (Alvarenga et al., 2014; de Oliveira et al., 2013). However, there is no report about *P. decaturense* isolation from marine algae, regarding either its biological properties or chemical profile from ocean environment. In previous chemical studies *P. waksmanii* was associated with some tryptofan-containing alkaloids production (Kozlovskii et al., 1997). This penicillin derivate species has already been isolated from brown algae *Sargassum ringgoldianum* Harvey, in which two metabolites showed significant cytotoxicity in the P388 lymphocytic leukemia system (Amagata et al., 1998). In addition, marine *P. waksmanii* was responsible by the production of antifungal compound griseofulvin, detected by ESI-IT/MS (Petit et al., 2004). There are no data about antibacterial action of *P. waksmanii*.

The *n*-hexane fraction of *Trichoderma atroviride* exhibited weak antifungal activity against *C. sphaerospermum* and ethyl acetate fraction showed more than 80% inhibition growth of tumor cell HL-60. *Trichoderma* species is known by producing several fungitoxic cell-wall-degrading enzymes (and probably also peptaibol antibiotics – unusual linear polypeptides) and have also been adopted as agents of biological control of plant pathogenic fungi (Daniel and Filho, 2007). Peptaibols have also been showing cytotoxic properties: atroviridins and derived compounds showed cytotoxicity to human cancer cell lines *in vitro* and unprecedented 17- and 19-residue peptaibiotics produced by marine-derived *T. atroviride* showed weak *in vitro* cytotoxity against KB cells (Carroux et al., 2013). *Trichoderma* genera are already reported as a marine-derived endophytic from sponges (El-Bondkly and El-Gendy, 2012), however not as seaweed marine endophyte. Besides, neither *T. atroviride* was mentioned in literature as marine endophytic fungi.

The *n*-hexane fraction of *Phomopsis* sp. showed a weak antibacterial potential against *S. aureus* ($400\,\mu g\,ml^{-1}$) and showed displayed moderate antifungal activity against *C. sphaerospermum*. Dicerandrol C isolated from *P. longicolla* (endophyte from marine red macroalgae *B. radicans*) displayed notable antibacterial/bacteriostatic effect against *S. aureus* and *S. saprophyticus* (Erbert et al., 2012). *Phomopsis* endophytic strains are prolific producers of phomoxanthones, xanthone dimers derived from secalonic acid, compounds remarkably antimicrobial, cytotoxic, pro-apoptotic and immunostimulatory (Choi et al., 2013; Ding et al., 2013; Ronsberg et al., 2013). Moreover, *Phomopsis* strains are very versatile regarding biosynthesis of secondary metabolites, producing bioactive compounds from a variety of chemical structures scaffold (Choi et al., 2013).

Eurotium sp. ethyl acetate fraction demonstrated moderate tumor growth inhibition SF-295 and HCT-8. *Eurotium cristatum*, endophyte from the marine alga *Sargassum thunbergii* (Mertens ex Roth) Kuntze, provided isolation of anthraquinones derivatives and antimicrobial indole alkaloids against *Escherichia coli* and *Staphylococcus aureus* (Du et al., 2012, 2014). A marine algal associated *Eurotium herbariorum* strain has produced a cytotoxic pyrrolidinoindoline diketopiperazine against K562 tumor cell line (Li et al., 2013).

Acremonium implicatum n-hexane fraction showed antibacterial properties against *S. aureus* ($100\,\mu g\,ml^{-1}$). From literature, *A. chrysogenum* is a producer of cephalosporin C, a pharmaceutical compound inhibitor of peptidoglycan biosynthesis in bacteria (Martin et al., 2010). Although *Acremonium* sp. was isolated from samples of marine macroalga *Plocamium* genus in Germany (Pontius et al., 2008), *A. implicatum* has no report regarding occurrence as marine endophyte.

In our studies, *Acremonium* sp. extract and fractions were inactive for evaluated assays, as well as *Nigrospora oryzae* and Xylariaceae sp. strains. *Nigrospora* genera was reported as marine endophyte from marine and mangrove plants providing a rich chemistry and range bioactivity compounds (Chen et al., 2012; Shang et al., 2012), but there is no register about being a marine seaweed endophyte.

The Xylariaceae (Xylariales, Ascomycotina), a large family covering of around forty genera, has received notable attention over the past decades regarding production of secondary metabolites (Whalley and Edwards, 1995). For instance, the Xylariaceae family is closely related to azaphilones, a fungi metabolite class, that exhibits a wide range of interesting biological activities, such as antimicrobial, antifungal, antiviral, antioxidant, cytotoxic, nematicidal and anti-inflammatory activities (Schultze et al., 2010). In our studies, despite Xylariaceae strain presented no positive results for evaluated assays, *Xylaria* sp. crude extract and ethyl acetate fraction showed moderate inhibition growth for tumor cell SF-295 and HCT-8.

Xylaria is the largest genus of the family Xylariaceae (Xylariales, Sordariomycetes) and presently includes 300 accepted species of

stromatic pyrenomycetes. *Xylaria* species are widespread from the temperate to the tropical zones in the terrestrial globe, and besides, fungi of this genus have been shown to be potential sources of novel secondary metabolites, and many of them possess biological activities relevant for drug discovery, including cytotoxic, antimalarial, and antimicrobial activities (Song et al., 2014). Despite widespread occurrence of *Xylaria* in marine environment, there are no reports regarding being marine seaweed endophytic fungi.

Isolation of macrolide cytochalasin D (**1**) from *Xylaria* sp. has evidenced pharmaceutical potential of seaweed endophytic fungi. Cytochalasans comprise a diverse group of fungal polyketide-amino acid hybrid metabolites with a wide range of distinctive biological functions (Scherlach et al., 2010). Cytochalasin D was first described from *Metarhizium anisopliae* (Aldridge and Turner, 1969), and despite being an isomeric metabolite of cytochalasin B and C, it had shown antibiotic and antitumor activity, by acting in actin polymerization (Scherlach et al., 2010). Cytochalasin D has already been reported as marine derived fungus, but literature has not data about this compound from marine seaweed endophytes.

We can conclude that *B. tenella* presents a microbiological potential and, not only this species but also Brazilian marine macroalgae deserve more exploratory and deep investigations. Proposed SSM were efficient once promoted the achievement of 45 endophytic strains. Cytotoxic, antifungal and antibacterial activities were revealed for CE and organic fractions, demonstrating the importance in developing a deep natural products investigation aiming to determine which substances are responsible by biological activities. Our work complains first report of *A. implicatum* as marine endophyte and *Xylaria* sp., *T. atroviride* and *N. oryzae* as marine seaweed endophytes. In addition, we are reporting first data about cytotoxic and antifungal properties of *P. decaturense* and antibacterial for both *P. decaturense* and *P. waksmanii*. Cytochalasin D isolation and characterization (first time for this kind of organic source) came out in order to illustrate how chemically prospective marine seaweed endophytic fungi can be. On this way, it is valuable to mention that natural products chemistry approaches aiming discovery of bioactive metabolites have been applied using promising strains. In a short future, reports regarding chemical potential of these endophytes will come to improve and enrich literature data concerning chemistry of marine fungi secondary metabolites. Furthermore, this study comes to encourage more microbiological exploration in Brazilian marine coast, comprising a challenge for current and next generation of researchers.

Authors' contributions

RF (PhD student), GBP (MSc student), ALLO (PhD student) and CE (PhD student) contributed in collecting alga sample, running the laboratory work and analysis of the data. NAJCF, EGF (PhD student), LVC and MCMY contributed to biological studies. NSY contributed in algal collection and identification, and manuscript revision. RC and MTP contributed to design and supervised microbiological isolation experiment. HMD designed the whole study, supervised the laboratory work and contributed to critical reading and major correction of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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