Revista Brasileira de Farmacognosia 25 (2015) 641–650

Revista Brasileira de Farmacognosia
BRAZILIAN JOURNAL OF PHARMACOGNOSY
www.sbfgnosia.org.br/revista

Original Article

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

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ARTICLE INFO

Article history:
Received 16 April 2015
Accepted 17 August 2015
Available online 12 September 2015

Keywords:
Brazilian seaweed
Cytochalasin D
Penicillium
Xylaria

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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http://dx.doi.org/10.1016/j.bjp.2015.08.003
0102-695X/

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