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Evaluation of the cytotoxicity of extracts of the freshwater sponge *Drulia cristata* (Porifera: Metaniidae) from the Tapajós River

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ABSTRACT

Extracts from marine sponges and associated microorganisms have already been shown to be important for the development of new drugs. The knowledge about bioactive metabolites of freshwater sponges, however, is still discrete. In this study, the research was carried out using the sponge *Drulia cristata* and its associated bacteria. The collections were made in Maracanã Beach, Tapajós River, in Santarém. Crude extracts of sponges were obtained and the culture of their bacteria was carried out. After the fermentation of isolated strains, their crude extracts were obtained. The MTT assay was used to evaluate the cytotoxicity of these extracts against HCT-116 line (colorectal carcinoma). The extracts of the sponges had no cytotoxic activity. Extracts from the two strains of bacteria (DTR1 and DTR2) isolated showed moderate cytotoxic activity. From the DTR2 strain, the activity reached 65% inhibition of cancer cells, a promising result for future bioprospecting studies with freshwater sponges.

Keywords: *Drulia*; bioprospecting; MTT assay; cancer.

Avaliação da citotoxicidade de extratos da esponja dulcícola *Drulia cristata* (Porifera: Metaniidae) do Rio Tapajós

RESUMO

Extratos de esponjas marinhas e microrganismos associados têm demonstrado importância para o desenvolvimento de novas drogas terapêuticas. Entretanto, o conhecimento sobre metabólitos bioativos de esponjas de água doce é ainda bastante discreto. Neste estudo, a pesquisa foi realizada utilizando a esponja *Drulia cristata* e suas bactérias associadas. As coletas foram feitas na Praia Maracanã, Rio Tapajós, no Município de Santarém. Os extratos brutos da esponja foram obtidos e a cultura de suas bactérias foi realizada. Após a fermentação de cepas isoladas, seus extratos brutos também foram obtidos. O ensaio do MTT foi usado para avaliar a citotoxicidade desses extratos contra a linhagem HCT-116 (carcinoma colorretal). Os extratos da esponja não apresentaram citotoxicidade. Já os extratos das duas cepas de bactérias (DRT1 e DRT2) isoladas apresentaram atividade citotóxica moderada. O extrato da cepa DRT2 atingiu 65% de atividade inibitória sobre as células cancerosas, um resultado promissor para futuros estudos de bioprospecção com esponjas de água doce.

Palavras-chave: *Drulia*, bioprospecção, ensaio do MTT, câncer.

Introduction

Since the earliest, times man has used nature as the source of bioactive products. The extensive chemical diversity of plant, animal, fungal and microorganism species is a result of the evolution in the selection and conservation of important defense mechanisms used to repel, paralyze or kill predators (CRAGG; NEWMAN, 1999; FAULKNER, 2000).

Research shows that, among animals, the most abundant sources of bioactive natural products are the invertebrates of the corals, sponges, mollusks, bryozoans and ascidians groups. Also deserving special attention are the associations between invertebrates and microorganisms (RADJASA et al., 2011). Cytotoxicity is the most commonly found biological activity and usually presents with high potency, which is consistent with the proposed ecological function of these compounds to favor competition for space, eliminate pathogenic microorganisms and paralyze or kill predators, overcoming the diluent effects of the aquatic environment (MUNRO et al., 1999). However, it is recognized that the ability to biosynthesize a broad diversity of metabolites resulting from such distinct biogenetic pathways is not a characteristic of animals, but rather an activity of microorganisms associated with them (PEREIRA, 2009). On the other hand, knowledge about the ecology of freshwater microorganisms is very poor compared to the marine ones. In Brazil, this knowledge can be considered incomplete and fragmented (LOGUE et al., 2008).

Some advantages of using microorganisms attract attention, such as ease of handling, maintenance, and cultivation. For these reasons, microbial biotechnology is very promising at the beginning of the century (MORAIS et al., 2014). Thus, the objective of this study was to investigate the cytotoxic activity of crude extracts of the freshwater sponge *Drulia cristata* and of sponge-associated bacteria against in vitro colorectal cancer cells.

Materials and Methods

Sponge samples were collected in their natural habitat, Maracanã Beach, on the banks of the Tapajós River, in the municipality of Santarém, State of Pará (02° 26' 35" S e 54° 42' 30" W). Authorization of access to genetic resources: SisGen – A195CE0. Specimens were washed with distilled water, sprinkled with alcohol to remove dirties and immersed in organic solvents or bagged under cooling to be transported to the Marine Bioprospection and Biotechnology Laboratory (LaBBmar) of the Federal University of Ceará. Solvents used were Methanol (Vetec®) and ethyl acetate (Vetec®). In the laboratory, 20 g of the dried sponge were macerated and cultured in Petri dishes, which were observed for two months. During this period of growth of the colonies, the appearance of Gram negative bacteria was observed, being two strains isolated and identified the DTR1 and DTR2 (Strains 1 and 2 from Drulia of the Tapajós River) (Figure 1).

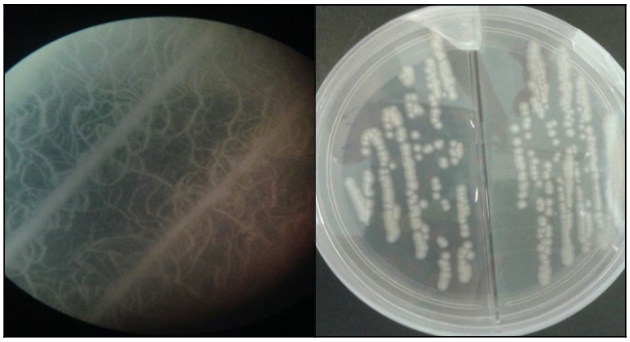

Figure 1. Isolated strains. (A) Strain DTR1; (B) Strain DTR2. / **Figura 1.** Cepas isoladas. (A) Cepa DTR1; (B) Cepa DTR2.

After confirmation of purity, the strains were inoculated in liquid culture medium (medium A1), maintained in an orbital shaker at approximately 26 °C for one week, for better bacterial growth and fermentation (SAHM, 2014). The bacterial metabolites present in the liquid medium were extracted with ethyl acetate (Vetec®).

Human colon cancer *cells* (*HCT-116*) were maintained in RPMI-1640 medium (Gibco™) supplemented with 10% FBS and 1% antibiotics (penicillin/streptomycin [Sigma]). The cells were cultured at 37 °C in a 5% CO_2 humidified incubator. All extracts (at concentrations of 5 μg mL^{-1} and 50 μg mL^{-1}) were tested for in vitro cytotoxicity, using HCT-116 cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (MOSMANN, 1983). As a positive control, it was used the chemotherapy drug doxorubicin (Sigma). The results were analyzed by GraphPad Prism 4.0 (Intuitive Software for Science). The same software was used for graphical representation, as percent inhibition of cell growth.

Results and Discussion

After 72 hours of incubation, crude extracts of *D. cristata* were considered non-cytotoxic against HCT-116 cells, since the MTT assay revealed that they inhibited less than 1% of cell growth (Figure 2). Regarding bacterial extracts, only significant results, above 50% inhibition, were observed at the concentration of 50 μg mL^{-1} (Figure 3).

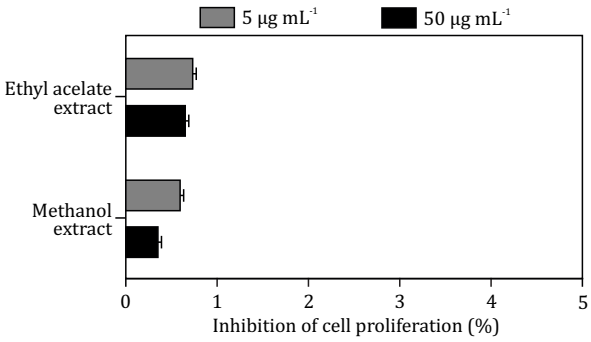

Figure 2. Percent inhibition of HCT-116 cell line growth after 72h incubation with crude extracts of sponge *D. cristata* in the MTT assay. / **Figura 2.** Percentagem de inibição de crescimento da linhagem HCT-116 após 72h de incubação com os extratos brutos da esponja em *D. cristata* no ensaio do MTT.

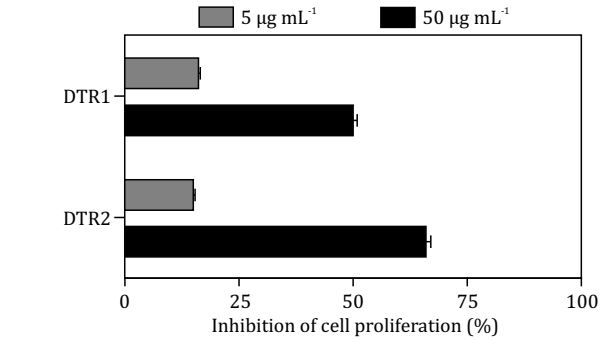

Figure 3. Percent inhibition of HCT-116 cell line growth after 72 h incubation with ethyl *acetate crude extract* of bacterial strains isolated from the *D. cristata*, in the MTT assay. / **Figura 3.** Percentagem de inibição de crescimento da linhagem HCT-116 após 72h de incubação com o extrato acetato de etila das cepas bacterianas isoladas de *D. cristata*, no ensaio do MTT.

There are in the literature several publications on extracts or bioactive substances isolated from marine sponges. Table 1 lists some publications with Brazilian marine sponges. However, the same does not occur with freshwater sponges, which, in addition to representing a smaller number about marine species, have not received the same attention on the part of the researchers. The present study, to our knowledge, is the first bioprospecting investigation of natural cytotoxic products from freshwater sponges.

Table 1. Activities of extracts and substances isolated from marine sponges. / **Tabela 1.** Atividades de extratos e substâncias isoladas de esponjas marinhas.

Activities tested	Comments	References
Cytotoxic, hemolytic and neurotoxic	13 of the 24 species studied demonstrated medium to high toxicity in one or more bioassays.	Rangel et al. (2001)
Cytotoxic, antibacterial, antifungal and antichemotactic	Eight of the ten species studied demonstrated activity in one or more bioassays.	Monks et al. (2002)
Cytotoxic, hemolytic and antimitotic.	Seven of the eight species studied demonstrated activity in one or more bioassays.	Jimenez et al. (2004)
Cytotoxic and antimitotic.	Nine cytotoxic species in tumor cell lines and 19 that inhibited the division of eggs of the sea urchin *Lytechinus variegatus*.	Ferreira et al. (2007)
Cytotoxic and antibacterial.	The most active extracts were obtained from the genera *Aaptos, Aplysina*, *Callyspongia, Haliclona, Niphates, Cliona, Darwinella, Dysidea, Ircinia, Monanchora* and *Mycale*.	Seleghim et al. (2007)
Cytotoxic and genotoxic.	Ingenamine G, alkaloid isolated from *Pachychalina alcaloidifera*, presented moderate cytotoxic activity against lymphocytes in culture, besides clastogenic effect.	Cavalcanti et al. (2008).
Cytotoxic.	Extracts and fractions isolated from *Polymastia janeirensis* induced cell death in a human glioma cell line.	Frota Júnior (2008)
Cytotoxic.	Two of five guanidine alkaloids isolated from *Monanchora arbuscula* showed activity against four tumoral lines.	Ferreira et al. (2011)
Cytotoxic and antimitotic.	Six of ten substances isolated from *Plakortis angulospiculatus* promoted cycle arrest in HCT -116 human colorectal cancer cell lines.	Santos et al. (2015)
Cytotoxic.	Aqueous extract from *Polymastia janeirensis* was highly cytotoxic to glioma and neuroblastoma cell lines.	Biegelmeyer et al. (2016)

A variation in criteria for evaluating the results of cytotoxicity tests of crude extracts is observed in the literature. Almeida et al. (2014) tested extracts of *Annona vepretorum* against the HCT-116, OVCAR-8 and SF-295 tumor cell lines and considered the results of 50 to 75% inhibition as moderate cytotoxic activity, while extracts with percent inhibition greater than 75% were considered high cytotoxic activity. In this criterion, the cytotoxicity of DTR1 and DTR2 extracts from the present

study would be considered moderate. However, in LaBBmar, the tests should only follow for bioguided fractionation when the cytotoxicity of extracts is ≥75% (SAHM, 2014), which did not occur with any of the extracts obtained in the present study.

On the other hand, Guimarães (2013) worked with filamentous actinomycetes from the sediment of a beach in the State of Ceará and adopted a less conservative perspective, considering as active the extracts from bacteria that inhibited above 65% of the cell growth of the HCT-116 line, and selecting them for fractionation and continuation of studies. In the present study with the *D. cristata*, DTR2 extract (50 μg mL^{-1}) showed 65% inhibition on HCT-116 cells and might merit further analysis.

The cytotoxic activity of the extracts of the DTR1 and DTR2 strains was much higher than that of the extracts of the sponge, which was expected. According to Gerwick and Moore (2012), since the last decade it has been realized that microorganisms are, in fact, the most promising source of secondary metabolites. Aquatic microorganisms are of particular interest because, in addition to being still little explored, they have a great potential for the production of bioactive compounds due to the fact that in these environments there is the diluting effect (SIBANDA et al., 2010).

Finally, another interesting aspect is the possibility that the same microbial extract exerts different percentages of inhibition according to the exposed cell lineage. Sahm (2014), for example, reports that the extract of a strain identified as BRA60, although not demonstrating a potent effect on the PC-3/M line (slightly less than 50%), was tested against two other lines, HCT-116 and HL-60, and the results were excellent (almost 100% inhibition). Therefore, we cannot rule out the possibility that the DRT2 extract has greater activity against other lines.

Conclusion

The present study corresponds to the first analysis of cytotoxicity of natural products from the freshwater sponge and its associated bacteria. The most interesting result was observed in the DTR2 extract with 65% inhibition against the HCT-116 cell line, which may be considered promising for future bioprospecting studies with freshwater sponges.

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