Arquivos de Ciências do Mar

CYTOTOXIC AND ANTIMICROBIAL ACTIVITIES OF HYDRO-METHANOLIC EXTRACTS OF SPONGES (PORIFERA) FROM CEARÁ STATE, BRAZIL

Atividade citotóxica e antimicrobiana de extratos hidrometanólicos de esponjas (Porifera) do Estado do Ceará, Brasil

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ABSTRACT

The present study evaluated the cytotoxic potentials in hydro-methanolic extracts (HME) of eight sponge species (Amphimedon sp., Amphimedon viridis, Chondrilla *aff.* nucula, Echinodictyum dendroides, Halichondria *sp.*, Placospongia intermedia, Tedania ignis *and* Tethya *sp.) collected in Ceará State, Brazil. The cytotoxicity of methanol extracts were rated at the following bioactivities: inhibition of the development of sea urchin eggs, hemolytic activity on mice erythrocytes, inhibition of in vitro cellular proliferation on tumor cell lines and antimicrobial potential. Seven, out of the eight species investigated, showed some kind of activities on any of the assays performed, being the inhibition of the development of sea-urchin eggs and the reduction of the* in vitro *proliferation of tumor cells the most commonly observed. The HME of* Amphimedon *sp. showed the highest antimicrobial potential (MIC = 0.15 mg/mL) against both* Staphylococcus aureus *and* Pseudomonas aeruginosa *strains and the strongest antiproliferative action on tumor cell lines, with* IC_{50} *of 2.8, 31.9 and 13.0 μg/mL towards CEM, HL-60 and HCT-8, respectively. On the assay with sea urchin eggs, this extract showed a stronger inhibition on the 3rd cleavage* (IC_{50} *= 107.8 μg/mL) and blastulae* (IC_{50} *< 62.5 μg/mL) stages.*

Key words: *sponges, Porifera, cytotoxicity, hemolysis, sea urchin embryos, tumor cell lines, antimicrobial assay.*

RESUMO

Este estudo avaliou a atividade citotóxica e antimicrobiana dos extratos hidro-metanólicos das esponjas Amphimedon *sp.*, Amphimedon viridis, Chondrilla *aff.* nucula, Echinodictyum dendroides, Halichondria *sp.*, Placospongia intermedia, Tedania ignis *e* Tethya *sp. coletadas no litoral cearense. A citotoxicidade foi avaliada quanto ao potencial hemolítico, à inibição do desenvolvimento de embriões de ouriço-do-mar e ao efeito antiproliferativo sobre células tumorais, enquanto que a atividade antimicrobiana foi avaliada pelo método de difusão em placa em quatro cepas de bactérias e uma de levedura. Das oito espécies estudadas, sete apresentaram algum tipo de atividade biológica, sendo as mais comumente observadas a inibição do desenvolvimento dos ovos de ouriço-do-mar e redução do crescimento celular* in vitro. *O EHM de* Amphimedon *sp. foi o mais ativo dentre os testados. Apresentou um forte efeito inibitório do crescimento das bactérias* Staphillococcus aureus *e* Pseudomonas aeruginosa *(CIM = 0,15 mg/mL). A sua atividade sobre o desenvolvimento dos embriões de ouriço-do-mar foi mais acentuada sobre a 3ª clivagem* (CI_{50} *=107,8 μg/mL) e a blástula* (CI_{50}*< 62,5 μg/mL). A inibição do crescimento celular* in vitro *também foi evidente, sendo a* CI_{50} *2,8, 31,9 e 13,0 μg/mL para CEM, HL-60 e HCT-8, respectivamente.*

Palavras-chaves: *esponjas, Porifera, citotoxicidade, hemólise, ovos de ouriço-do-mar, linhagens celulares tumorais, atividade antimicrobiana.*

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INTRODUCTION

Nature has always been an attractive source of chemical compounds with therapeutic applications. Besides the classical plant and microbial sources, a new field that has been gaining the attention of chemists and pharmacologists, especially in the last few decades, is that of marine natural products chemistry (Munro *et al.*, 1999; Faulkner, 2000).

The marine invertebrates are strong candidates for the investigation of interesting secondary metabolites (Pawlik, 1993). In fact, studies performed at the United States National Cancer Institute have shown that these animals present a higher incidence of cytotoxic compounds than any other group of living organisms (Garson, 1994; Munro *et al.*, 1999).

The work of Bergman and Feeney in the beginning of the 1950s, which resulted in the isolation of two arabinonucleosides from the Caribbean sponge *Cryptotethya crypta* (Tethyidae), brought out the marine natural products as well as their biomedical applications to the interests of the scientific community and pharmaceutical industries (Munro, 1999; Schwartsmann *et al.*, 2001). These nucleosides inspired a new class of arabinonucleoside analogs, ARA-A and ARA-C. Currently, these are the only marine-derived products in clinical use for the treatment of virus infection and cancer (König & Wright, 1996). Other compounds are already found in clinical or pre-clinical studies for the most varied applications, only to mention, antibiotic and anti-inflammatory agents, immune suppressants, antiviral and anticancer (Ireland *et al.*, 1993; Faulkner, 2000; Blunt *et al.*, 2003).

Data suggest that Porifera species are the most suitable animals for the search of bioactive compounds from their secondary metabolite arsenal (Ireland *et al.*, 1988; Faulkner, 1994), a fact that could be related to the defensive role of these chemicals against predators, competitors and parasites (Pawlik, 1993). Many of these compounds have presented notable antibiotic, antiviral and cytotoxic activities in previous studies (Kashman *et al.*, 1999).

It is worthwhile mentioning that only a few data concerning the chemical or pharmacological characterization of marine organisms from the northeastern Brazilian coast are available. This work is part of a more ambitious project that focuses on a screening for cytotoxicity in selected groups of marine organisms occurring in the intertidal waters of the seashore of Ceará State, Northeast of Brazil. Recently, Jimenez *et al.* (2003) brought an overview of the cytotoxic potentials of the peculiar ascidian fauna from this region. In the present study, eight sponge species collected in Ceará were screened for cytotoxic and antimicrobial activities. The increasing number of unique chemicals isolated from this group of organisms, as well as the pioneer motive of this project argues for the importance of this study.

MATERIALS AND METHODS

Species collection

The sponge species studied in this paper were collected in crevices or on the underside of beach-rocks in the intertidal zone at Flexeiras Beach (03°13.597'S; 39°14.965'W, Trairí, Ceará State Brazil. Voucher samples of each species were deposited at the Porifera Collection of Museu Nacional do Rio de Janeiro (MNRJ). The animals were homogenized in methanol (1/5, w/v) and filtered. After filtration the extracts were dried in a rotary vacuum evaporator and the residue reconstituted in an appropriate buffer before testing.

Inhibition of the development of sea-urchin eggs assay

The assay was performed following the method described in Jimenez *et al.* (2003). Adult sea urchins, *Lytechinus variegatus*, were collected at Pecém beach, Ceará State, Brazil. Gamete elimination was induced by injecting 3.0 mL of 0.5M KCl into the urchin's coelomic cavity. For fertilization, 1 mL of a sperm suspension (0.05 mL of concentrated sperm in 2.45 mL of filtered sea water) was added to every 50 mL of egg solution. The assay was carried out in 24-multiwell plates. The extracts were added immediately after fecundation (within 2 min) to get concentrations of 62.5, 125, 250, 500 and 1000 µg/mL in a final volume of 2 mL. At appropriate intervals, aliquots of 200 µL were fixed in the same volume of 10% formaldehyde to obtain first and third cleavages and blastulae. One hundred eggs or embryos were counted for each concentration of test substance to obtain the percentage of normal cells (as described in Jimenez *et al.*, 2003).

MTT assay

The cytotoxicity of the extracts was tested against B16 (murine melanoma), HCT-8 (human colon cancer), CEM (human leukemia) and HL-60 (human leukemia) cell lines from the Children's Mercy Hospital, Kansas City, USA. Cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 µg/mL streptomycin and 100 U/mL penicillin, and incubated at 37 °C with a 5% CO_2 atmosphere. For experiments, cells were plated in 96-well plates (10^5 cells/well for adherent cells or 0.5 x 10^5 cells/well for suspended

cells in 100 µL of medium). After 24 h, the extract (1.5 to 100.0 µg/mL) dissolved in distilled water was added to each well and incubated for 3 days (72 h). Control groups received the same amount of distilled water. 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 bromide (MTT) to a purple formazan product. At the end of the incubation, the plates were centrifuged and the medium was replaced by fresh medium (200 µL) containing 0.5 mg/mL MTT. Three hours later, the MTT formazan product was dissolved in 150 µL DMSO, and absorbance was measured using a multiplate reader (Spectra Count, Packard, Ontario, Canada) (Mosmann, 1983). Drug effect was quantified as the percentage of control absorbance of reduced dye at 550 nm.

Hemolytic assay

The test was performed in 96-well plates using a 2% mouse erythrocyte suspension in 0.85% NaCl containing 10 mM $CaCl_2$, following the method described in Jimenez *et al.* (2003). The extracts were tested at concentrations ranging from 3.9 to 1000.0 µg/mL. After incubation at room temperature for 30 min and centrifugation, the supernatant containing hemoglobin was measured spectrophotometrically as the absorbance at 540 nm (as described in Jimenez *et al.*, 2003).

Antimicrobial assay

The extracts were tested against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella cholerae-suis* and *Candida albicans* obtained from the Reference Material Laboratory of Fundação Oswaldo Cruz (Rio de Janeiro, Brazil). The microorganisms were grown in BHI culture media on a 0.5 MacFarland Standard (1.5×10^8 colony forming units (CFU)/mL) and seeded on agar Mueller Hinton (Merck, New Jersey, United States) for the bacterial strains and agar Sabouraud (Merck, New Jersey, United States) for the yeast. Antimicrobial assays were conducted using the plate diffusion method (Bauer *et al.*, 1966). The extracts (0.07 to 20 mg/ 25 µL) were applied to the seeded agar in perforated wells (diameter 6 mm). After 18 h of incubation at 37 °C, zones of growth inhibition were measured. Cloranfenicol (30 µg), Amikacin (30 µg) and Itraconazole (4 µg) were used as standard antimicrobial agents against *S. aureus, S. cholerae-suis* and *C. albicans* respectively, while Ampicilin (10 µg) was used for *P. aeruginosa* and *E. coli*. The minimal inhibitory concentration (MIC) was determined by the lowest concentration tested able to induce an inhibitory halo.

Statistical analysis

Data are presented as means ± S.E.M. The IC_{50} (mean inhibitory concentration) or EC_{50} (mean effective concentration) and their confidence intervals were obtained by nonlinear regression using the GRAPHPAD program (Intuitive Software for Science, San Diego, USA).

RESULTS AND DISCUSSION

Studies performed during the last decades have revealed that marine natural products possess promising biomedical potentials and this fact has brought the ocean to the interest of various research groups in the search of new drugs (Munro *et al.*, 1999).

Among the eight species investigated in this study, seven presented some kind of activity on any of the assays performed, being the inhibition of the development of sea-urchin eggs and the reduction of the proliferation of cultured tumor cells the most commonly observed.

The extract derived from *Amphimedon* sp. (Order Haplosclerida), exhibited, among those tested, the greatest range of activities: inhibition of the development of sea-urchin embryos and of the proliferation of tumor cells and antimicrobial activity (Tables I, II and III). This broad spectrum of activity presented by the extract of *Amphimedon* sp., cannot be attributed, in its totality, to the presence of halitoxin or amphitoxin, two guanidinium alkaloids that possess a well documented hemolytic action (Jakowska & Nigrelli, 1960; Middlebrook *et al.*, 1974; Schmitz *et al.*, 1978; Muricy *et al.*, 1993; Berlinck *et al.*, 1996), since it did not exhibit any kind of lytic activity on mouse erythrocytes, even on higher concentrations (data not shown). On the sea-urchin assay, a trace activity could be noticed on the first division, but its greatest action occurs later, on the third division and blastulae, where a large number of abnormal cells could be observed.

The toxicity of the sponge extracts could also be related to their antimicrobial substances (Amade *et al.*, 1987), fact that may be confirmed by their being the main source for marine antibiotics (Nigrelli *et al.*, 1959; Burkholder & Ruetzler, 1969). The extract derived from *Amphimedon* sp. showed an equally intense antimicrobial activity against *P. aeruginosa* and *S. aureus*, gram-negative and gram-positive bacteria, respectively. These results confront those previously obtained by Bergquist & Bedford (1978), in which they suggest the sponge extracts to be more active against gram-negative bacteria. On the other hand, Burkholder & Ruetzler (1969) present the exact opposite, where the sponge extracts were found to be more active against the gram-positive bacteria.

Table I – Antimitotic activity of the hydro-methanolic extracts of the sponges in the sea urchin's (*Lytechinus variegatus*) embryos (1st and 3rd cleavages and blastulae). The IC_{50} values and their 95% confidence interval (CI 95%) were obtained by non-linear regression. Experiments were performed in quadruplicate.

Species	1st cleavage IC_{50} (μg/mL)	3rd cleavage IC_{50} (μg/mL)	Blastulae IC_{50} (μg/mL)
Amphimedon sp. (MNRJ 6087)	938.5 (842.4-1046)	107.8 (91.3-127.3)	< 62.5
Amphimedon viridis (MNRJ 6085)	> 1000.0	>1000.0	> 1000.0
Chondrilla aff. *nucula* (MNRJ 6076)	>1000.0	432.5 (388.3-481.7)	319.4 (252.6-403.8)
Echinodictyum dendroides (MNRJ 6078)	> 1000.0	>1000.0	> 1000.0
Halichondria sp (MNRJ 6081)	689.4 (613.2-775.2)	218.3 (201.1-236.9)	261.0 (211.4-322.3)
Placospongia intermedia (MNRJ 6079)	>1000.0	798.5 (652.5-977.2)	602.0 (525.2-690.1)
Tedania ignis (MNRJ 6082)	> 1000.0	>1000.0	> 1000.0
Tethya sp. (MNRJ 6080)	>1000.0	426.7 (281.2-647.5)	376.5 (292.6-484.6)

Table II – Cytotoxic activity of the hydro-methanolic extracts of the sponges on leukemia (HL-60 and CEM), colon carcinoma (HCT-8) and melanoma (B-16) tumor cell lines. Data are presented as IC_{50} values and 95% confidence interval obtained by non-linear regression. Experiments were performed in triplicate.

Species	CEM IC_{50} (μg/mL)	HL-60 IC_{50} (μg/mL)	HCT-8 IC_{50} (μg/mL)	B-16 IC_{50} (μg/mL)
Amphimedon sp.	2.8 (1.1 - 7.4)	31.9 (25.6 – 39.7)	13.0 (6.5 – 26.0)	> 100.0
Amphimedon viridis	20.5 (2.0 – 35.1)	29.5 (17.1 – 51.3)	69.1 (51.3 – 93.0)	> 100.0
Chondrilla aff. *nucula*	> 100.0	> 100.0	>100.0	34.4 (14.6 – 81.0)
Echinodictyum dendroides	> 100.0	> 100.0	33.6 (16.7 – 67.7)	> 100.0
Halichondria sp	> 100.0	> 100.0	> 100.0	> 100.0
Placospongia intermedia	> 100.0	> 100.0	>100.0	> 100.0
Tedania ignis	> 100.0	> 100.0	> 100.0	> 100.0
Tethya sp.	3.5 (2.1 – 5.8)	13.3 (10.9 – 16.3)	16.9 (16.9 – 23.4)	> 100.0

Table III – Minimal inhibitory concentration (MIC) of the hydro-methanolic extracts of the sponges determined by the diffusion in solid medium method evaluated in different microbial strains after incubation at 37 °C for 18 hours. Volume of the samples applied in each well: 25 mL. Well diameter = 6 mm.

Species	Minimal inhibitory concentration (mg/mL)				
	S. aureus ATCC 6538	*P.aeruginosa* ATCC 15442	*E. coli* ATCC 11229	*S. cholerae-suis* ATCC 10708	*C. albicans* ATCC 10231
Amphimedon sp	0.15	0.15	> 20.0	> 20.0	10.0
Amphimedon viridis	> 20.0	> 20.0	> 20.0	> 20.0	> 20.0
Chondrilla aff. *nucula*	> 20.0	> 20.0	> 20.0	> 20.0	> 20.0
Echinodictyum dendroides	> 20.0	> 20.0	> 20.0	> 20.0	> 20.0
Halichondria sp.	> 20.0	> 20.0	> 20.0	> 20.0	> 20.0
Placospongia intermedia	> 20.0	> 20.0	> 20.0	> 20.0	> 20.0
Tedania ignis	> 20.0	> 20.0	> 20.0	> 20.0	> 20.0
Tethya sp	> 20.0	> 20.0	> 20.0	> 20.0	> 20.0

Observation: inhibition halos of positive controls: Chloramphenicol (30 µg) = 33 mm (*S. aureus*); Amikacin (30 µg) = 22 mm (*S. cholerae-suis*); Ampicillin (10 µg) = 11 mm (*P. aeruginosa*); 15 mm (*E. coli*) and Itraconazole (4 µg) = 17 mm (*C. albicans*). Negative control: deionized water = 0 mm.

A. viridis (Order Haplosclerida) also originated a highly cytotoxic extract, strongly inhibiting the growth of the human tumor cell lines, but having no effect on the murine melanoma (Table II). This selectivity was also observed with the extracts derived from *Tethya* sp. and *Amphimedon* sp. (Table II).

As in *Amphimedon* sp., halitoxin was also isolated from polar crude extracts of *A. viridis*. The pronounced hemolytic activity of this alkaloid was not observed in the assay with mouse erythrocytes (data not shown) nor was an antimitotic activity in sea-urchin eggs detected (Table I). Previous studies by Muricy *et al.* (1993) and Berlinck *et al.* (1996) with crude or partially fractioned extracts of *A. viridis* specimens collected on the southeastern Brazilian coast have encountered, among others, antimicrobial, hemolytic and antimitotic activities. Since none of theses activities was observed with the extract derived from the northeastern specimen, one can wonder about the geographic location or ecological pressures for which the organism requires to produce or store the toxin.

The extract from *Echinodictyum dendroides*, showed, on the MTT assay, a selective toxicity towards the HCT-8 cell line, and no other activity on the remaining assays (Table II). On the other hand, the extract derived from *Chondrilla nucula* strongly inhibited the growth of the B-16 cells (Table II), the only one from a murine origin. As this activity was not observed against any other cell line from the panel, we could be led to wonder about a more specific binding to receptors exclusive to this cell. On the sea-urchin assay, the inhibition was not observed in the first cleavage of the eggs, but only from the third cleavage on, suggesting a time-dependent activity (Table I).

The Order Hadromerida became well known for an illustrious representative sponge species, *Cryptotethya crypta*, from which Bergman and Feeney (1951) isolated the c-nucleosides spongouridin and spongotimidin, that led to the synthesis of ARA-C (citarabin) and ARA-A (vidarabin) and found a broad clinical use in the treatment of leukemias and viral infections, respectively (Newman *et al.*, 2000).

The extract derived from *Halichondria* sp. showed no effect against the proliferation of tumor cells (Table II), but presented a fair antimitotic effect on the sea urchin eggs, mainly on 3rd cleavage and blastulae stages (Table I). Halichondrin B, a complex polyether macrolide first isolated from a species belonging to this same genus, *H. okadai*, was screened for cytotoxicity against a 60 cell line panel in the United States National Cancer Institute (NCI-US) and the results indicated

that this compound would be an antimitotic agent with antitubulin activity (Bai *et al.*, 1991).

Up to the present, no other pharmacological characterization of the sponge fauna from the intertidal waters of the northeastern coast of Ceará has been reported. Although deeper chemical and biological investigations on the active compounds of these extracts are needed in order to make further considerations, this study highlights the pharmaceutical potentials of the sponges from Ceará's coast, virtually unexplored by research groups studying natural products.

CONCLUSION

The sponges collected on the Northeastern Brazilian coast have demonstrated to be a rich source of chemicals with cytotoxic and antibiotic compounds. *Amphimedon* sp. and *Tethya* sp. are the most promising species among those in the present study and the activities exhibited by their crude extracts are suitable for a better chemical and pharmacological characterization.

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