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Original Article

Trypanocidal activity of organic extracts from the Brazilian and Spanish marine sponges

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ABSTRACT

Chagas' disease is a parasitic infection caused by protozoan *Trypanosoma cruzi* that affect millions of people worldwide. The available drugs for treatment of this infection cause serious side effects and have variable efficacy, especially in the chronic phase of the disease. In this context, natural compounds have shown great potential for the discovery of new chemotherapies for the treatment of this infection and various other diseases. In present study, we evaluated the *in vitro* antiprotozoal activity of five species of Brazilian and Spanish marine sponges (*Condrosia reniformes*, *Tethya rubra*, *Tethya ignis*, *Mycale angulosa* and *Dysidea avara*) against *T. cruzi*. By GC–MS data, we observed that in these extracts were present the major classes of the following compounds: hydrocarbons, terpenes, steroids and alcohols. The extracts showed activity against the three forms of this parasite and did not induce toxicity in mammalian cells. Better activities were observed with the extracts of marine sponges, *C. reniformes* (EC_{50} = 0.6 μg/ml), *D. avara* (EC_{50} = 1.1 μg/ml) and *M. angulosa* (EC_{50} = 3.8 μg/ml), against trypomastigote forms. In intracellular amastigote forms, the extract of *T. ignis* showed IC_{50} of 7.2 μg/ml and SI of 24.65. On this basis, our results indicate that these extracts can be promising chemotherapeutic agents against *T. cruzi*.

Introduction

Chagas' disease or American trypanosomiasis is caused by unicellular parasite *Trypanosoma cruzi*. Estimates indicate that it affects about 6–7 million people worldwide, mainly in Latin America (WHO, 2015). This infection is characterized by two clinical phases: acute phase, defined by high parasitemia, and a long and progressive chronic phase that can manifest symptoms after some years (Annang et al., 2015). Two drugs are used to treat infected patients, benznidazole and nifurtimox (Maya et al., 2007). Both feature high toxicity and limited therapeutic potential (Maya et al., 2007). These facts make the search for new therapeutic alternatives that are more effective as an urgent need (Valdez et al., 2012).

Many studies show that natural products have great potential for the treatment of infectious diseases (Izumi et al., 2012). These products have in their composition a richness of secondary metabolites, as terpenes, steroids, polyketides, peptides, alkaloids and porphyries (Torres et al., 2014). Among the natural products, the marine biodiversity stands out for possessing substances with activity of interest; although, oftentimes, little is known about them (Ferreira et al., 2014).

The marine sponges exhibit many biological activities that are of potential pharmacological importance, such as antiviral, anticancer, antiprotozoal, antifungal and anti-inflammatory (Mehbub et al., 2014). These organisms are primitive metazoa, sessile and so exhibit defense chemical substances that protect them from predators (Sepcic et al., 2010). Symbiotic associations between marine sponges and microorganisms can lead to the production of secondary metabolites that are biologically active, making sponges promising candidates for the treatment of various diseases (Thomas et al., 2010).

Based on this context, the purpose of the present study was to evaluate the trypanocidal activity *in vitro* of crude extracts of five species of Brazilian and Spanish marine sponges (*Condrosia*

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Box 1 Marine sponges collected in Brazilian and Spanish Coasts for biological assays.

Specie	Collection local	Taxonomy (Order, Family)
T. rubra	Baía de Todos os Santos, Salvador, Bahia, BR	Hadromerida, Tethyidae
C. reniformes	Punta Santana, Blanes, Cataluña, SP	Chondrosida, Chondriliidae
D. avara	Medas, Cataluña, Mediterrâneo, SP	Dictyoceratida, Dysideidae
T. ignis	Praia do Bonfim, Angra dos Reis, Rio de Janeiro, BR	Poecilosclerida, Tedaniidae
M. angulosa	Praia do Bonfim, Angra dos Reis, Rio de Janeiro, BR	Poecilosclerida, Mycalidae

reniformes, *Tethya rubra*, *Tethya ignis*, *Mycale angulosa* and *Dysidea avara*) in order to find more effective and less toxic alternative therapies for Chagas' disease.

Materials and methods

Sponges collection and identification

Five species of sponges were collected through free diving and SCUBA diving, from tide zone to 19 m depth at Brazilian and Spanish coasts (Box 1).

Preparation of extracts

After collection, sponge species were immediately frozen and then lyophilized. Freeze dried materials (100 g) were extracted at room temperature by maceration with acetone three times for a period of 72 h. The crude extracts obtained were evaporated to dryness under low temperatures (<50 °C) on a rotary evaporator.

Gas chromatography–mass spectrometry (GC–MS) analyses

The acetone crude extracts of five species of Brazilian and Spanish marine sponges (*C. reniformes*, *T. rubra*, *T. ignis*, *M. angulosa* and *D. avara*) were analyzed by gas chromatography coupled with mass spectrometry (GC–MS) in the apparatus of Shimadzu QP 2010 in an operating system *via* electron impact (70 eV) equipped with gun Split (gas chromatography 260 °C). DB-5 MS column was used (30 m × 0.25 mm × 0.25 μm), Agilent J&W GC Columns, using helium as the carrier gas; the column flow was 1.3 ml/min, injection volume was 1 μl, injector temperature was 260 °C and pressure was 97.4 kPa. A mixture of (C_9–C_{20}, C_{21}–C_{40}) linear hydrocarbons was injected under the same conditions to identify the components. The spectra obtained were compared with the database of equipment (FFNSC1.3.lib, WILEY7.LIB, NIST08s.LIB, MY LIBRARY.lib).

Parasites and cells

Epimastigote forms of *T. cruzi* (Y strain) were maintained at 28 °C for 96 h in liver infusion tryptose medium (LIT) supplemented with 10% fetal bovine serum (FBS).

Epithelial cells from the kidney of the monkey *Macaca mulatta* ($LLCMK_2$ cells) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 10% FBS at 37 °C, and buffered with sodium bicarbonate in a humidified 5% CO_2 atmosphere. After monolayer formation, the cells were infected with 5×10^6 trypomastigotes/ml. Extracellular parasites were removed after 24 h, the cells washed, and these cultures were maintained in DMEM medium containing 10% FBS, until trypomastigotes emerged from the infected cells.

Antiproliferative activity on epimastigote forms

Epimastigote forms cultivated for 96 h (phase log) were adjusted to a final inoculum of 1×10^6 parasites/ml in LIT medium with 10% FBS. Afterwards, they were added to the wells of a 24-well microplate that contained increasing concentrations of compounds (10, 25, 50, 100, and 200 μg/ml), diluted in dimethyl sulfoxide (DMSO) and LIT medium. The assay was incubated at 28 °C for 96 h. After incubated, cell density was measured by counting in a Neubauer's chamber. Antiproliferative activity was expressed as the percentage of growth inhibition compared with control parasites grown in LIT medium. The concentration able to inhibit 50% of the parasites (IC_{50}) was expressed by linear regression.

Assay cytotoxicity in $LLCMK_2$ cells

The $LLCMK_2$ cells were distributed in 96-well microplate at a concentration of 2.5×10^5 cells/ml in DMEM medium supplemented with 10% FBS and then incubated at 37 °C in a humidified 5% CO_2 atmosphere for 24 h. Afterwards, the compounds were added in the concentration desired (50, 100, 150, 250 and 350 μg/ml), diluted in dimethyl sulfoxide (DMSO) and DMEM medium and incubated for 96 h at 37 °C in a humidified 5% CO_2 atmosphere. After incubation, the cells were washed with PBS and a solution of 2 mg/ml MTT was added. This assay was incubated for 4 h at 37 °C in 5% CO_2 atmosphere. DMSO was added to each well to stop the reaction, and absorbance was read at 492 nm using a BIO-TEK Power Wave XS spectrophotometer. Then, the selective index (SI), that indicates the toxicity of the parasite compared to the host, was calculated.

Assay cytotoxicity in erythrocytes

Human blood A+ type without anticoagulant was collected and homogenized in Erlenmeyer flask with glass beads. The blood was centrifuged in saline and the cells were distributed into Eppendorf tubes with the desired concentration of the compounds (10, 50, 100, 500, 1000 μg/ml). The samples were incubated at 37 °C for 120 min. Afterwards, the test sample was centrifuged and the supernatants were transferred to 96-well plates. The absorbance was read at 540 nm using a BIO-TEK Power Wave XS spectrophotometer.

Evaluation of trypomastigote motility

Trypomastigote forms, in concentration of 1×10^7 parasites/ml, were resuspended in DMEM medium and added in duplicate to each well of a 96-wellmicro plate, in presence of different concentrations of the compounds (0.1, 1, 5, 10 and 25 μg/ml). The assay was incubated for 24 h at 37 °C in a humidified 5% CO_2 atmosphere. The results were obtained by observing motility, allowing the determination of the viability of the parasites, using the Pizzi–Brener method (Brener, 1962). For this, an aliquot of 5 μl of each sample was placed on slides plus coverslips and immediately counted by optical microscopy; subsequently, the concentration that lysed 50% of the parasites value (EC_{50}) was calculated.

Activity against intracellular amastigote forms

To evaluate the effects of compounds on the intracellular amastigote forms, a suspension of 2.5×10^5 cells/ml in DMEM medium supplemented with 10% FBS was seeded in 24-well microplates that contained round coverslips and then maintained at 37 °C in a 5% CO_2 atmosphere for 24 h until a monolayer was