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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

Table 1
Compound GC–MS data observed in bioactivity crude extracts of five species of Brazilian and Spanish marine sponges (*C. reniformes*, *T. rubra*, *T. ignis*, *M. angulosa* and *D. avara*).

Peak	RT	Compounds	IS (%)	Area (%)	Observed (m/z)	Molecular formula	Da.	Ma.	Cr.	Tr.	Tig.
1	26.1	dodecanal	96	1.60	184	$C_{12}H_{24}O$					+
2	26.2	4-ethyloctanoic acid	87	2.75	172	$C_{10}H_{20}O_2$				+	
3	27.4	hexadecan-1-ol	98	3.61	242	$C_{16}H_{34}O$					+
4	2 8.1	octadecanal	96	2.95	268	$C_{18}H_{36}O$					+
5	40.0	(*Z*)-octadec-13-enal	95	1.85	266	$C_{18}H_{34}O$		+			
6	42.08	(3*E*)-3-ethyl-2-methyl-1,3-hexadiene	81	9.35	124	C_9H_{16}	+				
7	42.4	2-furoic acid, 2-methyloct-5-*yn*-4yl ester	80	87.13	234	$C_{14}H_{18}O_3$	+				
8	42.7	octadecanamide	91	2.50	283	$C_{18}H_{37}NO$					+
9	44.0	4,6-cholestadien-3β-ol	93	7.71	384	$C_{27}H_{44}O$			+	+	+
10	46.7	(3*S*,8*S*,9*S*,10*R*,13*R*,14*S*,17*R*)-17-[(*E*,2*R*,5*S*)-5-ethyl-6-methylhept-3-en-2-yl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1*H*-cyclopenta[a]phenanthren-3-ol	92	1.54	412	$C_{29}H_{48}O$		+	+	+	
11	46.8	1-hexadecyne	82	1.98	222	$C_{16}H_{30}$					+
12	47.2	(3β)-cholest-5-en-3-ol	93	60.47	386	$C_{27}H_{46}O$		+	+	+	+
13	47.7	24-methyl cholest-5,22-dien-3β-ol	88	5.57	398	$C_{28}H_{46}O$		+	+	+	+
14	47.9	(20*R*)-cholesta-3,5-diene	96	4.83	368	$C_{27}H_{44}$			+		
15	47.9	cholesterol, pentafluoropropionate	90	2.67	532	$C_{30}H_{45}F_5O_2$					+
16	48.3	3,5-cholestadien-7-one	92	14.36	382	$C_{27}H_{42}O$			+	+	+
17	48.4	gorgost-5-en-3-ol,(3β)-	82	2.20	426	$C_{30}H_{50}O$			+		
18	48.5	stigmasta-5,24(28)-dien-3-ol; 5,24[28]-Stigmastadien-3β-ol; 3β-hydroxy-5,24[28]-stigmastadiene	85	29.35	412	$C_{29}H_{48}O$		+			
19	48.5	22-dihydrobrassicasterol	88	1.35	400	$C_{28}H_{48}O$			+		
20	48.8	(7α)-7-hydroxycholest-4-en-3-one	88	1.05	384	$C_{27}H_{44}O$			+		
21	49.2	4,6-cholestadien-3-one	80	1.92	382	$C_{27}H_{42}O$			+		
22	49.6	17-(5-ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol	95	10.88	414	$C_{29}H_{50}O$			+	+	+
23	50.7	stigmasta-3,5-dien-7-one	83	7.49	410	$C_{29}H_{46}O$					+
24	50.78	(1α,2α,5α)-1,2-epoxycholestan-3-one	80	2.51	400	$C_{27}H_{44}O_2$			+		
25	51.00	5-cholesten-3β-ol-7-one	81	3.90	400	$C_{27}H_{44}O_2$			+		
26	51.0	(3*S*,8*S*,9*S*,10*R*,13*R*,14*S*,17*R*)-17-[(2*R*,5*R*)-5,6-dimethylheptan-2-yl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[*a*]phenanthren-3-ol	92	8.89	400	$C_{28}H_{48}O$		+		+	

RT, retention time; IS (%), index of similarity; (+), occurrence, Cr., *C. reniformes*; Tr., *T. rubra*; Tig., *T. ignis*; Ma., *M. angulosa*; Da., *D. avara*.

obtained. Afterwards, trypomastigote forms were added to the wells at a concentration of ten parasites by host cell. After 24 h, non-internalized parasites were removed and the cells were incubated in presence of different concentrations of the compounds (12.5, 25, 50, 75 and 100 μg/ml) for 96 h at 37 °C in a 5% CO_2 atmosphere. The coverslips were fixed with methanol and stained with Giemsa, and permanently mounted with Entellan (Merck, Darmstadt, Germany). A total 200 cells were counted using light microscope and the percentage of infected cells and number of intracellular parasites were estimated. The survival index (percentage of infected cells × number of amastigotes per cell) and IC_{50} value were then determined.

Results

Compound GC–MS data

From compound identification using GC–MS data, 26 chemical compounds of the following classes were identified: hydrocarbons, terpenes, steroids and alcohols, as major compounds, in extracts of

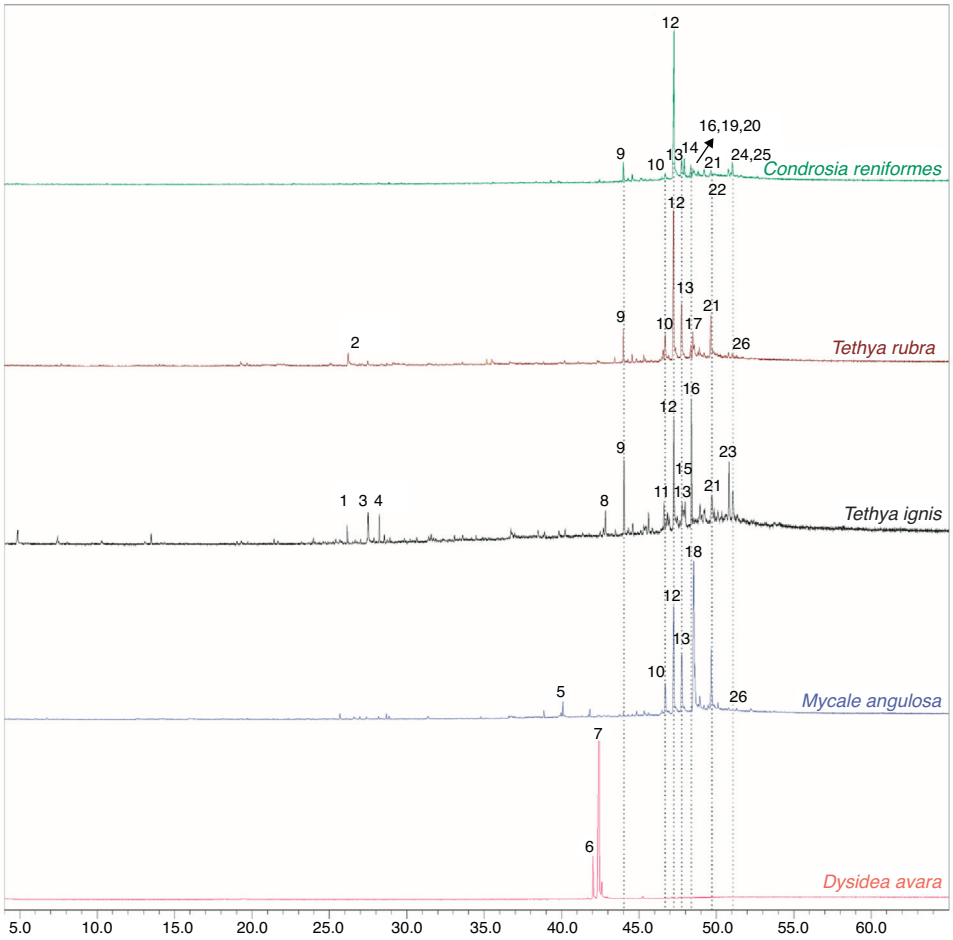

Fig. 1. Chromatogram GC–MS of compounds identified from five species of Brazilian and Spanish marine sponges (*C. reniformes*, *T. rubra*, *T. ignis*, *M. angulosa* and *D. avara*).

five species of Brazilian and Spanish marine sponges (*C. reniformes*, *T. rubra*, *T. ignis*, *M. angulosa* and *D. avara*) (Fig. 1).

Among the chemical compounds identified are: 4,6-cholestadien-3β-ol; (3*S*,8*S*,9*S*,10*R*,13*R*,14*S*,17*R*)-17-[(*E*,2*R*,5*S*)-5-ethyl-6-methylhept-3-en-2-yl]-10,13-dimethyl-2,3,4,7,8,9,11,12, 14,15,16,17 dodecahydro-1*H*-cyclopenta[*a*]phenanthren-3-ol (stigmasterol); 17-(5-ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,11,12,14, 15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol (β-sitosterol); (3β)-cholest-5-en-3-ol; 24-methyl cholest-5,22-dien-3β-ol (brassicasterol) (Table 1).

Effect of extracts on growth of epimastigotes T. cruzi

All the extracts tested showed activity against epimastigote forms (Table 2). The extracts of *D. avara*, *M. angulosa* and *C. reniformes* were most active, with IC_{50} values of 23.4, 67.3 and 28.6 μg/ml, respectively. Greater concentrations of *T. ignis* and *T. rubra* were needed to inhibit parasite proliferation in 50%, 124.7 and 109.9 μg/ml, respectively.

Effect of extracts on toxicity in mammalian cells

The extracts of marine sponges showed no toxic effects in $LLCMK_2$ cells at concentrations that inhibited 50% of the parasites. The most toxic extract was *C. reniformes* with CC_{50} of 79.8 μg/ml. The extracts of *T. ignis*, *T. rubra* and *D. avara* showed CC_{50} of 177.5, 172.5 and 144.2 μg/ml, respectively. However, the less toxic extract was of *M. angulosa* with CC_{50} of 302.7 μg/ml (Table 2).

In human red blood cells, it was observed that the concentration to haemolyse 50% of the erythrocytes was higher than 1000 μg/ml.

Effect of extracts on viability of trypomastigotes T. cruzi

The non-proliferative infective forms of *T. cruzi*, trypomastigotes, were sensitive to the presence of different concentrations of marine sponge extracts (Table 2). The extracts of *T. ignis*, *D. avara*, *M. angulosa* and *C. reniformes* showed EC_{50} of 6.3, 1.1, 3.8 and 0.6 μg/ml, respectively. For marine sponge *T. rubra*, the concentration that lysed 50% of trypomastigotes was 22.3 μg/ml.

Effect of extracts on growth of amastigotes T. cruzi

All the extracts of the marine sponge studied showed inhibitory activity on amastigote forms (Table 2). The extract of *T. rubra*, *D. avara*, *M. angulosa* and *C. reniformes* showed IC_{50} of 44.5, 40.3, 55.5 and 82.6 μg/ml, respectively. However, the better IC_{50} observed (7.2 μg/ml) was with the extract obtained from *T. ignis*.

Discussion

The marine biodiversity is a promising source of natural products with remarkable biological activity (Napolitano et al., 2009). Studies with marine sponges are yielding 200 new pharmacologically active metabolites every year (Turk et al., 2013). These organisms are ancient and some of them contain diverse groups of metabolically active compounds (Santalova et al., 2004). In that way, the identification of biological activity of extracts obtained

Table 2
Activity values of extracts of marine sponges on epimastigote, trypomastigote and amastigote forms of *T. cruzi*, cytotoxicity in mammalian cells and selectivity index.

Extracts	Epimastigote (IC_{50})	Trypomastigote (EC_{50})	Amastigote (IC_{50})	$LLCMK_2$ (CC_{50})	SI (CC_{50}/EC_{50} Trypo)	SI (CC_{50}/IC_{50} Ama)
T. ignis	124.7 ± 10.2	6.3 ± 0.1	7.2 ± 5.2	177.5 ± 73.5	28.0	24.6
T. rubra	109.9 ± 3.4	22.3 ± 1.1	44.5 ± 11.6	172.5 ± 14.5	7.7	3.9
D. avara	23.4 ± 0.4	1.1 ± 0.2	40.3 ± 0.3	144.2 ± 0.9	127.6	3.6
M. angulosa	67.3 ± 12.2	3.8 ± 2.2	55.5 ± 8.5	302.71 ± 17.5	79.6	5.4
C. reniformes	28.6 ± 1.6	0.6 ± 0.1	82.6 ± 10.3	79.8 ± 8.5	133.0	1.0

Values of concentration are represented in μg/ml. IC_{50}, inhibitory concentration of 50%; EC_{50}, effective concentration of 50%; CC_{50}, cytotoxic concentration of 50%; SI, selectivity index between host cell $LLCMK_2$ and parasites.

from marine sponges and other marine organisms is important for isolate compounds with potential biomedical action (Sepcic et al., 1997).

Some compounds, identified by GC–MS in marine sponges studied (*C. reniformes*, *T. rubra*, *T. ignis*, *M. angulosa* and *D. avara*), are widely known in the literature for the biological activities, as antiprotozoal activity (Torres et al., 2014), which justifies this study. For example, the steroids, stigmasterol, β-sistosterol and brassicasterol, are found in larger quantities in organic extracts and show activity against *T. cruzi* (Herrera et al., 2008; Moreira et al., 2009; Santalova et al., 2004; Visbal et al., 2011).

Here, the extract studies revealed potent activity in epimastigote, trypomastigote and amastigote forms of *T. cruzi*. The antitrypanosomal activity in epimastigote forms showed great variations in the IC_{50} values. These variations in activity also were observed against *T. brucei rhodesiense*, *T. cruzi*, *Leishmania donovani* and *Plasmodium falciparum* treated with sponge-derived compounds (Orhan et al., 2010). Furthermore, the extracts showed pronounced activity on trypomastigote forms, the same as observed by Izumi et al. (2011), in which the isolation of methanolic extract of *Piptadenia africana* (Mimosaceae) showed a EC_{50} of 4.0 μg/ml. The SI found was considered excellent, similar to the results obtained in literature with organic extracts of several marine sponges (Hoet et al., 2004; Perdicaris et al., 2013). Interestingly, *T. ignis* exhibited a remarkable IC_{50} against amastigote forms, which can be better exploited, due the lack of efficacy of chemotherapeutic agents available in the chronic phase of Chagas' disease (Garcia et al., 2005). Additionally, the SI showed that the extracts were more toxic to the parasites than to $LLCMK_2$ cells. Although, acetone extracts can have high levels of hemolytic activity (Sepcic et al., 2010), the extracts studied showed hemolysis in human cells only in high concentrations (>1000 μg/ml).

Conclusion

In conclusion, the marine sponge's species tested in the current study showed relevant activity against *T. cruzi* opening the way for bioguided fractionation and isolation of bioactive components. Thus, these extracts may lead to important advances in the development of new chemotherapies in the treatment of patients with Chagas' disease.

Authors' contributions

JCP, VCD, EBG and SCM contributed to the biological analysis and elaboration of the manuscript. GGO contributed for the gas chromatography assay and identification of lead compounds. SMR and EMB contributed for collection, identification and elaboration of the organic extracts of the marine sponges. TUN, SOS and CVN designed the study, supervised the laboratory work and contributed to critical reading 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.

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