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Original Article

Natural products from marine red and brown algae against *Trypanosoma cruzi*

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ABSTRACT

Various extracts obtained from the red alga *Plocamium brasiliense* (Greville Howe & Taylor), including a fraction containing crude 5-chloro-1-(*E*)-chlorovinyl-2,4-dibromo-1,5-dimethylcyclohexane (**1**) and another containing a mixture of halogenated monoterpenes (**F**), as well as atomaric acid meroditerpene (**2**) isolated from brown alga *Stypopodium zonale* (J. V. Lamouroux) Papenfuss, were evaluated for their activity against *Trypanosoma cruzi*. The cytotoxic and trypanosomicidal effects of these extracts were evaluated in Vero cells and clinically relevant forms of *T. cruzi* (amastigotes and trypomastigotes). All extracts from *P. brasiliense* presented low cytotoxicity and moderate trypanosomicidal effects, except for the hydroalcoholic extract. The crude **1** and **F** fractions had enhanced trypanocidal activity but showed low selectivity. Moreover, atomaric acid (**2**) was identified as a hit, demonstrating a potent trypanocidal effect reaching an IC_{50} <10 μM against two different DTU (Yand high selectivity index (<10). These results identify marine natural products as promising candidates against Chagas disease.

Introduction

Bioactive molecules can be found in green, red, and brown seaweeds, but each group of algae produces different secondary metabolites as a defensive mechanism. Rhodophyceae produce terpenes, phenols, and polyether (e.g., Liu et al., 2011), with high levels of halogenations in their products (Teixeira, 2013). In Brazil, multiple research groups have isolated halogenated monoterpenes (Supporting Information **1**, **3**-**15**) from *P. brasiliense* collected from the Brazilian coast (Ferreira et al., 2010; Vasconcelos et al., 2010; Fonseca et al., 2012; Da Silva et al., 2015).

Studies have demonstrated the bioactivity of natural products of *P. brasiliense* against the human herpes virus HSV-1 (Ferreira at al., 2010) and anti-herpes virus bovine Type 5 activity (Pinto et al., 2014), as well as antioxidant activities (Martins et al., 2013), anti-snake venom effects (Da Silva et al., 2015), and allelopathic potential (Fonseca et al., 2012).

Phaeophyceae produce particularly terpenoids (Teixeira, 2013). In previous studies, *S. zonale*yielding various meroditerpenes (Supporting Information, **2**, **16**-**21**) (*e.g.,* Soares et al., 2007; 2015; 2016).

Many pharmacological activities have been attributed to meroditerpenes isolated from *S. zonale*, such as high cytotoxicity against pulmonary and colon cancer cells (Dorta et al., 2002), strong anti-HSV-1 activity (Soares et al., 2007), and activity against *Leishmania amazonensis*. The isolated compounds showed high selectivity against intracellular forms of *L. amazonensis*, as well as low cytotoxicity (Soares et al., 2016).

Promising results of seaweed bioactive molecules and extracts were also shown against *T. cruzi*, which causes Chagas disease (*e.g.,* Torres et al., 2014), a silent disease which affects approximately 6–7 million people worldwide and is endemic in Latin America (World Health Organization, 2015).

The present study investigated the trypanocidal activity (vs. *T. cruzi*) of extracts and fractions of the red seaweed *P. brasiliense* and the isolated product, atomaric acid, from the brown algae *S. zonale*.

Materials and methods

The project obtained a permit for scientific purposes on 01/07/2012 (SISBIO/IBAMA number 3534). *Plocamium brasiliense* (Greville Howe & Taylor) and *Stypopodium zonale* (J. V. Lamouroux)

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Table 1
In vitro Cytotoxicity of PE, DCM, EtOAc and HID extracts, fraction **F** and compound **1** from *Plocamium brasiliense* and the meroditerpene **2** isolated from *S. zonale* against Vero cells and *Trypanosoma cruzi* (Dm28c-luc) intracellular amastigote forms.

Extracts, fraction **F** and Compounds **1** and **2**	Vero cells CC_{50} (μg/ml)[a]	Amastigotes (Dm28cluc) IC_{50} (μg/ml)[b]	SI[c]
P. brasiliense			
PE	126 ± 9.1	39.2 ± 3.6	3.2
DCM	137.8 ± 20.0	58.9 ± 6.7	2.3
EtOAc	154.0 ± 24.9	50.6 ± 9.5	3.0
HID	>200	>200	Nd
Fraction **F**	20.3 ± 6.7	4.9 ± 3.7	4.0
1	21.0 ± 0.9	7.1 ± 2.1	2.9
Meroditerpene **2** from *S.zonale*	40.2 ± 4.9 (90.8 μM)	2.4 ± 1.8 (5.4 μM)	16.8
Bz	>100 (>200 μM)	1.0 ± 0.3 (2 μM)	>100

nd = not determined.

[a] CC_{50}(Cytotoxicity concentration that kills 50% of the Vero cells) calculated using dose response curve. Results of mean and standard deviation of the assay triplicates. Control corresponds of 100% of living cells without the compounds.

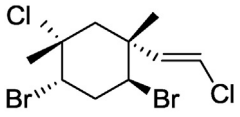
[b] IC_{50} (Concentration that inhibits the growth of 50% of *T. cruzi* Dm28c luc intracellular forms within 72 h) calculated using response curve. Results represent the mean and standard deviation of the assay triplicates.

[c] SI (Selective Index = CC_{50}/IC_{50}).

Papenfuss were collected by snorkeling at a depth of 2–5 m at Enseada do Forno, Armação de Búzios, RJ, Brazil (22° 44′ 49″S and 41° 52′ 54″W). The algae were cleaned and dried at room temperature for 10 days and then ground in an industrial blender and reduced to powder. The exsiccated *P. brasiliense* and *S. zonale* were deposited in the Herbarium of the Rio de Janeiro State University (HRJ 10331-32 and HRJ 8643, respectively).

The petroleum ether (PE), dichloromethane (DM), ethyl acetate (EtOAc), and hydroalcoholic (HID, ethanol/water 7:3) extracts of *P. brasiliense* were obtained according to the method used by Fonseca and collaborators (2012).

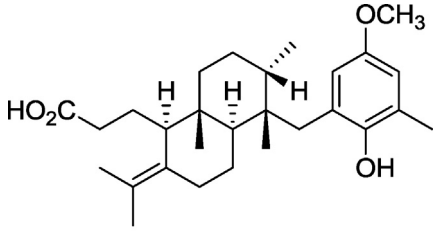
To obtain pure monoterpenes, an aliquot of the DCM extract from *P. brasiliense* (80 mg) was subjected to silica gel column chromatographies according to the method used by Ferreira et al.(2010). The combined fraction was filtered in pure PE to produce crude **1**. However, the low biomass in the sample made it impossible to obtain a pure product.



1

The partial crude PE extract (80 mg) was subjected to silica gel column chromatography (Pasteur pipette of 23.5 cm) eluted with pure PE to produce 45 fractions. The fractions F4-F6 were combined, yielding a mixture of halogenated monoterpenes (**F**).

The partial extract from *S. zonale* (2.5 g) was fractionated by silica gel column chromatographies using hexane/EtOAc, yielding crude atomaric acid (**2**). The material (155 mg) was fractioned by column chromatograph Sephadex (LH-20) using MeOH, yielding 90 fractions. The fractions F_{51}-F_{89} (117.5 mg) provided pure atomaric acid (**2**).



2

For biological assays, we used clusters of 5 mg of each extract, 0.8 mg of crude **1**, 10 mg of the fraction **F**, and 10 mg of **2**. Solutions were made with DMSO for all samples, except EBPB-HID, which was solubilized in distilled water. The results were 10 mg/mL for the four extracts and **1** and 20 mg/mL for **F** and **2**.

Confluent monolayers of Vero and LLC-MK2 cells were dissociated with Trypsin-EDTA solution (0.025%). The cell lines were cultivated in RPMI 1640 medium containing 10% fetal bovine serum (FBS). Cytotoxicity and trypanocidal assays were performed with Vero cells.

Trypomastigotes were isolated from the supernatant of LLC-MK2 cells infected with different stocks of *T. cruzi*, including genetically modified Dm28c clone of *T. cruzi* expressing luciferase (Dm28c-Luc) and Y strain. Briefly, cells were infected with *T. cruzi*, Y strain, or Dm28c-Luc, in a ratio of 10:1 parasites/host cell. After 4 days post-infection (dpi), free parasites in the supernatant were harvested, counted in a Neubauer chamber, and used in the experimental assays.

Vero cells were used to analyze the cytotoxic effects of marine products, according to the modified procedures described previously (Lechuga et al., 2016). Trypomastigotes (1×10^6 parasites/well) of *T. cruzi*, Y strain, were treated according to Lechuga et al. (2016). For intracellular amastigotes, *T. cruzi*-infected Vero cells (Dm28c-Luc) were treated (72 h at 37 °C) with algae extracts (0.4–100 μg/ml), isolated substances (**1** and **2**), and fraction **F** (0.08–20 μg/ml), as well as Bz (0.4–100 μM) as a positive control. Luminescent signal was measured after addition of luciferin (300 μg/ml) using a FlexStation® 3 reader. The concentration that reduces the number of viable parasites by 50% (IC_{50}) was calculated by linear regression. Additionally, the effect of the most active compound was also analyzed against *T. cruzi* Y strain. After treatment (72 h at 37 °C) with the promising algae compound and Bz (1–10 μM), cultures were fixed in Bouin's solution and stained with Giemsa. The IC_{50} value was calculated by microscopic determination of infection index (the percentage of infected cells × the number of the intracellular parasites). At least three different assays were performed in duplicate.

Results and discussion

The assessment of the chemical profile of *P. brasiliense* extracts included TLC analyses, NMR spectra, and comparison with NMR data of previously isolated products (Ferreira et al., 2010; Vasconcelos et al., 2010).

The crude **1** was identified as 5-chloro-1-(*E*)-chlorovinyl-2,4-dibromo-1,5-dimethylcyclohexane by comparing to ^{1}H NMR spectroscopic data from the literature (*e.g.*, Ferreira et al., 2010). Fraction **F** was identified as a complex mixture of halogenated

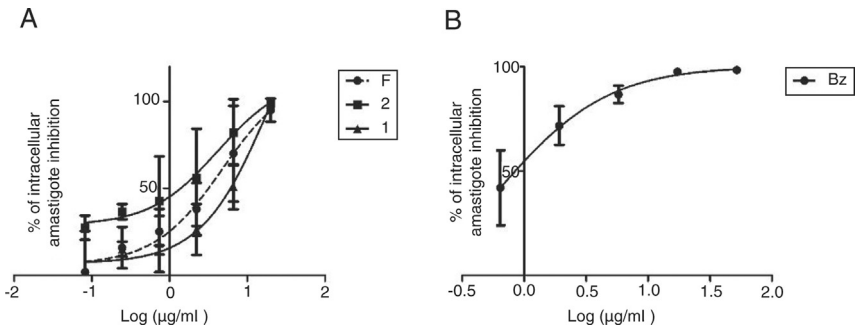


Fig. 1. Sigmoidal dose-response curves of fractions and compounds. Antiproliferative evaluation of different concentrations (20–0.08 μg/ml) of fraction **F** and compounds **1**, and **2** against intracellular forms of Dm28c-luc, after 72 h of treatment (A). Dose response curve showing the activity of different concentrations of Bz (100–0.4 μM) against amastigotes after 72 h (B). Results represent mean and standard deviation of experiments repeated at least three times.

monoterpenes, which includes **3**, **4**, **5**, and **6** (Supporting Information), previously isolated by our group (Ferreira et al., 2010; Vasconcelos et al., 2010). The atomaric acid (**2**) was identified by ^{1}H and ^{13}C NMR spectra, by comparison with spectroscopic data of literature (*e.g.*, Dorta et al., 2003; Soares et al., 2007). The Supplementary Information presents the ^{13}C and ^{1}H NMR data (300 MHz in CDCl3) of atomaric acid (δ in ppm, *J* in Hz).

The toxic effects of the extracts **1** and **F** of *P. brasiliense* were evaluated on mammalian cells using the Cell Titer Viability Assay. A small cytotoxic effect was detected after treatment of Vero cells with EP (CC_{50} = 126 μg/ml), DCM 2015 (CC_{50} = 137 μg/ml), EtOAc (CC_{50} = 154 μg/ml), and HID (CC_{50} >200 μg/ml) extracts, as well as with Bz (CC_{50} >100 μg/ml). Atomaric acid (**2**) had a moderate toxic effect on Vero cells (CC_{50} = 40.2 μg/ml/90.8 μM), but **1** and the fraction **F**, both with halogenated monoterpenes, showed elevated cytotoxicity, achieving CC_{50} values of approximately 20 μg/ml (Table 1).

Considering this cytotoxic profile, we next evaluated the efficacy of the marine extracts and products against human-disease relevant stages of *T. cruzi*. The screening was first performed against intracellular amastigotes of *T. cruzi*, clone Dm28c-luc (TcI). All extracts of *P. brasiliense* had low activity against amastigotes (IC50 > 30 μg/ml), while the fraction **F** and crude **1** presented an enhanced effect with IC_{50} values of 4.9 and 7.1 μg/ml, respectively (Table 1).

Interestingly, the highest activity against amastigotes was achieved with meroditerpene (**2**), reaching an IC_{50} value of 2.4 μg/ml (5.42 μM) and a selectivity index of 16.75. However, Bz inhibited amastigote growth at a lower concentration (IC_{50} = 1 μg/ml/2 μM). The effect of **2** against *T. cruzi* Y strain intracellular amastigotes also revealed a high activity (IC_{50} = 1.77 μg/ml/4 μM), but this was still lower than Bz (IC50 < 0.5 μg/ml/1 μM) (Table 1).

In high concentrations, Bz (10 μM) mostly cleared a *T. cruzi* infection, while the same concentration of **2** greatly reduced an intracellular amastigote nest. However, a lower concentration of **2** (1 μM) showed a parasite load similar to the control group (Fig. 1).

Seaweed natural products were also screened against trypomastigotes of the *T. cruzi*, Y strain. No trypanocidal effect (IC_{50} >50 μg/ml) was observed with *P. brasiliense* extracts. Crude **1** (IC_{50} = 10.8 μg/ml) and fraction **F** (IC_{50} = 20.3 μg/ml) displayed better trypanocidal activity than *P. brasiliense* extracts but lower efficacy compared to Bz (IC_{50} = 8.52 μg/ml; 16.4 μM). At 50, 25, and 12.5 μg/ml, **1** and **F** were more effective than the *P. brasiliense* EP and DCM extracts from which they respectively originated. In contrast, at 6.25 μg/ml, only the **F** fraction had a trypanocidal activity that statistically differed from the crude extract (Fig. 2).

All *P. brasiliense* extracts had low cytotoxicity (CC_{50} >100 μg/ml). Also, the HID extract, which is rich in sulfated polysaccharides (CC_{50} >200 μg/ml), had no trypanocidal activity.

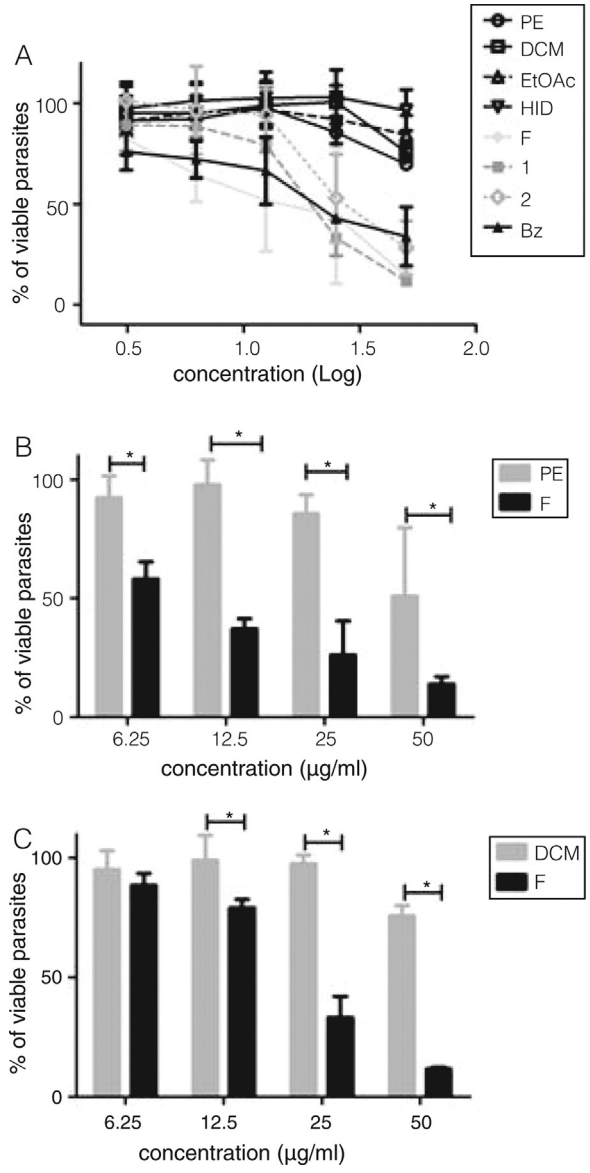


Fig. 2. Trypanocidal activity of extracts, fractions and compounds against trypomastigotes. Viability of parasites was demonstrated by resazurin assay after 24 h of treatment with different concentrations (50, 25, 12.5, 6.25 and 3.1 μg/ml) of extracts, fractions and compounds (A). Comparison of the activity on trypomastigotes of *Plocamium brasiliense* extracts in dichloromethane (DCM) and petroleum ether (PE) versus crude **1** (B) and **F** (C). Note that the fractions enriched with halogenated monoterpenes had improved activity against the parasite. Results represent the mean and standard deviation of three independent experiments. *Statistically significant, $p \leq 0.05$.

In contrast, an evaluation of isolated sulfated polysaccharides from marine seaweeds showed high cytotoxicity and leishmanicidal activity, with low selectivity (Lehnhardt Pires et al., 2013). The best activity was reached with the apolar extract, or the PE extract, which achieved an IC_{50} value of 39.2 μg/mL against intracellular amastigotes. As expected, the halogenated monoterpenes increased the trypanocidal activity approximately five-fold for amastigotes and more than two-fold for trypomastigotes. However, they were also toxic to Vero cells, maintaining low selectivity values. Our results demonstrated that cyclic halogenated monoterpene **1** and the mixture of halogenated monoterpenes **F** are not good examples for prototypes against the parasite *T. cruzi.*

Our results demonstrate the activity of atomaric acid (**2**) against different discrete type units (DTU), the clone Dm28c-luc (TcI), and the Y strain (TcII). The DTU, along with TcV and TcVI, are predominantly associated with human infections (Miles et al., 2009). The meroditerpene **2** demonstrated a potent effect against the clinically relevant intracellular form of both Y and Dm28c-luc, achieving an IC_{50} <10 μM and a selectivity index of 16.75 for the Dm28cluc clone, thus fitting the parameters of a Chagas disease HIT compound (Don and Ioset, 2014). Despite the high activity against amastigotes, **2** was ineffective against culture-derived trypomastigotes, with an IC50 similar to the CC_{50}.

Unlike our results, a previous study showed that **2** had strong effects against both promastigotes and intracellular amastigotes of *Leishmania amazonensis* (Soares et al., 2016). It seems that **2** acts on the redox metabolism, modulating nitric oxide production and eliciting the production of reactive oxygen species in infected and uninfected host macrophages (Soares et al., 2016; Lira et al., 2016).

The use of chemical transformations of **2**, its synthesis, and the cultivation of the *S. zonale*, as well as the isolation of similar products in other seaweeds, might be good strategies for improving current (and discovering new) active compounds against *T. cruzi*.

Authors' contributions

JCRL and CJBR (PhD students) contributed to chromatographic analysis. GCL (PhD student) contributed to biological assay. MCSP, CMC, SCB NO, and VLT designed the study, supervised the laboratory work, and contributed to the critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflict of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bjp.2019.08.003.

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