

SUPPLEMENTARY MATERIAL

pH-dependent cytotoxic effects of extracts of the marine sponge *Polymastia janeirensis* on cancer cell lines

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The purpose of this work was to study the cytotoxic effects of marine sponge *Polymastia janeirensis*, which has been observed in the field to release an orange substance that is toxic to fish. The result showed that aqueous extract (pH 7.0) was highly cytotoxic to glioma (U87) and neuroblastoma (SHSY5Y) cancer cell lines (IC₅₀ < 1.0 µg/ml). In addition, this extract showed potent antioxidant and procoagulant (decreased the clotting time by 1.7 fold) activities. Interestingly, the cytotoxic effects were pH-dependent since the viability of the cancer cells was not affected with the extract (pH 5.5). The close similarity between the aqueous extract (pH 7.0) and the orange liquid that is released by the sponge indicates that this potential chemical defense of *P. janeirensis* deserves further investigation.

Keywords: *P. janeirensis*; chemical defense; cytotoxic; pH-dependent activity.

Experimental

Reagents

3-(4,5-dimethyl)-2,5-diphenyl tetrazolium bromide (MTT), HEPES and Trolox were obtained from Sigma-Aldrich (St. Louis, Mo., USA). Methanol, ethyl acetate and hexane (high-performance liquid chromatography [HPLC] grade) were purchased from Tedia (Fairfield, Ohio, USA).

Sponge collection

Samples of the marine sponge *Polymastia janeirensis* were collected by hand at depths of between 10-15 m from locations along the coastline of Santa Catarina, Brazil. The specimen was deposited by the number MCN 8922 at the Museum of Natural Sciences, Porifera collection of the Fundação Zoobotânica do Rio Grande do Sul, Brazil.

Preparation of extracts and fractions

The marine sponge samples were initially freeze-dried. The extracts were prepared using animal samples by the Ultra-Turrax system with methanol. Considering that *P. janeirensis* releases an orange substance when severed, extracts were also prepared with distilled water aliquots that were maintained at distinct pH levels (5.5, 6.0, 6.5, 7.0, 7.5 and 8.0). The pH was adjusted with solution of NaOH 1.0 M or HCl 1.0 M.

To obtain a polarity based fractionation, the raw methanol extracts were partitioned with other solvents to obtain the initial aqueous, hexane and ethyl acetate (EtOAc) fractions. First, the methanol extracts were concentrated and adjusted to 9:1 (MeOH:H₂O). The MeOH:H₂O suspensions were partitioned against hexane. Next, the methanol was evaporated from each sample using a rotary evaporator, and the remaining aqueous suspension was partitioned against EtOAc.

Evaluation of cytotoxic effects

Cell cultures

The human glioma (U87) and human neuroblastoma (SHSY5Y) cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). The glioma cells were grown and maintained in low-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Carlsbad, USA), which contained 0.1% fungizone and 100 U/l gentamicin and was supplemented with 10% fetal bovine serum (FBS). The neuroblastoma cells were grown and maintained in a 1:1 mixture of Ham's F12 and

DMEM that was supplemented with 10% heat-inactivated FBS, 2 mM of glutamine, 0.28 µg/µl of gentamicin and 250 µg of amphotericin B. Cells were plated in 96-well plates (10^4 /well) and were incubated at 37°C in a humidified atmosphere with 5% CO₂. The media were replaced every 3 days, and all treatments were performed when the cells reached confluencies of 70-80%.

Treatments

The aqueous extracts and fractions were dissolved in water and dimethylsulfoxide (DMSO) to final concentrations of 10 and 50 mg/ml (dried extract weight, *w/v*), respectively. Successive dilutions were made with DMEM medium to final concentrations ranging from 0.1 to 100 µg/ml in multi-well plates. The cultures were incubated with the sponge extracts and fractions for 24 h. The final concentration of DMSO (0.25%) was proven not to affect the experiments. The control cultures were treated identically of samples (with the same solvent), except that the extracts were not used.

Assessments of glioma and neuroblastoma cell viabilities

Cell viabilities were determined by the MTT assay. Following the treatments, the medium was discarded, and fresh medium containing 0.5 mg/ml MTT was added. The cells were incubated for 45 min at 37°C in a humidified atmosphere with 5% CO₂. After, this medium was removed, and DMSO was added for 30 min to solubilize the formazan crystals. Absorbances were measured at 550 nm (test) and 690 nm (reference) using a SoftMax Pro Microplate Reader (Molecular Devices®, USA). The half maximal inhibitory concentration values (IC₅₀) were estimated from a semilog plot of the fractions and extract concentrations versus the percentages of tumor cell line growth inhibition.

Total reactive antioxidant potential (TRAP) method

The TRAP method, as described by Dresch et al. (2009), was used to estimate the *in vitro* antioxidant capacity with a liquid scintillator counter (Wallac 1409, Perkin Elmer, Boston, MA, USA). The samples were diluted with glycine buffer to final concentrations of 1.0-100 µg/ml (dried extract weight, *w/v*). The final percentage of DMSO (0.25%) was proven not to affect the system. The results were expressed as a plot of the percentage of counts per minute (% cpm) versus the time (s) and area under

the curve (AUC). The Trolox equivalent antioxidant capacity (TEAC) was estimated using the standard curve of Trolox (between 0.05 and 0.4 μM) and the AUC.

Clotting assay

Recalcification times (RTs) were assessed using a SpectraMax microplate ELISA reader as described by Ribeiro et al. (1995), with some modifications. Briefly, 50 μl of human citrated platelet-poor plasma was incubated for 5 min with 80 μl of 20 mM HEPES, pH 7.4, with or without (control) varying amounts of extracts and fractions: 1.0-100 $\mu\text{g}/\text{ml}$ (dried extract weight, *w/v*). Coagulation was triggered by adding CaCl_2 to a final concentration of 10 mM, and clot formation was monitored at 37°C using the SpectraMax system at a wavelength of 650 nm at 15 s intervals for 20 min. Samples were diluted with HEPES buffer to their final concentrations, and the final percentage of DMSO (0.25%) was proven not to affect blood coagulation. Clotting was indicated by a rapid and sharp increase in absorbance following a lag phase. A variation of 0.05 in the absorbance value (onset time) was defined as the measurement of the recalcification time, using the module that was included in the instrument software.

Statistical analysis

The *in vitro* procedures were carried out with $n = 3$, while cell culture experiments were conducted with $n=3$ in at least three independent experiments. Data were expressed as mean \pm standard error of the mean (SEM). The results were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. The differences were considered statistically significant if $P < 0.05$. Data analyses were performed using the GraphPad software®, San Diego, CA; version 5.00.

Table S1. Cytotoxic effects in human neuroblastoma and glioma cell lines of *P. janeirensis* aqueous extracts in different pH levels.

<i>P. janeirensis</i> Aqueous Extract	IC ₅₀ (µg/ml) ^a	
	glioma (U87)	neuroblastoma (SH-SY5Y)
pH 5.5	na	na
pH 6.0	1.89 ± 1.38	1,76 ± 1.40
pH 6.5	0.75 ± 0.35	0.35 ± 0.32
pH 7.0	0.78 ± 0.28	0.22 ± 0.20
pH 7.5	0.74 ± 0.10	0.25 ± 0.24
pH 8.0	0.80 ± 0.20	0.28 ± 0.33

^aAll values given are the mean ± SEM of dried extract/fraction weight; na: not active.

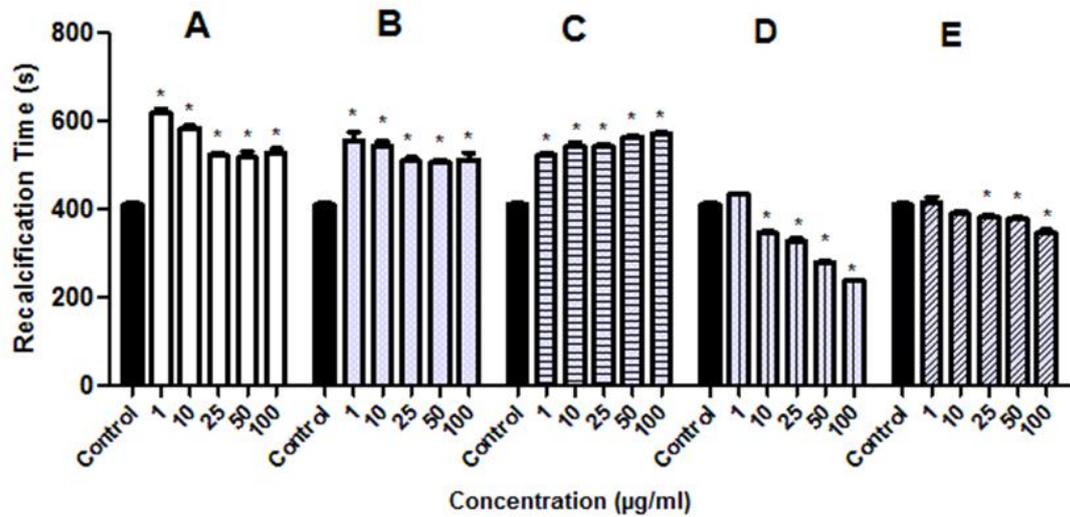


Figure S1. Effect of extracts of the marine sponge *P. janeirensis* on blood coagulation, as represented by recalcification time: anticoagulant activity of the EtOAc fraction (A), hexane fraction (B) and aqueous fraction (C) and the procoagulant activity of aqueous extract at pH 7.0 (D) and aqueous extract at pH 5.5 (E). Bars represent means \pm SEM. Asterisks denote significance at $p < 0.05$ compared with control (one-way ANOVA followed by Tukey's test).

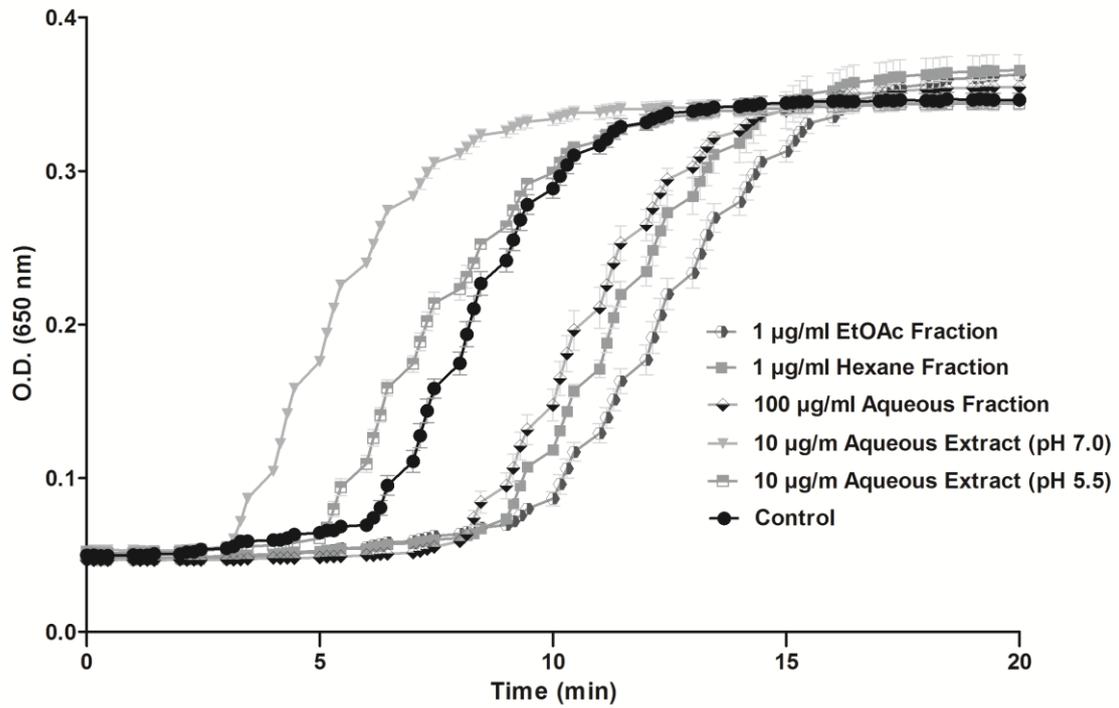


Figure S2. Profile of changes in blood coagulation of *P. janeirensis* fractions and extracts. The concentrations that exhibited the highest anticoagulant or procoagulant activities are shown. Bars represent means \pm SEM.

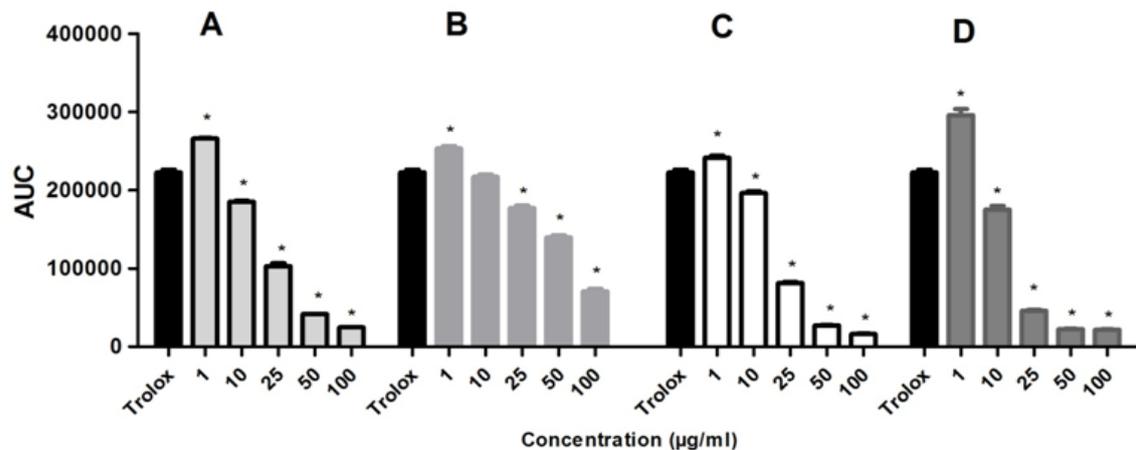


Figure S3. TRAP of *P. janeirensis*: EtOAc fraction (A), aqueous fraction (B), aqueous extract at pH 7.0 (C) and aqueous extract at pH 5.5 (D). Effects of different concentrations of fractions and extracts on free radical-induced CL were measured as AUC values. Trolox (0.05 µg/ml) was used as standard antioxidant. Bars represent means \pm SEM. Asterisks denote significance at $p < 0.05$ compared with control (one-way ANOVA followed by Tukey's test).

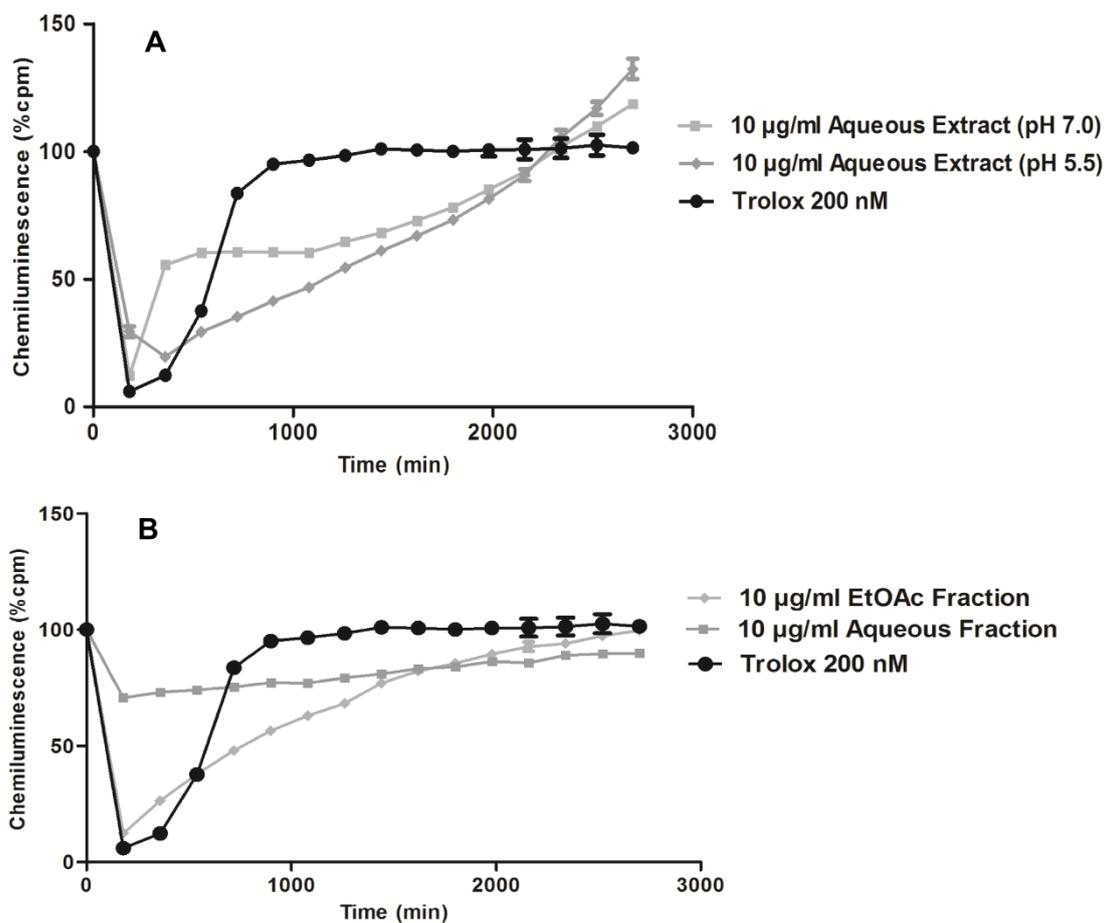


Figure S4. CL intensity (% cpm) measured after addition of extracts (A) and fractions (B) of *P. jancirensis*. CL profiles are shown for samples at concentrations that exhibited similar AUC values to those of the Trolox standard.

References

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