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Cytotoxic activity of the crude extract and derived fractions from the sea anemone *Telmatactis panamensis* against cancer cell lines

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Abstract

Sea anemones are considered a source of bioactive compounds with important pharmacological properties. In this study, we assessed the cytotoxic activity of *Telmatactis panamensis* and derived gel filtration fractions against the MCF-7 breast cancer and C6 rat glioma cell lines. The crude extract induced a concentration- and time-dependent response, reducing the viability of C6 cells from 38.76 to 1.90% with concentrations of 50–500 µg/mL at 24 h and from 11.81 to 0.73% at 48 h. In MCF-7 cells, the extract reduced viability from 61.71 to 20% and from 12.07 to 0.61% at 24 and 48 h, respectively. Fraction 1 provoked the highest cytotoxic activity in the C6 cell line, followed by fraction 3 and fraction 2. Lower sensitivity to the fractions was shown in MCF-7 cells, with only fraction 3 reducing viability by up to 50%. Both the extract and the fractions displayed low or no cytotoxicity in the normal breast cell line Hs 578Bst, suggesting that they present selectivity towards breast cancer cells over normal cells. The results support that *T. panamensis* represents a potential source for the discovery of biologically active compounds against tumor cell lines.

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Introduction

Sea anemone venoms are a complex mixture of polypeptides such as cytolysins, neurotoxins (Na_V and K_V channel toxins), enzymes (PLA_2), and non-proteinaceous compounds including serotonin, histamine and purines.[1,2] The hemolytic,[3,4] cardiostimulatory,[5] anticoagulant,[6] antimicrobial,[7] antiparasitic,[8] and anti-carcinogenic activities of sea anemone cytolysins have been demonstrated.[9-11] Therefore, due to the different properties of these toxins, including a wide range of biological activities, pore-forming ability and selective binding to sphingomyelin on the cell membrane surface, high stability to temperature and proteolytic cleavage, they are currently being explored as new drug lead compounds.[12,13]

Cancer is the second leading cause of death worldwide. In 2022, 20 million new cases of cancer were diagnosed and 9.7 million people died from cancer according to the World Health Organization, with increasing numbers in developing countries, being breast cancer the second most frequently diagnosed malignancy worldwide and the most common cancer among women.[14] Resistance to conventional chemotherapeutic drugs and the absence of fully effective treatments led to the introduction of innovative natural compounds for cancer treatment.[15-17] For this purpose, cytolysins extracted from sea anemones are being explored as promising anti-tumor agents, due to their lytic capacity and the possibility to address them to specific tissues.[18,19]

The cytotoxic and cytolytic properties of extracts and toxins of several sea anemone species against cancer cell lines have been studied. For instance, the crude extract and a UcI protein isolated from *Urticina piscivora* produce toxic effects against tumor cell lines KB (oral squamous carcinoma), and L1210 (mouse lymphocytic leukemia).[4] Sticholysins I and II (StnI and StnII) from *Stichodactyla helianthus* induce hemolytic activity and a cytotoxic effect against human Raji B-cell lymphoma,[2,20] and EqtII and Bc2 actinoporins from *Bunodosoma caissarum* were found to be effective anticancer agents affecting fibrosarcoma and glioblastoma cultured cells.[8]

Telmatactis panamensis is a sea anemone (Anthozoa: Actiniaria)

that inhabits from the Gulf of Mexico to the coast of Ecuador.[21] The studies on the isolation and characterization of its toxins were not performed, however, some research has specified the potent toxicity of other species of the genus *Telmatactis*, such as *T. australiensis* and *T. decora*.[22] Hence, the aim of the present study is to evaluate the cytotoxic activity of the crude extract and chromatographic fractions from the sea anemone *T. panamensis* in order to determine the use of this species as a source of anticancer compounds.

Materials and Methods

Specimen collection

Specimens of *T. panamensis* were collected from Club de Yates beach, Manzanillo, Colima, Mexico, by SCUBA diving at depths of 4 to 10 m. The organisms were immediately frozen and transported to the Toxicology Laboratory where they were stored at -70°C until subsequent use. The specimen's taxonomical identification was carried out based on the original description of Verrill.[23]

Crude extract preparation

Toxins extraction by nematocyst discharge was performed following the modified method by Kem *et al*.[24] Briefly, the organisms were added to distilled water and kept under mechanical agitation for 1 hour. Subsequently, the tissue was separated from the supernatant and refrigerated at 4°C in order to repeat the procedure, whereas the supernatant was frozen at -20°C for two hours for lyophilisation. This procedure was repeated nine times. Between each cycle the samples were sonicated (Cole-Parmer® model 08895-52, Vernon Hills, IL, USA) for 2 h at a temperature below 15°C and the rupture of nematocysts was monitored by microscopy until most nematocysts were discharged. The supernatant was centrifuged at 3,190 g for 10 min (Eppendorf® 5810 R, Hamburg, Germany) and frozen at -20°C. Finally, the crude extract (CE) was lyophilized (Freeze Dry Labconco System 77500, Kansas City, MO, USA) and kept at -20°C (Figure 1).

Fractionation of crude extract

For the fractionation, the extract was passed through a gel filtration column (Sephadex G-50 M, 5.0 cm × 82.5 cm). The extract (3 g) was dissolved in distilled water (50 mL) and eluted with 0.3 M acetic acid at a flow rate of 2 mL/min. The absorbance was monitored at 280 nm in an ÄKTA purifier system detector (GE Healthcare, Chicago, IL, USA) and fractions 1 (F1), 2 (F2), 3 (F3), 4 (F4) and 5 (F5) (18 mL) were collected and separated according to the peaks observed in the chromatogram using the PrimeView® 5.0 software. The fractions were pooled and concentrated under reduced vacuum (rotavapor BUCHI® R-215, Flawil, Switzerland), dialyzed using a pore size limit of 3,500 Da (Fisherbrand® Dialysis tubing, Waltham, MA, USA), and lyophilized.

Protein quantification

The protein concentration was quantified by the Bradford method[25] with the Assay Bio-Rad® Quick Start™ kit (Hercules, CA, USA), using Bovine Serum Albumin (BSA) as standard. The absorbance was measured at 595 nm with a microplate reader (Stat Fax 4200®, Awareness Technology, Palm City, FL, USA).

Cell culture

Rat glioma cells (C6 ATCC® CCL-107™), human breast adenocarcinoma (MCF-7 ATCC® HTB-22™), and normal breast cells (Hs 578Bst ATCC® HTB-125™) were cultured in monolayers at 37°C in a humidified atmosphere with 5% CO_2 in F12K (ATCC® 30-2004™, Manassas, VA, USA), EMEM (ATCC® 30-2003™, Manassas, VA, USA) and Hybri-Care (ATCC® 46-X™, Manassas, VA, USA) medium supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin-steptomycin and 0.1% amphotericin B (ATCC® OCS-999-002™, Manassas, VA, USA), respectively. Additionally, 10 µg/mL of insulin (Sigma-Aldrich®, I9278-5ML, Saint Louis, MO, USA) were added to the EMEM medium to promote and increase cell proliferation and growth due to the overexpressed insulin-like growth factor receptors in MCF-7 cells, and 30 ng/mL mouse Epithelial Growth Factor (EGF) (Sigma-Aldrich®, E5160-100UG, Saint Louis, MO, USA) were added to the Hybri-Care medium to induce EGF expression, which stimulates cell growth and regulates normal breast cell development. For propagation, cells were detached and harvested with 0.25% trypsin solution when they reached 70-90% confluency.

Cytotoxicity assay

The cytotoxic effects of the CE and fractions of *T. panamensis* were evaluated on various cell lines by measuring the reduction in cell viability and observing morphological changes. In all experiments, the cells were seeded in 96-well plates (Corning® No. 3696, Williamsburg, NY, USA) at 5×10^3 per well and cultured to 80% confluence. The cells were then treated with different protein concentrations of the CE and five fractions (50, 150, 300 and 500 µg protein/mL) and incubated for 24 and 48 hours at 37°C, in 5% CO_2

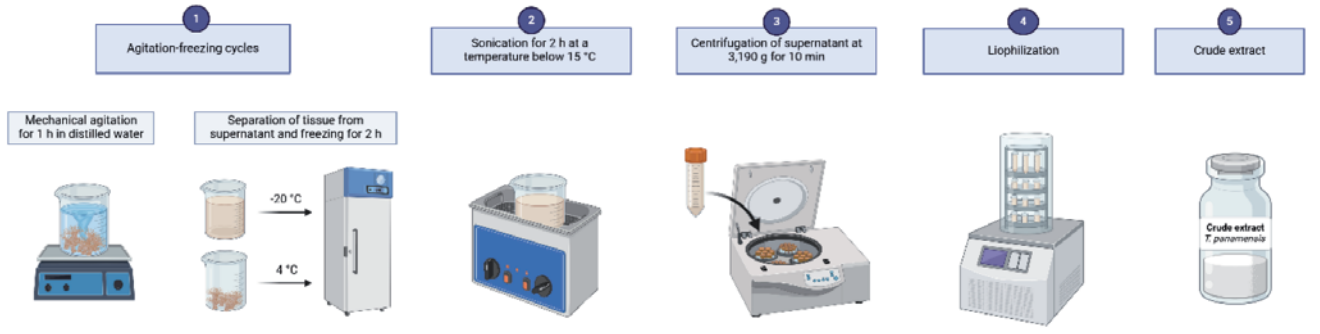

Figure 1. Schematic diagram of the crude extract preparation from *Telmatactis panamensis*. Created with Biorender.com.

atmosphere. Medium without sample was used as a negative control. Four independent experiments were performed for each concentration.

Cell viability assay

To assess the cytotoxicity effects of CE and fractions, two colorimetric bioassays were used. Crystal Violet (CV) assay was performed according to the method of Chiba *et al*.[26] Briefly, 50 µL of CV (Sigma CO775, Saint Louis, MO, USA) were added to each well and after 15 min of agitation the microplates were destained with 10% acetic acid and the absorbance (A) was measured at 570 nm with a microplate reader (Stat Fax 4200®, Awareness Technology, Palm City, FL, USA). Neutral Red (NR) assay was performed using the protocol of Repetto *et al*.[27] Hence, 100 µL of 40 µg/mL NR-Medium (Sigma-Aldrich N4638, Saint Louis, MO, USA) were added to each well. After 2 h of incubation the solution was discarded and replaced with an ethanol solution to extract the dye and record the absorbance (A) at 540 nm with a microplate reader (Stat Fax 4200®, Awareness Technology, Palm City, FL, USA). Cell viability (% CV) was calculated using Equation [1]:

$$Cell\ viability\ (\%) = \left[\frac{A_{treated}}{A_{control}}\right] \times 100 \quad [1]$$

Where $A_{treated}$ is the absorbance of the treated cells with CE and fractions and $A_{control}$ is the absorbance of the control.

Statistical analysis

Data were expressed by mean ± SD, followed by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison tests using MINITAB® 17 software. All tests were considered statistically significant at $p < 0.05$.

Results

Extraction and fractionation of crude extract from *T. panamensis*

The extraction procedure allowed to obtain 23.26 g of CE, which represents 6.64% of total yield. The protein concentrations of CE and fractions are shown in Table 1. The first purification step using gel filtration chromatography in a Sephadex G-50 column resulted in the isolation of five fractions, F1-F5 (Figure 2).

Cytotoxicity assays

The results indicate that the CE caused a higher decrease in cell viability in the MCF-7 and C6 tumor cell lines compared to the fractions. The neutral red method showed that CE induced a concentration and exposure time-dependent decrease in cell viability in C6 rat glioma cells (Figure 3A) and MCF-7 breast cancer cells (Figure 3B). There was a significant difference between 24 and 48 h at different concentrations ($p < 0.05$). CE reduced C6 cell viability from 38.8 to 1.9% and MCF-7 from 61.7 to 20% at concentrations ranging from 50 to 500 µg/mL after 24 h. After 48 h, the values decreased from 11.8 to 0.73% for C6 and 12.07-0.61% for MCF-7. Low cytotoxicity was shown in normal breast cells Hs 578Bst (Figure 3C). The crystal violet method showed a reduction in cell survival of C6 and MCF-7 cells from 65.44 to 14.37% and from 53.73 to 11.62% after 24 h, and from 28.80 to 11.77% and from 17.29 to 8.46%, respectively, after 48 h. The viability of treated Hs 578Bst cells was not significantly different from the control group ($p > 0.05$), with values up to 88.47 and 85.74%, and 92.27 and 95.95% for neutral red and crystal violet, respectively (Tables 2 and 3). Additionally, in the presence of CE, C6 and MCF-7 cells changed their typical fusiform (Figure 3D) and polygonal morphology (Figure 3F), respectively, (as observed in the control group) to a round shape, reduced in size, and decreased their density (Figure 3E-G). No significant morphological changes were observed in Hs 578Bst breast cells; they maintained their fusiform shape with membrane protrusions (Figure 3H), although they shrank slightly (Figure 3I).

Table 1. Protein concentration of crude extract and fractions of *T. panamensis*.

Sample	Protein concentration (mg/mg)[a]
CE	0.085
F1	0.143
F2	0.181
F3	0.034
F4	0.065
F5	0.074

[a]mg of protein per mg of crude extract or fraction.

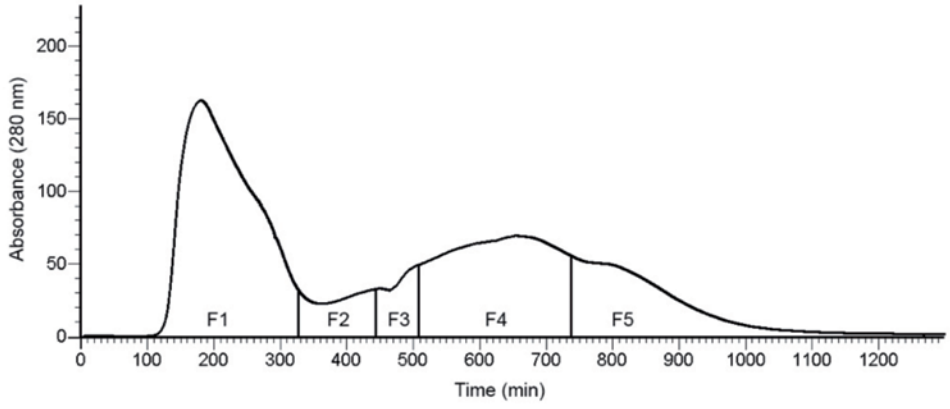

Figure 2. Sephadex G-50 elution profile of crude extract from *Telmatactis panamensis*.

Table 2. Viability percent values of different cell lines treated with 50 and 500 µg/mL of crude extract and fractions of *T. panamensis* for 24 and 48 h using neutral red staining.

	C6[1]				MCF-7[2]				Hs 578Bst[3]			
	24 h		48h		24 h		48h		24 h		48h	
	50	500	50	500	50	500	50	500	50	500	50	500
CE	38.76*	1.90*	11.81*	0.73*	61.71*	20.00*	12.07*	0.61*	94.69*	88.47*	91.39*	85.74*
F1	100	2.09*	100	0.82*	100	70.57*	100	86.94*	100	93.24*	99.00	89.85*
F2	100	17.92*	100	22.02*	84.66*	83.23*	95.81	89.99*	100	90.65*	100	85.22*
F3	100	11.64*	93.17	2.65*	68.37*	52.40*	94.22	71.64*	100	87.65*	100	89.23*
F4	N/A	N/A	N/A	N/A	91.05*	69.01*	100	97.22	99.78	88.59*	98.48	88.41*

CE, crude extract; F1, fraction 1; F2, fraction 2; F3, fraction 3; F4, fraction 4; [1]rat glioma; [2]breast cancer; [3]normal breast cells; N/A not available, untested sample; ANOVA post-test Dunnett, comparing the control with the different concentrations: *p <0.05. The table only shows the minimum and maximum concentration tested. The other concentrations can be appreciated in the graphs.

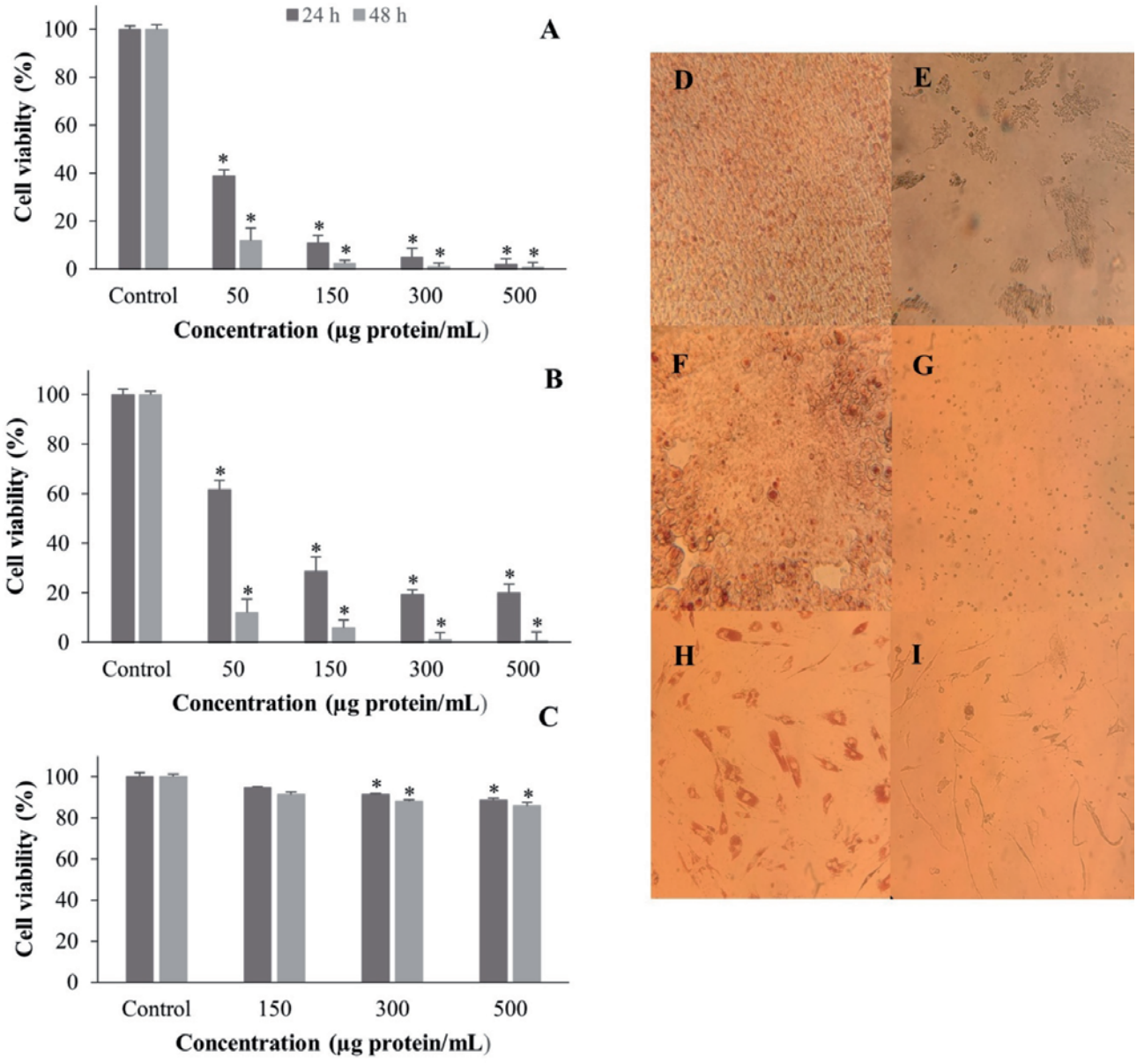

Figure 3. Cytotoxic effect of the crude extract from *T. panamensis* on the cell viability of tumor and normal cell lines during 24 and 48 h: (A) C6; (B) MCF-7; (C) Hs 578Bst. Morphological changes after 48 h of incubation with 500 µg/mL of CE using neutral red staining: (D) control MCF-7; (E) MCF-7 with CE; (F) control Hs 578Bst; (G) Hs 578Bst with CE; (H) C6; (I) C6 with CE. Data are expressed as mean±SD, (n=4). Asterisks show significant differences (p <0.05).

The cytotoxic effects displayed by the fractions were variable against the cell lines. The major inhibitory effect on tumoral cell lines was observed on the C6 rat glioma cells, followed by the MCF-7 breast cancer cells as shown in Table 2. The tested fractions (F1, F2 and F3) showed a significant inhibitory effect on C6 cells at concentrations of 150, 300 and 500 µg/mL for both exposure times ($p < 0.05$). F1 showed the highest cytotoxic activity at a concentration of 500 µg/mL, reducing the viability to 2.09% and 0.82% after 24 and 48 h, respectively (Table 2). The effect of F2 on cell viability was independent of concentration and time; only the concentration of 300 µg/mL caused values below 12% ($p < 0.05$) (Figure 4A). F3 elicited a significant concentration- and time-dependent cytotoxic response, resulting in a 100 to 11.64% decrease at 24 h and 93.17 to 2.64% with 50-500 µg/mL at 48 h ($p < 0.05$) (Figure 4B). The MCF-7 cell line showed lower sensitivity to fractions. F3 showed the highest cytotoxic activity in a dose-dependent manner, reducing the viability from 68.37 to 52.40% after 24 h exposure when increasing the concentration from 50 to 500 µg/mL ($p < 0.05$) (Figure 4C). F4 decreased the viability from 91.05 to 69.01% at 24 h (Figure 4D). The effects of F1 and F2 on MCF-7 cells were lower compared to the other fractions (>70%) and were not dependent on concentration or time (Table 2). Hs 578Bst cell viability was up to 85%, therefore, the cytotoxic activity of the fractions against this particular cell line is lower than against tumor cell lines (Figure 4E-F; Table 2).

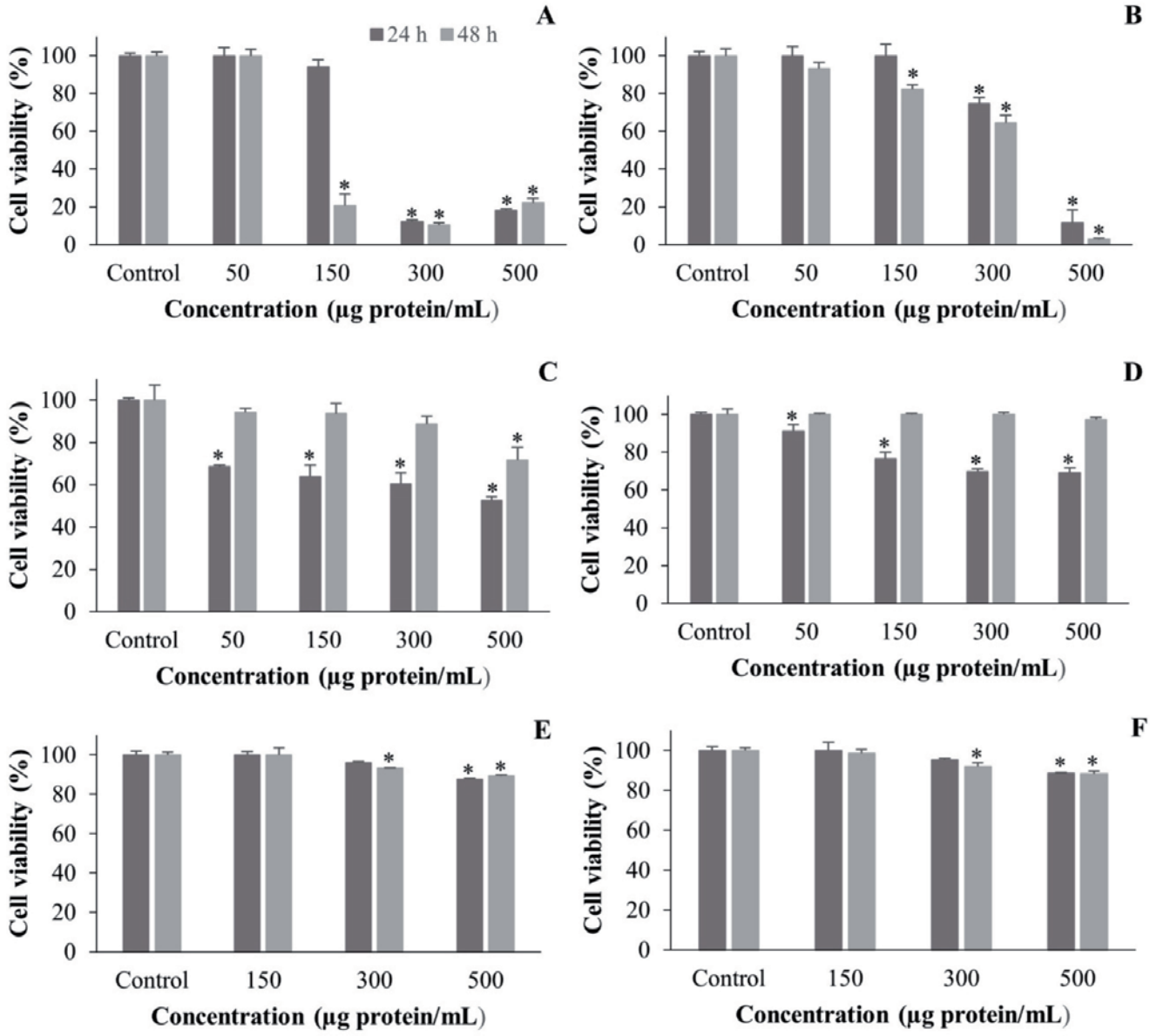

Figure 4. Cytotoxic effect of fractions from *T. panamensis* on the cell viability of tumor and normal cell lines; (A) C6 with F2; (B) C6 with F3; (C) MCF-7 with F3; (D) MCF-7 with F4; (E) Hs 578Bst with F3; (F) Hs 578Bst with F4. Cell viability was determined by neutral red method after 24 and 48 h. Data are expressed as mean±SD, (n=4). Asterisks show significant differences ($p < 0.05$).

Table 3. Viability percent values of different cell lines treated with 50 and 500 µg/mL of crude extract and fractions of *T. panamensis* for 24 and 48 h using crystal violet staining.

	C6[1]				MCF-7[2]				Hs 578Bst[3]			
	24 h		48h		24 h		48h		24 h		48h	
	50	500	50	500	50	500	50	500	50	500	50	500
CE	65.44*	14.37*	28.80*	11.77*	53.73*	11.62*	17.29*	8.46*	98.20	92.27	98.16	95.95
F1	100	33.56*	100	10.15*	100	76.67*	100	100	100	98.56	100	96.03
F2	N/A	N/A	N/A	N/A	100	85.00*	88.08	86.57*	100	97.92	100	93.82*
F3	100	48.49*	100	20.13*	100	76.86*	95.52	76.82*	98.93	98.38	100	100
F4	N/A	N/A	N/A	N/A	93.65	94.18	95.89	90.82*	100	98.84	100	98.40

CE, crude extract; F1, fraction 1; F2, fraction 2; F3, fraction 3; F4, fraction 4; [1]rat glioma; [2]breast cancer; [3]normal breast cells; N/A not available, untested sample; ANOVA post-test Dunnett, comparing the control with the different concentrations: *p <0.05. The table only shows the minimum and maximum concentration. tested. The other concentrations can be appreciated in the graphs.

Cell viability values obtained with the crystal violet method, shown in Table 3, were consistent with the results of the neutral red method (Table 2). However, the percentages of viability evaluated with crystal violet are higher than those obtained with neutral red. Similarly, both methods showed significant differences (p <0.05) in the C6 and MCF-7 tumor cell lines after 24 and 48 h.

Discussion

The investigation of cnidarian toxins mainly focuses on characterizing their pharmacological properties to identify new molecules with potential therapeutic activity, being the anticancer agents the most relevant to date.[1,28] In the present study the cytotoxic properties of CE and derived fractions from *T. panamensis* were evaluated for the first time. Based on recent evidence, cytolysins are of particular interest as promising antitumor agents.[29] However, research into the potential of sea anemone toxins against cancer cells has not been extensively explored. The current study demonstrates the cytotoxicity of CE and fractions from *T. panamensis* on two tumor cell lines, C6 rat glioma and MCF-7 breast cancer.

The cytotoxicity of chemical compounds involves a set of effects on cell integrity, which includes altered cell morphology, failure of the cell to attach to surfaces, changes in growth rate, cell disintegration and death.[30] These responses are consistent with those observed in the CE and some fractions of *T. panamensis* against MCF-7 and C6 cells, including loss of typical morphology, reduced cell size and irregular density due to loss of the adherent phenotype. Additionally, the results showed that CE induced a concentration and exposure time dependent decrease in cell viability on C6 rat glioma cells and MCF-7 breast cancer cells. In previous studies evaluating the cytotoxicity of *Heteractis crispa*, *H. magnifica*, *H. malu*, *Cryptodendrum adhaesivum* and *Entacmaea quadricolor* CEs on lung (A549), breast (T47D) and skin (A431) cancer cell lines, a significant reduction in cell viability was observed in A549 for all extracts at 40 µg/mL. Only *H. malu* and *C. adhaesivum* extracts had an inhibitory effect on T47D and A431 cells at 40 µg/mL.[31] Similarly, exposure to 50 µg/mL of *Bunodeopsis globulifera* extract induced a 50% reduction in A549 cell survival after 24 h.[32] These results are consistent with the present study showing a similar cytotoxic response to that of *T. panamensis* CE, which had a significant effect on C6 and MCF-7 cells at 50 µg/mL protein. Furthermore, CE had a higher cytotoxic effect against MCF-7 and C6 tumor cell lines compared to the fractions. It has been previously reported that bioactivity differs between total extracts and fractions,[33] which can be explained by different factors, including synergistic effects of components in extracts, osmotic alterations due to high salt content in crude extracts which may cause false positive cytotoxic responses and loss of unstable compounds during the purification process.[34]

Evaluation of fractions against cancer cells showed a greater cytotoxicity against C6 rat glioma cells. F1, F2 and F3 induced a significant reduction in cell viability to 0.82%, 18% and 2.64% respectively on C6 at a concentration of 500 µg/mL. In contrast, only F3 significantly decreased the viability of MCF-7 cells to 52% with 500 µg/mL, whereas the other fractions showed viability values above 70%. Consistent with previous findings showing that tumor cell lines have different sensitivities to sea anemone crude extracts or to isolated compounds,[31,35] the low susceptibility of MCF-7 cells to T. *panamensis* fractions can be explained by the biology of this cell line. Tighter junctional complexes (desmosomes and adherens junctions) and many transmembrane proteins involved in cell-substrate and cell-cell adhesion make MCF-7 cells more resistant to chemical compounds.[36]

Hs 578Bst normal breast cells showed no significant cytotoxic responses induced by the CE and fractions, as their morphology, density and cell viability remained constant (above 85%) at the ranging concentrations. Several studies have demonstrated the selectivity of crude extracts or isolated compounds from anemones against cancer cells. Crude extract of *Heteractis magnifica* induced a survival rate of 5% and 50% in MCF-7 breast cancer cells and 184B5 normal breast cells, respectively. The study suggested that the cytolysins were responsible for the cytotoxic effect.[11] The selective binding to sphingomyelin membranes that can lead to an ionic imbalance and to cell death due to pore formation, is a relevant property in cancer therapy because it has been demonstrated that the lipids of tumor cell membranes present a significantly altered composition, particularly with a high concentration of sphingomyelin.[37] Based on this evidence, it was found that the CE and fractions of *T. panamensis* exhibited selectivity for breast cancer cell lines over normal breast cells.

Conclusions

The present study shows that crude extract of *T. panamensis* and its fractions contain bioactive molecules with cytotoxic activity. A significant cytotoxicity against C6 rat glioma and MCF-7 breast cancer was shown. *T. panamensis* extract had higher effect compared to the fractions. C6 was more sensitive to CE and fractions, whereas MCF-7 cells were only affected by CE and F3. In contrast, CE and fractions exerted considerably less effect on Hs 578Bst normal breast cells, indicating selectivity for breast cancer cells compared to

normal cells. These findings show that *T. panamensis* represents a potential source of antitumor compounds. Further purification and characterization of the active fractions of *T. panamensis* are necessary to identify the compounds responsible for the anticancer activity and to elucidate their specific mechanisms of action.

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