Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 28(2): 147 – 159 (2024)
www.ejabf.journals.ekb.eg

Cytotoxic Profiling of the Marine Gastropod (*Conus textile*) Venom Extracts on Human Cancer Cell Lines Unveiling its Therapeutic Side as Anti-Cancer Therapeutic Agents

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ARTICLE INFO
Article History:
Received: Jan. 7, 2024
Accepted: Feb. 6, 2024
Online: March 17, 2024

Keywords:
Marine gastropod,
Conus textile,
Marine venom,
Conotoxins,
Cytotoxicity,
Cancer cell lines,
In-vitro assay

ABSTRACT

Conus marine snails can be identified by their venom compositions, which are complex and comprise a wide variety of pharmacologically potent peptides known as conotoxins. Due to their possible therapeutic usages, these peptides have attracted a lot of attention, especially in the field of oncology. This work aimed to study the cytotoxic effects of venom gland and venom tube extracts from *Conus textile* on three human cancer cell lines (namely HepG2, hepatocellular carcinoma, Caco2, colorectal cancer, Mcf7, and breast cancer). The present study assessed the venoms' half-maximal inhibitory concentrations (IC_{50}), which represented their effectiveness in lowering cell viability, using an *in-vitro* experiment. With IC_{50} values ranging between 94 and 381.97µg/ ml, the results showed that both venom extracts had strong cytotoxic effects on all examined cell lines. When compared to the gland extract, the venom tube extract consistently showed greater potency, which may indicate a higher concentration of active cytotoxic chemicals. When treated with venom tube extract, the Mcf7 cell line showed the lowest IC_{50} value, suggesting a promising potential for breast cancer therapies. These findings supported the hypothesis that *Conus textile* venom contains bioactive components with selective toxicity towards cancer cell lines. The insignificant standard deviations reported support the regularity and dependability of the cytotoxic effects. The present work provided the foundation for future purifying and mechanistic investigations of conotoxins and advanced the investigation of marine natural products as a source of potential anti-cancer drugs.

INTRODUCTION

One of the most fatal diseases in the world is cancer. The World Health Organization **(WHO, 2020)** studied projects that by 2030, there would be 13 million cancer-related deaths and 21 million additional cases of the disease. Therefore, the development of novel, selective, and effective chemotherapeutic drugs derived from natural sources as opposed to synthetic ones remains necessary. Natural products are an essential part of today's pharmaceutical collection and a major source of new chemical variety **(Fenical & Jensen, 2006; Bohlin *et al*., 2010; Fattorusso *et al*., 2012; Gerwick & Moore, 2012; Hill, 2013)**.

Nowadays, it's believed that marine natural products offer the greatest platform for providing an innovative, efficient, and distinctive chemical structure that may have significant potential for treating or preventing cancer or acting as a scaffold for more potent chemotherapy drugs. The lengthy evolutionary history and the harsh maritime environment

have encouraged the synthesis of several chemicals with distinctive structures that aid in the survival of these marine animals **(Khalifa *et al*., 2019)**.

Animal toxins are more potent and selective than pharmaceuticals intended for therapeutic use, but they nevertheless face numerous obstacles, including low oral bioavailability, lack of membrane permeability, and short circulation half-life **(Chen *et al*., 2018)**. *Conus textile*, a marine cone snail, is one of the toxic animal species with the highest toxin diversity. These marine cones are a species of sea snail that are venomous and carnivorous **(Favreau & Stöcklin, 2009)**. There are currently eight approved drugs derived from marine organisms, five of which are utilized in various treatments for cancer **(Jimenez *et al*., 2003)**. The five marine-derived pharmaceuticals that have been approved come from the mangrove tunicate "*Ecteinascidia turbinate*", sea squirt "*Aplidium albicans*" and sponges "*Cryptotheca crypta*, *Halichondria okadai*, and *Tethya crypta*" **(Lindequist, 2016)**.

Venom from cone snails has received a lot of focus in recent years as genuine pharmacological assets due to its biological activity, remarkable diversity, and molecular studies that provided a portal for biomedicine study, especially on its anticancer activities **(Carroll *et al*., 2019)**. Peptides extracted from certain cone snail venoms could serve as useful models for the creation of new pharmaceuticals, as some of the conotoxins are currently being developed as FDA-approved medications (such as a synthetic version of the peptide x-conotoxin MVIIA) from *Conus magus* to treat chronic pain in humans. In fact, several of these compounds are now in clinical trials **(Sudewi *et al*., 2019)**.

Hence, the present study was aimed to evaluate the antitumor potential of venom extracts from *Conus textile* on different cancer cell lines. This finding could create a new path in the pharmacological properties' toxins from cone snail, and it could be an alternative source for the chemotherapy of breast cancer "Mvf7", hepatocellular carcinoma "HepG2" and colorectal cancer "Caco2".

MATERIALS AND METHODS

1. Site of collection and specimen collection

The specimens of *Conus textile* were collected from different sites along the coast of the Gulf of Aqaba in the Red Sea. Snails tend to be located along the coast in relatively shallow water (< 10 meters) consisting of sea-grass, a rock-sand and/or reef environment. A shallow water diving was conducted along the coast, the Gulf of Aqaba in the Red Sea coast of Egypt. Cone snails were obtained from sea-grass, rocks, coral, and sand; it was collected from flipping rocks in the inter-tidal region during low tide. The specimens were kept in ice and then transported to Marine Biology Laboratory at Zoology Department, Faculty of Science, Al-Azhar University, until the extraction of venom was conducted.

2. Venom gland and venom tube extracts preparation

The isolation of crude venom from *Conus textile* was performed by the method outlined by **McIntosh *et al*. (1995)**. The cone shell was cracked and shell pieces were removed. The interior body was entirely left intact. Although this method destroyed the shell

without causing damage to the interior venom gland and the venom duct. Once the interior body was removed, the body was carefully dissected to reveal the venom gland and venom duct. The ducts were then cut into small pieces 10- 15mm. These were suspended in 2% acetic acid and centrifuged (5000rpm for 10min, 5°C) to extract the peptide components from venom. The pellet was re-extracted three times with 2% acetic acid. The supernatant was lyophilized and stored at −80°C prior to use.

3. Viability assay (MTT protocol)

3.1. Determination of sample cytotoxicity on cells

The 96 well tissue culture plate was inoculated with 1 X 10^5 cells/ ml (100µl/ well) and incubated at 37^oC for 24 hours to develop a complete monolayer sheet. Growth medium was decanted from 96 well micro titer plates after confluent sheet of cells were formed; cell monolayer was washed twice with wash media. Two-fold dilutions of tested sample were made in RPMI medium with 2% serum (maintenance medium). Each extract concentration (0.1ml) was tested in different wells leaving 3 wells as control (which receiving only maintenance medium). The plate was incubated at 37^oC and then examined. Cells were checked for any physical signs of toxicity, e.g. partial or complete loss of the monolayer, rounding, shrinkage, or cell granulation. The MTT solution was prepared (5mg/ ml in PBS, BIO BASIC CANADA INC). Then, 20µl of MTT solutions were added to each well and placed on a shaking table at 150rpm for 5 minutes to thoroughly mix the MTT into the media. Subsequently, the plate was incubated at 37°C with 5% CO2 for 4 hours to allow the MTT to be metabolized. After incubation, the media were dumped off. The formazan (MTT metabolic product) was then resuspended in 200µl of DMSO and placed on a shaking table at 150rpm for 5 minutes to thoroughly mix the formazan into the solvent. Finally, the optical density was read at 560nm, and the background at 620nm was subtracted. The optical density was directly correlated with cell quantity.

3.2. Morphological assay

Large-scale morphological changes occurred at the cell surface or in the cytoskeleton and were followed by and related to cell viability. Damage was identified by large decreases in volume, secondary to losses in protein and intracellular ions due to altered permeability to sodium or potassium. Necrotic cells displayed nuclear swelling, chromatin flocculation, and loss of nuclear basophilia, while apoptotic cells exhibited cell shrinkage, nuclear condensation, and nuclear fragmentation.

RESULTS

1. Cytotoxicity of venom gland and venom tube extracts

The results of an *in-vitro* cytotoxicity assay that measured the efficacy of venom gland and venom tube extracts from the marine snail *Conus textile* on three different human cancer cell lines: breast cancer “Mcf7”, hepatocellular carcinoma “HepG2”, and colorectal cancer “Caco2” are presented Table (1) and illustrated in Figs. (1, 2). The assay aimed to determine the half-maximal inhibitory concentration (IC_{50}) values of these two venom extracts, which indicated the potency of the venom as an anti-cancer agent.

The IC_{50} values were calculated based on the dose-response relationship between the concentrations of the venom extracts (in µg/ ml) and the percentage of cell viability as measured by optical density (O.D) at a certain wavelength. The negative control (-ve control) represents the baseline cell viability with no venom treatment, against which the effects of the venom samples were compared. Cell viability percentage reflects the proportion of live cells remaining after venom treatment relative to the control, while toxicity percentage represents the proportion of cells that have been killed by the venom. For instance, a viability of 2.4% corresponds to a toxicity of 97.59%, indicating that almost all cells were nonviable at the highest concentration of venom gland extract tested in Mcf7 cells.

Table 1. Viability, toxicity and half-maximal inhibitory concentrations (IC_{50}) of *Conus textile* venom extracts for different cancer cell lines

Cancer cell lines	Sample	Extract concentration (µg/ml)	Optical denesity	Viability (%)	Toxicity (%)	IC_{50}± SD
Mcf7	**-ve Control**	-------	0.775 ± 0.004	100	0	
	Venom gland extract	1000	0.018 ±0.0006	2.4	97.591	**114.72 ± 1.69**
		500	0.023 ± 0.001	3.05	96.946	
		250	0.024 ± 0.001	3.18	96.817	
		125	0.336 ± 0.007	43.44	56.559	
		62.5	0.639 ± 0.006	82.53	17.462	
		31.25	0.752 ± 0.007	97.07	2.924	
	Venom tube extract	1000	0.028 ± 0.002	3.69	96.301	**94 ± 1.59**
		500	0.042 ± 0.003	5.41	94.58	
		250	0.124 ± 0.005	16.04	83.956	
		125	0.23 ± 0.009	29.72	70.279	
		62.5	0.545 ± 0.009	70.32	29.677	
		31.25	0.711 ± 0.007	91.82	8.172	
HepG2	**-ve Control**	-------	0.641 ± 0.001	100	0	
	Venom gland extract	1000	0.017 ± 0	2.75	97.243	**188.19 ± 2.05**
		500	0.018 ± 0	2.91	97.087	
		250	0.142 ± 0.008	22.25	77.743	
		125	0.542 ± 0.007	84.55	15.444	
		62.5	0.624 ± 0.004	97.45	2.548	
		31.25	0.637 ± 0.003	99.42	0.572	
	Venom tube extract	1000	0.018 ± 0	2.8	97.191	**84.37 ± 1.22**
		500	0.019 ± 0	3.06	96.931	
		250	0.084 ± 0.006	13.15	86.843	
		125	0.164 ± 0.011	25.58	74.414	
		62.5	0.368 ± 0.008	57.46	42.537	
		31.25	0.584 ± 0.004	91.1	8.892	
Caco2	**-ve Control**	-------	0.669 ± 0.002	100	0	
	Venom gland extract	1000	0.031 ± 0.002	4.73	95.266	**215.08 ± 3.64**
		500	0.093 ± 0.003	14	85.999	
		250	0.244 ± 0.006	36.57	63.428	
		125	0.593 ± 0.004	88.68	11.31	
		62.5	0.657 ± 0.006	98.3	1.694	
		31.25	0.665 ± 0.002	99.5	0.498	
	Venom tube extract	1000	0.05 ± 0.001	7.47	92.526	**381.97 ± 7.36**
		500	0.181 ± 0.007	27.15	72.845	
		250	0.507 ± 0.007	75.83	24.165	
		125	0.662 ± 0.005	98.95	1.046	
		62.5	0.667 ± 0.002	99.8	0.199	
		31.25	0.667 ± 0.001	99.7	0.298	

The IC_{50} values suggested that both extracts of venom gland and venom tube of *C. textile* had a potent cytotoxic effect on the tested cancer cell lines, with varying degrees of potency. For Mcf7 cells, the venom gland extract showed an IC_{50} of 114.72µg/ ml, while the venom tube extract was slightly more potent with an IC_{50} of 94µg/ ml. For HepG2 cells, the gland venom extract showed higher IC_{50} value (188.19µg/ ml) compared to the venom tube extract (84.37µg/ ml). While Caco2 required higher IC_{50} for both extracts of venom gland and venom tube (215.08 and 381.97µg/ ml, respectively).

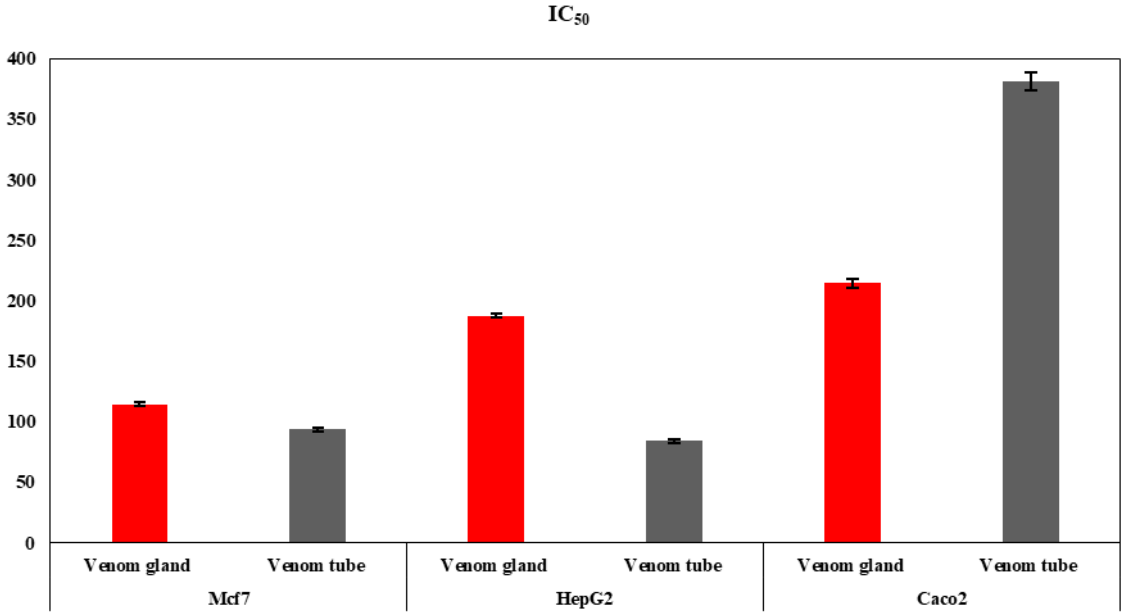
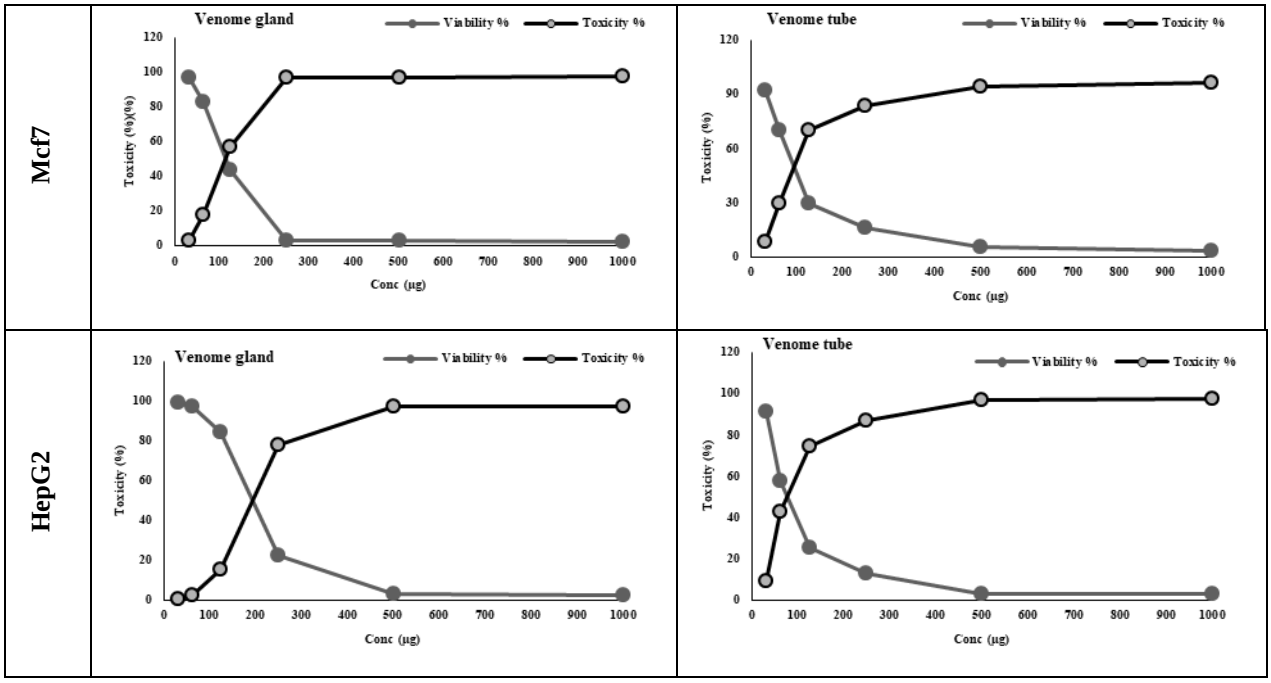


Fig. 1. IC_{50} for *in-vitro* assay of venom gland and venom tube extracts of *C. textile*



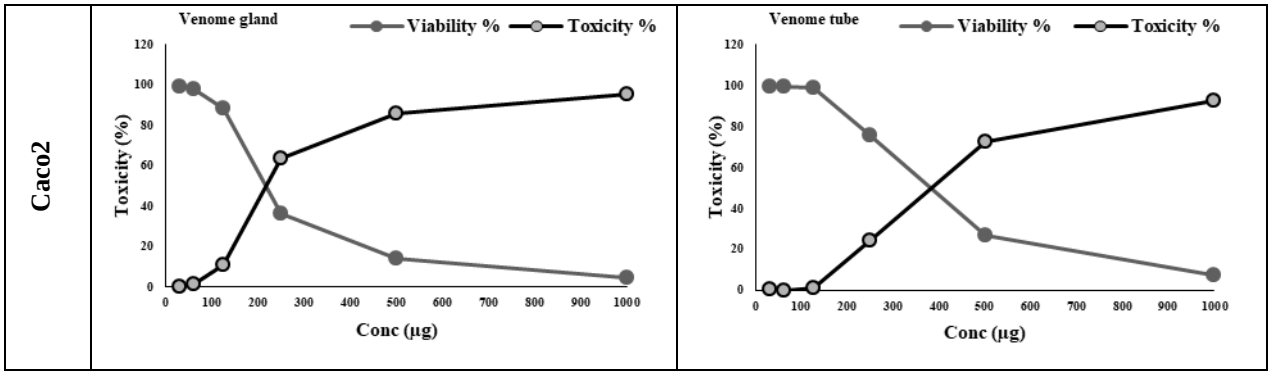


Fig. 2. Viability and toxicity for *in-vitro* assay of gland and tube venom of *C. textile*

The findings of this assay were statistically significant since they contributed to the growing body of evidence that marine snail venom contains complex mixtures of bioactive compounds with potential therapeutic applications, including cancer treatment. The differences in IC_{50} values across different cell lines underscore the specificity and selective cytotoxicity that could be harnessed for targeted cancer therapies. Further purification and identification of the active compounds within these venoms could lead to the development of new oncological drugs. It is also worth noting that the standard deviations are relatively small, indicating the consistency and reliability of the results.

The venom of *Conus textile* exhibited significant cytotoxic effects in *in-vitro* models, which warrants further investigation into its components and mechanisms of action as a potential source of novel anti-cancer agents.

2. Morphological *in-vitro* assay

The micrograph series of *in-vitro* assay (Plates I, II and III) provided a striking visual representation of the morphological changes induced in three human cancer cell lines "Mcf7, HepG2, and Caco2" following treatment with venom gland and venom tube extracts from *Conus textile*. The control groups for each cell line exhibited typical morphology, with Mcf7 cells showing their characteristic clustered growth, HepG2 cells displaying a polygonal shape, and Caco2 cells maintaining their well-defined borders and uniform appearance.

Upon exposure to venom gland extracts, notable alterations were observed. Mcf7 cells demonstrated a significant reduction in cell density and disorganization, indicating cell death and detachment. HepG2 cells, known for their robustness, showed signs of morphological stress, with changes in cell shape and a reduction in confluence. In Caco2 cells, the impact of the gland venom was manifested in cell shrinkage and the appearance of spaces between cells, suggesting loss of adhesion and apoptosis.

The effects of the venom tube extracts are even more pronounced. Mcf7 cells under this treatment displayed extensive cell lysis and a drastic decrease in viable cell count, highlighting the venom's potent cytotoxicity. HepG2 cells exhibited a scattered distribution, loss of typical morphology, and clear signs of cellular disintegration. In Caco2 cells, the venom tube extract led to severe morphological disruptions, with almost complete loss of cellular structure, indicative of high cytotoxic activity but at higher concentration.

Overall, the micrographs in Plate I visually underscore the potent cytotoxic effects of *C. textile* venom on these cancer cell lines. The distinct morphological changes induced by the venom, especially from the venom tube, align with the quantified cytotoxicity data, suggesting the presence of potent bioactive compounds with significant anti-cancer potential. This further supported the potential of marine-sourced bioactive compounds in cancer research and therapy.

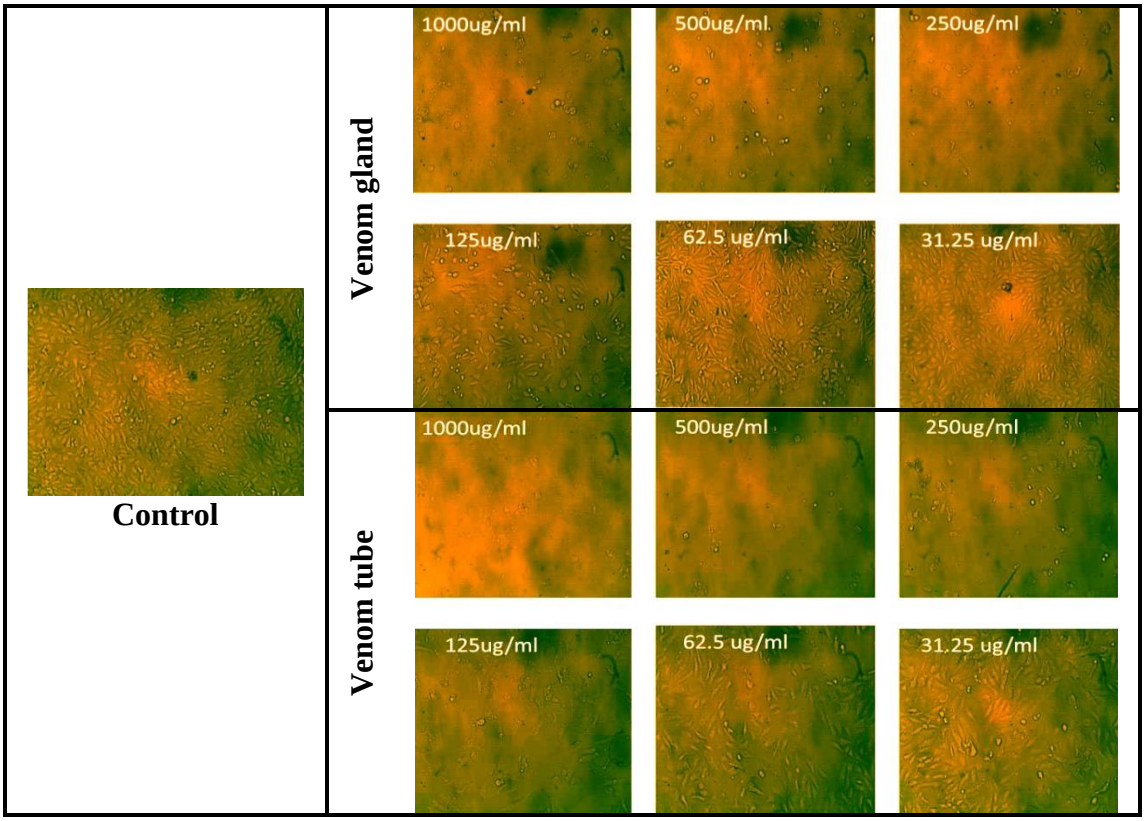
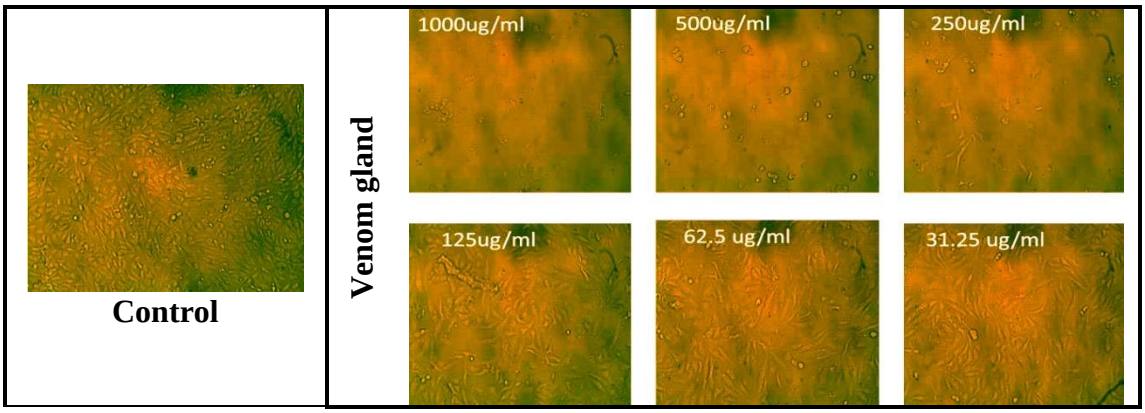


Plate I. Morphological *in-vitro* assay of venom gland and tube extracts of *C. textile* on Mcf7 cancer cell line



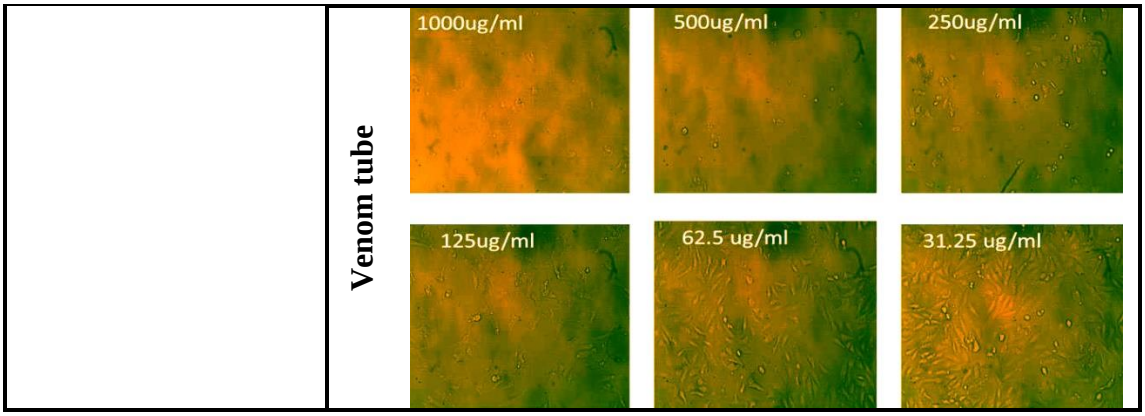


Plate II. Morphological *in-vitro* assay of venom gland and tube extracts of *C. textile* on HepG2 cancer cell line

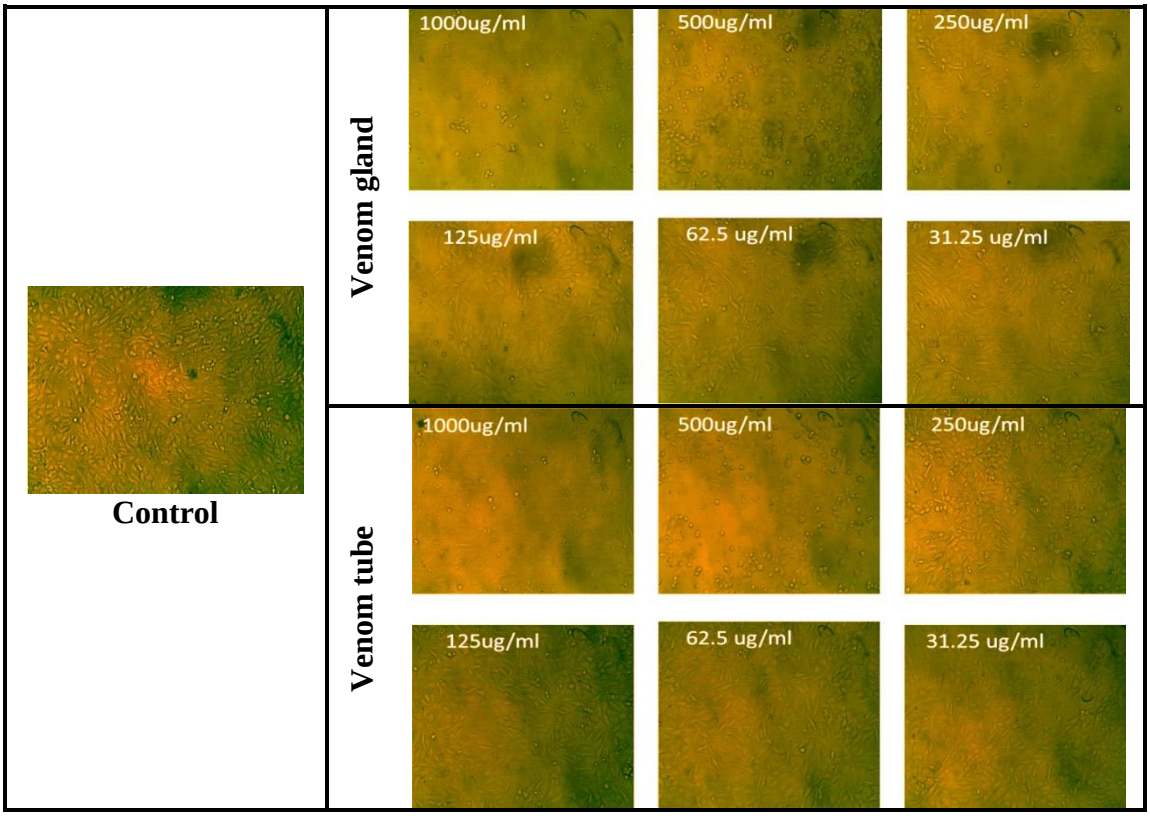


Plate III. Morphological *in-vitro* assay of venom gland and tube extracts of *C. textile* on Caco2 cancer cell line

DISCUSSION

A very rich availability of new isolable bioactive chemicals with a wide range of chemical structures can be found in marine ecosystem, making it a valuable resource for drug discovery **(Molinski *et al*., 2009)**. The last few years have seen a thorough evaluation of marine chemicals and a variety of projects, including anti-viral **(Dang *et al*., 2015)**, anti-bacterial **(Desbois *et al*., 2009)**, antifungal **(Plaza *et al*., 2009)**, anti-parasitic **(Wei *et al*., 2010)**, anti-tumor **(Nuijen *et al*., 2000; Zhang *et al*., 2017)**, and anti-inflammatory **(Asolkar *et al*., 2009)**, being reported.

Approximately 70% of the earth's surface and huge biodiversity have been found, consisting of up to 36 phyla **(Pomponi, 1999)**. There is a hypothesis that high taxonomic

diversity is correlated with a wide chemical diversity of natural products **(Williams, 2009)**. Due to their extraordinary complexity and diversity, venom from marine cone snails (*Conus*) has garnered much attention recently **(Safavi-Hemami *et al.*, 2011)**. Each cone species produces between 1100 to 1900 distinct peptide poisons in total; the genus *Conus* of snails is known to produce up to 500,000 distinct bioactive chemicals in total **(Davis *et al.*, 2009)**.

Many of these peptides have been shown to target specific ion channels, including ligand-gated and voltage-gated channels **(Ekberg *et al.*, 2008)**. *Conus* venom has been tested in a number of different cell lines for cytotoxic activity **(Oroz-Parra *et al.*, 2016; Ganesan *et al.*, 2022)**. Conolysin-Mt, the first cytolytic peptide, was discovered in the venom of the cone snail **(Biggs *et al.*, 2007)**. As a result of conolysin's characterization, membrane perturbation can be added to the list of mechanisms previously associated with conopeptides. The present study revealed that there is a variation in the activity of the *Conus* species (*C. textile*) from different locations on cancer cells. In the present study, findings highlighted the notable cytotoxic efficacy of venom gland and venom tube extracts from *Conus textile* against three human cancer cell lines: Mcf7 (breast cancer), HepG2 (hepatocellular carcinoma), and Caco2 (colorectal cancer).

The extracts demonstrated significant potential in reducing cell viability, indicating the presence of potent bioactive compounds with anti-cancer properties. Interestingly, the study revealed a variation in the potency of the venom extracts, with the venom tube extract consistently showing greater efficacy compared to the venom gland extract across all cell lines. This suggested a differential composition of bioactive substances between the two types of venom extract, with the venom tube possibly containing a higher concentration of effective cytotoxic agents.

This work was in agreement with **Alburae and Mohammed (2020)**, who studied the anticancer effect of *Conus geographus* venom (collected from Rabigh, KSA) against the ovarian cancer (SKOV-3), breast cancer (Mcf7, ER-positive) (MDA-MB231), and liver cancer (HepG2) cell lines and showed cytotoxic effect at IC_{50} values of 22.7, 68.7, 47, and 19mg/ ml, respectively. A related study was done by **Kumari *et al.* (2021)**, who tested the cytotoxic effect of *Conus loroisiivenom* (collected from the Bay of Bengal, Thoothukudi, India) against human breast cancer cells (Mcf7) and they showed cytotoxic effect at IC_{50} 32mg/ ml.

The anticancer effect of *C. textile* venom (collected from Larak Island in Persian Gulf, Iran) was evaluated by **Salimi *et al.* (2021)**. This study was performed against human glioma cells (U87MG), and it demonstrated a cytotoxic effect at IC_{50} 10mg/ ml compared to normal human embryonic kidney 293 cells (HEK293).

The present work agreed with **Alburae and Mohammed (2020)**, which revealed that *C. geographus* extract exhibited a potent cytotoxic effect against HepG2 cells via a mechanism involving G0/G1 cell cycle arrest.

Thus, *C. geographus* is a potential source of a new anticancer agent. Additionally, the results exhibited promising apoptosis inducing activity of *C. flavidus* venom through up regulation of apoptosis-related genes of P53, Bax, Casp-8, and Casp-9 with caspase-3 activation in HepG2 cells. These results corroborated the findings of **Salimi *et al.* (2021)**, who illustrated the significant cytotoxic and selective activity of *Conus textile* crude venom on U87MG human glioma cells, with IC_{50} value of 10mg/ ml. Additionally, they aligned with **Magdy *et al.* (2022)**, who confirmed confirmed the cytotoxic activity of *C. flavidus* venom through apoptotic cell death in HepG2 cells.

Conus textile crude venom induced activation of caspase-3 and induction of cell apoptosis through a mitochondrial signaling pathway. Furthermore, these results agreed with **Alburae and Mohammed (2020)**, who confirmed that *C. geographus* venom showed apoptotic signaling that leads to caspase-3 activation through the intrinsic (mitochondrial) pathway. In the present study, *C. textile* venom extract exhibited a more cytotoxic effect on HepG2 with an IC_{50} value of 84.37µg/ ml and it was less toxic against Caco2 cells with an IC_{50} value of 318.97µg/ ml. Accordingly, for investigating the apoptotic activity in HepG2 cells, it up regulated the expressions of pro-apoptotic. These findings confirmed the cytotoxic activity of *C. textile* venom through apoptotic cell death in HepG2 cells.

Therefore, a detailed study for highlighting the proteomic characterization for this venom and studying the effective molecular target is highly recommended for developing new anticancer agents from natural sources. The IC_{50} values obtained provide a quantitative measure of this potency, offering a promising avenue for further exploration and potential therapeutic application. Overall, the study contributes valuable insights into the therapeutic potential of marine natural products, particularly highlighting *Conus textile* venom as a source of novel anti-cancer agents, warranting further research into its components and mechanisms of action.

CONCLUSION

The results of this investigation showed the remarkable cytotoxic activity of *Conus textile* venom gland and venom tube extracts against three human cancer cell lines: HepG2 (hepatocellular carcinoma), Caco2 (colorectal cancer), and Mcf7 (breast cancer). The extracts showed promise in lowering cell viability, suggesting the presence of strong bioactive substances with anti-tumor effects. It is interesting to note that the study found differences in the venom extracts' potencies, with the venom tube extract constantly demonstrating higher efficacy than the venom gland extract in all cell line. According to this, there may be a difference in the bioactive component contents of the two forms of venom, with the venom tube potentially having a larger concentration of potent cytotoxic chemicals. An avenue for further investigation and possible therapeutic application is presented by the obtained IC_{50} values, which offer a quantitative assessment of this potency. All things considered, the study offered insightful information on the medicinal potential of marine natural products. It specifically highlighted *Conus textile* venom as a source of novel anti-cancer compounds, indicating that more research into the components and methods of action of this venom is necessary.

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