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Evaluating Potential Anti-Cancer Therapeutics of the Marine Gastropod (*Conus virgo*) Venom Extracts on Human Cancer Cell Lines

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ABSTRACT

Marine snails of the genus *Conus* are known for their complex venom compositions, containing a rich diversity of pharmacologically active peptides, collectively termed conotoxins. These peptides have garnered significant interest for their potential therapeutic applications, particularly in the realm of oncology. This study investigated the cytotoxic effects of venom gland and venom tube extracts from *Conus virgo* on three human cancer cell lines: Mcf7 (breast cancer), HepG2 (hepatocellular carcinoma), and Caco2 (colorectal cancer). Employing an *in-vitro* assay, we determined the half-maximal inhibitory concentrations (IC_{50}) of the venoms, which reflected their potency in reducing cell viability. The results demonstrated that both of the venom extracts exerted significant cytotoxic effects across all tested cell lines, with IC_{50} values ranging from 173.89 to 481.27µg/ ml. The venom tube extract showed a consistently higher potency compared to the gland extract, suggesting a higher concentration of active cytotoxic compounds. The lowest IC_{50} value was observed in the Mcf7 cell line when treated with venom tube extract, indicating a promising potential for breast cancer therapeutics. These findings supported the hypothesis that *Conus virgo* venom contained bioactive components with selective toxicity toward cancer cells. The consistency and reliability of the cytotoxic effects were substantiated by the narrow standard deviations obtained. This study contributed to the exploration of marine natural products as a source for novel anti-cancer agents. Additionally, it set the ground work for future purification and mechanistic studies of conotoxins.

INTRODUCTION

Cone snails are part of the broad phylum of Mollusca and the very large superfamily of Conoidea, otherwise known as Toxoglossa (poison tongue). The several families that make up this group, the turrids (Turridae, ~3.0000 species), the auger snails (Terebridae, ~400 species), and the cone snails (Conidae, ~700 species), were divided into 13 new monophyletic families recently **(Bouchet *et al*., 2011; Kendel *et al*., 2013)**.

The genus *Conus* contains about 700 species of marine snails that are found in tropical and subtropical waters **(Catterall, 1992; Rockel *et al*., 1995; Lewis *et al*., 2012)**. The majority of cone snails are nocturnal feeders **(Kohn, 1959)**. They seek fish, worms, or mollusks more than other species. Cone snail species are classified as either piscivorous, vermivorous, or molluscivorous, while some of them can consume many types of prey **(Duda *et al*., 2001)**. In 1978, the first peptide to be extracted from Conus venom was described **(Cruz *et al*., 1978; Olivera *et al*., 1985)**. There has been a great deal of research focused on the analysis of specific components of cone snail venom. According to these initial studies,

Conus venom is a complex mixture of physiologically active substances, including a sizable number of neuroactive peptides known as conotoxins **(Kohn, 1956; Endean *et al.*, 1977)**.

Research on the compositional diversity of venom has direct applications in the fields of biology, biochemistry, pharmacology, envenomation management, and snail evolution. Venoms from cone snails are known to differ among species, within species, and over time within the same specimen (intra-specimen). Each cone species is genetically capable of producing more than 1000 different venom chemicals **(Biass *et al.*, 2009; Davis *et al.*, 2009)**, with the unique peptide count of each species being at least 100.

Based on the molecular targets, over 100 conotoxins that were extracted from venoms have been categorized into pharmacological families **(Terlau & Olivera, 2004; Chen *et al.*, 2008)**. There are more than 300 toxic species, forty of which are harmful and may poison humans **(Olivera *et al.*, 1990; Cruz & White 2017)**. Humans can die from the most deadly *Conus geographus* species **(Anderson & Bokar 2012)**.

Piscivores are considered more dangerous to humans than other cone snails **(Haddad Junior *et al.*, 2006)**. **Fegan and Andresen (1997**) subsequently recorded that several species in this genus, including *Conus geographus*, *C. textile*, and *C. marmoreus*, can kill and paralyze men. The poison of *C. geographus*, administered with a disposable hypodermic needle, has tragically killed a great number of gullible people.

Bingham *et al.* (2012) concluded that peptides of cone venoms are strong, extremely selective ion channel function blockers or modulators that are involved in some kinds of diseases. Cone snails are marine predators that use strong venom to kill their victims. A broad category of toxins with varying pharmacological and chemical properties, conotoxins are present in venom. It was a Dutch naturalist who first noticed the toxicity of conus snails, three centuries ago in the scientific literature **(Russell, 1965)**. Venom can be produced by a wide range of organisms, including bacteria and larger organisms. Venom has a wide range of biological functions, some of which are peptide-based and others not. It is used to either kill or digest prey or to protect against predators. Its composition allows for a variety of pharmacological applications **(Zufferey, 2007)**.

One of the major groups of these venomous or toxic animals, belonging to the Phylum Mollusca and class Gastropod, has an excellent venom pattern. Cone snail venom is a complicated mixture of 50– 200 individual peptides, each unique to its own species and ranging in length from 10 to 35 amino acids **(Olivera, 1999)**. These poisons function as a "cabal" or combined medication tactic by immobilizing the prey and then interfering with its neuromuscular transmission **(Olivera, 1997)**. A number of diseases, including chronic pain, epilepsy, heart disease, mental illness, mobility disorders, spasticity, cancer, and stroke, are being investigated in relation to conotoxin **(Olivera, 2002).**

Hence, the present study aimed to evaluate the antitumor potential of venom from *C. virgo* on different 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 Mvf7, HepG2 and caco2 chemotherapy.

MATERIALS AND METHODS

1. Site of collection and specimen collection

Conus virgo specimens were collected from various locations along the coast of the Gulf of Aqaba in the Red Sea. Snails tend to be found along the coast in relatively shallow waters (<10 meters) consisting of coral reef environment, rocky sand, and/or sea grass bed.

Diving in a shallow-water was conducted along the coast of the Gulf of Aqaba in the Red Sea coast of Egypt. Cone snails were obtained from sea grass, rock, coral, and sand. They were collected from the stirring rocks in the intertidal zone during low tide. The samples were preserved in ice and then transferred to the marine biology lab at the Department of Zoology at Al-Azhar University's Faculty of Science until the venom could be extracted.

2. Venom gland and venom tube extracts preparation

Isolation of the crude venom from *Conus virgo* was performed according to the manner of **McIntosh *et al*. (1995)**. After cracking the cone shell, pieces of the shell were removed. The inner body mass was left completely intact. This method destroyed the shell without causing damage to the internal venom gland and venom channel. Once the inner body was removed, the body was carefully dissected to reveal the venom gland and venom channel. The duct was then cut into small pieces of 10- 15 millimeters. They were suspended in 2% acetic acid and centrifuged (5000rpm for 10 minutes, 5°C) to extract the peptide components of the venom. The pellet was re-extracted three times using 2% acetic acid. The supernatant was dried and stored at -80°C before use.

3. Viability assay (MTT protocol)

3.1. Determination of sample cytotoxicity on cells

Inoculation with 1 × 105cells/ mL (100µL/ well) was carried out in the 96-well tissue culture plate, and it was incubated at 37°C for 24 hours to form a complete monolayer. The growth medium from 96-well microtiter plates was decanted well after the formation of homogeneous sheets of cells. The cell monolayer was washed twice with washing media. Double dilutions of the tested sample were made in RPMI medium with 2% serum (maintenance medium). Each extract concentration (0.1ml) was tested in different wells, and 3 wells were left as a control (which receives only maintenance medium). The plate was incubated at 37°C and then examined. Cells were examined for any physical signs of toxicity, e.g. partial or complete loss of monolayer, rounding, shrinking, or granulation of the cell. MTT solution (5mg/ mL in PBS, BIO BASIC CANADA INC) was prepared. 20µL of MTT solution was added to each well and placed on a shaking table at 150rpm for 5 minutes to ensure thorough mixing of the MTT into the media. The plate was then incubated at 37°C with 5% CO2 for 4 hours to allow MTT to be metabolized. Afterward, the media were discarded (note: the plate was dried on paper towels to remove residue if necessary). The formazan (MTT metabolic product) was resuspended in 200µL of DMSO. The plate was placed on a shaking table at 150rpm for 5 minutes to thoroughly mix the formazan with the solvent. The optical density was read at 560nm, and the background at 620nm was subtracted. The optical density was directly related to the amount of cells.

3.2. Morphological assay

Large-scale morphological changes that occured on the cell surface or in the cytoskeleton, was tracked and correlated with cell viability. Damage was determined by a significant reduction in volume secondary to the loss of protein and intracellular ions due to altered sodium or potassium permeability. Dead cells exhibited nuclear swelling, chromatin flocculation, loss of nuclear basophilia, while apoptotoc cells displayed cell shrinkage, nuclear condensation, and nuclear fragmentation.

RESULTS

1. Cytotoxicity of venom gland and venom tube extracts

Results of an *in vitro* cytotoxicity tests that measured the efficacy of venom gland and venom tube extracts from the marine snail *Conus virgo* on three different human cancer cell lines: Mcf7 breast cancer, HepG2 hepatocellular carcinoma, and Caco2 colorectal cancer are presented in Table (1) and illustrated in Figs. (1, 2). The assay aimed to determine the IC_{50} values of these two toxin extracts, indicating the effectiveness of the toxin as an anticancer agent.

The values of IC_{50} were calculated based on the dose-response relationship between venom extract concentrations (µg/ ml) and the percentage of cell survival measured by optical density (OD) at a given wavelength. The negative control (-ve control) represented the survival of the basal cell without toxin treatment, to which the effects of the toxin samples were compared. The cell viability ratio reflects the proportion of viable cells remaining after toxin treatment compared to the control, while the toxicity ratio represents the proportion of cells killed by the toxin. For example, 2.91% viability corresponds to 97.08% toxicity, indicating that almost all cells were non-viable at the highest concentration of toxin extract tested in Mcf7 cells.

The values of IC_{50} indicate that both the gland and tube venom of *C. virgo* have a strong cytotoxic effect on the cancer cell lines tested, with varying degrees of potency. For Mcf7 cells, the glandular venom showed an IC_{50} of 367.26µg/ ml, while the tubular venom was slightly more effective with an IC_{50} of 232.03µg/ ml. HepG2 had higher IC_{50} values for the gland venom (481.27 and 173.89µg/ ml, respectively) compared to the tube venom. Caco2 required a higher IC_{50} for both gland and tube venom (243.94 and 243.16µg/ ml, respectively).

The results of this assay are important since they contribute to the growing body of the evidence that sea snail venom contains a complex mixture of bioactive compounds with potential therapeutic applications, including cancer treatment. Differences in IC_{50} values across different cell lines emphasize the specificity and selective cytotoxicity that can be harnessed in targeted cancer therapies. Further purification and identification of active compounds within these toxins could lead to the development of new tumor drugs. It is also noteworthy that the standard deviations are relatively small, demonstrating the consistency and reliability of the results.

Conus virgo venom exhibited significant cytotoxic effects in laboratory models, warranting further research into its components and mechanisms of action as a potential source of novel anticancer agents.

Table 1. IC_{50} for *in-vitro* assay of gland and tube venom of *C. virgo*

Cell line	Sample	µg/ml	O.D	Viability %	Toxicity %	IC_{50}± SD
Mcf7	-ve control	-------	0.709 ± 0.002	100	0	
	Venom gland	1000	0.074 ± 0.005	10.48	89.51	367.26 ± 4.36
		500	0.187 ± 0.006	26.42	73.57	
		250	0.48 ± 0.006	67.79	32.2	
		125	0.706 ± 0.002	99.67	0.32	
		62.5	0.707 ± 0.001	99.71	0.28	
		31.25	0.709 ± 0.003	100	0	
	Venom tube	1000	0.02 ± 0.001	2.91	97.08	232.03 ± 2.23
		500	0.058 ± 0.002	8.18	91.81	
		250	0.306 ± 0.005	43.15	56.84	
		125	0.674 ± 0.003	95.15	4.84	
		62.5	0.707 ± 0.003	99.71	0.28	
		31.25	0.708 ± 0.002	99.95	0.04	
HepG2	-ve control	-------	0.661 ± 0.001	100	0	
	Venom gland	1000	0.058 ± 0.002	8.87	91.12	481.27 ± 5.79
		500	0.307 ± 0.004	46.44	53.55	
		250	0.637 ± 0.005	96.36	3.63	
		125	0.658 ± 0.004	99.59	0.4	
		62.5	0.66 ± 0	99.89	0.1	
		31.25	0.659 ± 0.002	99.79	0.2	
	Venom tube	1000	0.02 ± 0.001	3.12	96.87	173.89 ± 0.56
		500	0.059 ± 0.007	9.02	90.97	
		250	0.144 ± 0.007	21.78	78.21	
		125	0.422 ± 0.011	63.94	36.05	
		62.5	0.651 ± 0.004	98.58	1.41	
		31.25	0.658 ± 0.001	99.64	0.35	
Caco2	-ve control	-------	0.748 ± 0.002	100	0	
	Venom gland	1000	0.035 ± 0.001	4.67	95.32	243.94 ± 3.59
		500	0.112 ± 0.008	14.97	85.02	
		250	0.353 ± 0.007	47.28	52.71	
		125	0.688 ± 0.002	92.02	7.97	
		62.5	0.742 ± 0	99.28	0.71	
		31.25	0.747 ± 0.002	99.95	0.04	
	Venom tube	1000	0.023 ± 0.001	3.07	96.92	243.16 ± 3.31
		500	0.057 ± 0.004	7.7	92.29	
		250	0.354 ± 0.004	47.41	52.58	
		125	0.731 ± 0.005	97.72	2.27	
		62.5	0.745 ± 0.002	99.64	0.35	
		31.25	0.746 ± 0.001	99.82	0.17	

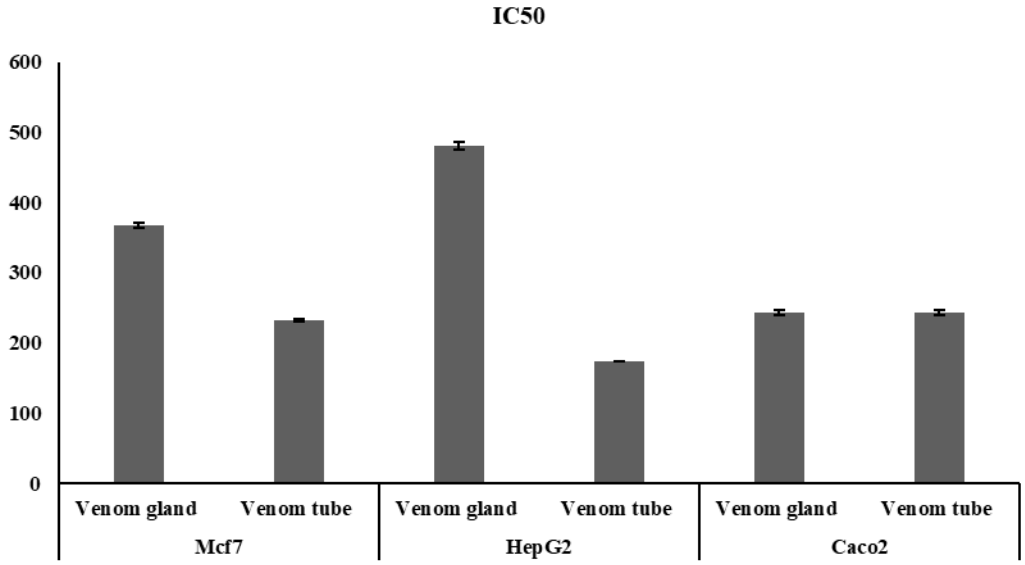

Fig. 1. IC_{50} for *in-vitro* assay of venom gland and venom tube extract of *C. virgo*

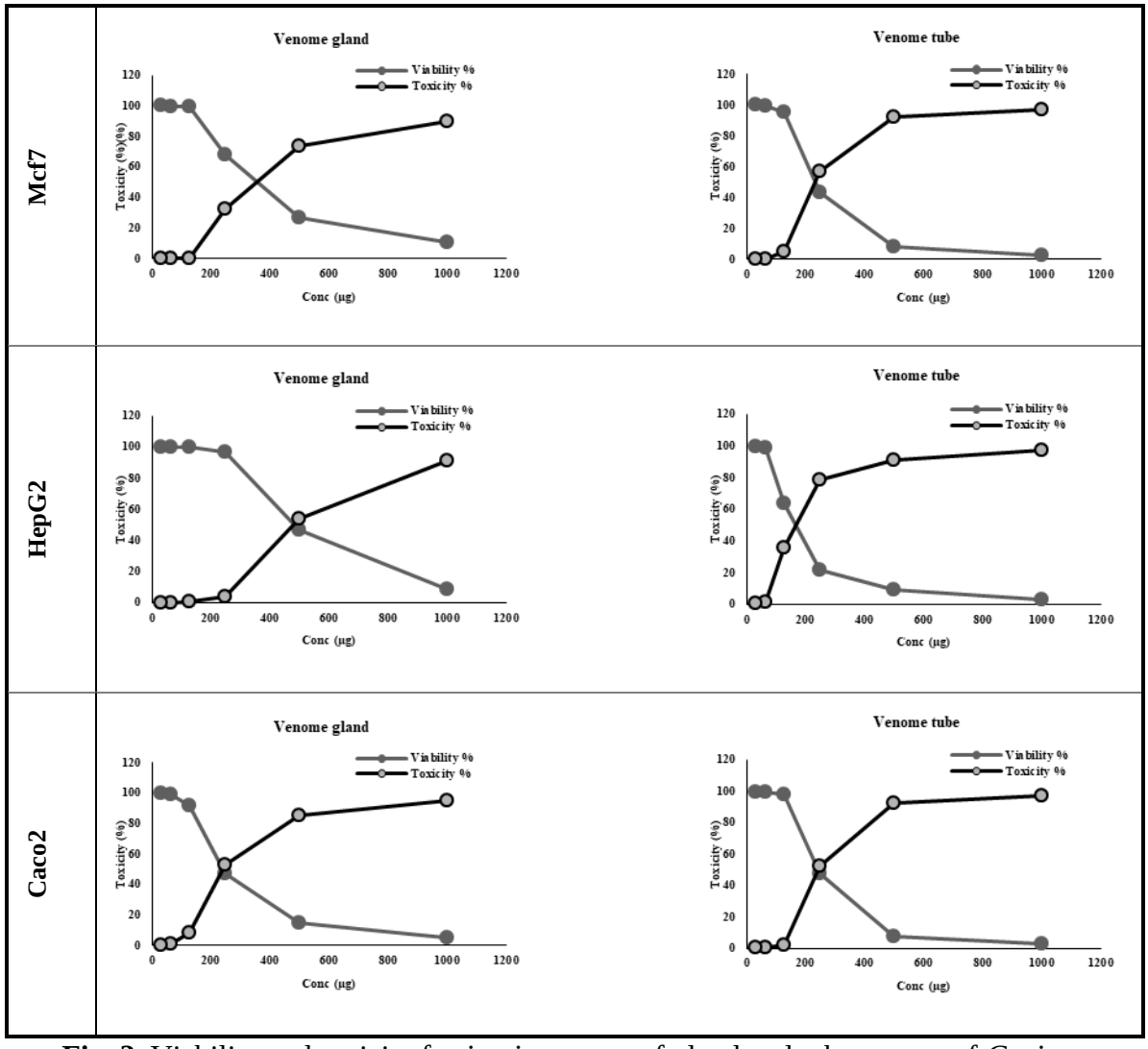

Fig. 2. Viability and toxicity for *in-vitro* assay of gland and tube venom of *C. virgo*

2. Morphological *in vitro* assay

The series of micrographs in Plates (I, II, III) provide a striking visual representation of the morphological changes induced in three human cancer cell lines - Mcf7, HepG2, and Caco2 - after treatment with *Conus virgo*'s gland and tube venom extracts. The control groups for each cell line exhibit typical morphology, with Mcf7 cells showing their characteristic cluster growth, HepG2 cells exhibiting a polygonal shape, and Caco2 cells maintaining their well-defined boundaries and uniform appearance.

By the exposure to toxic gland extracts, marked changes were observed. Mcf7 cells showed a significant decrease 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 decrease in confluence. In Caco2 cells, the effect of the gland toxin was manifested by cell shrinkage and the appearance of spaces between cells, indicating the loss of adhesion and apoptosis.

The effects of venom tube extracts were more pronounced. Mcf7 cells under this treatment showed extensive cell lysis and a drastic decrease in the number of viable cells, highlighting the strong cytotoxicity of the venom. HepG2 cells showed scattered distribution, loss of typical morphology, and clear signs of cellular disintegration. In Caco2 cells, the venom tube extract induceed severe morphological disturbances, with an almost complete loss of cellular structure, demonstrating high cytotoxic activity but at a higher concentration.

Overall, the micrographs in Plates (I, II, III) visually confirm the potent cytotoxic effects of *C. virgo* venom on these cancer cell lines. The characteristic morphological changes induced by the venom, especially from the venom tube, are consistent with quantitative cytotoxicity data, suggesting the presence of potent bioactive compounds with significant anticancer potential. This further supports the potential of bioactive compounds from marine sources in cancer research and treatment.

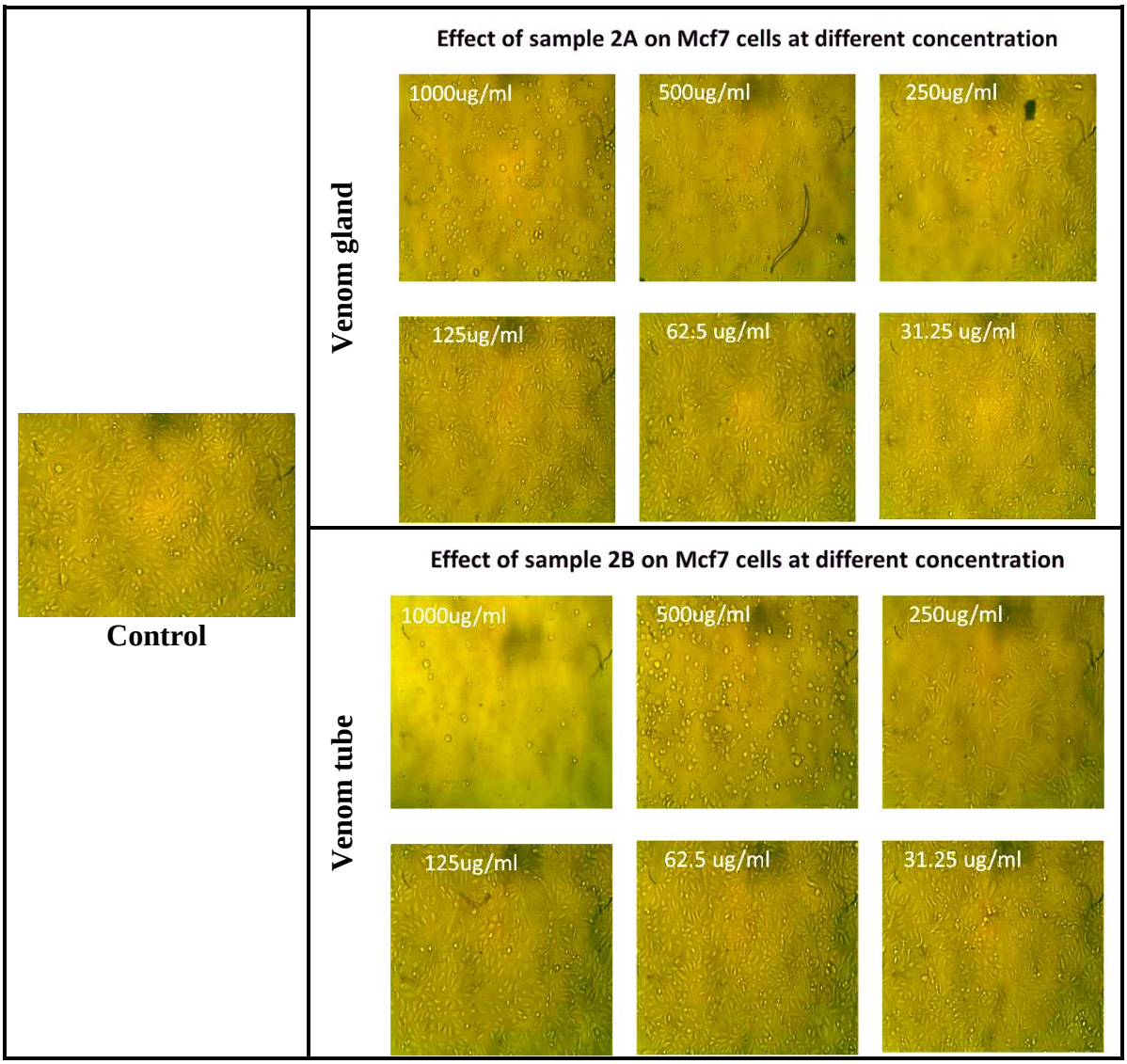

Plate I. Morphological *in-vitro* assay of venom gland and tube extracts of *C. virgo* on Mcf7 cancer cell line

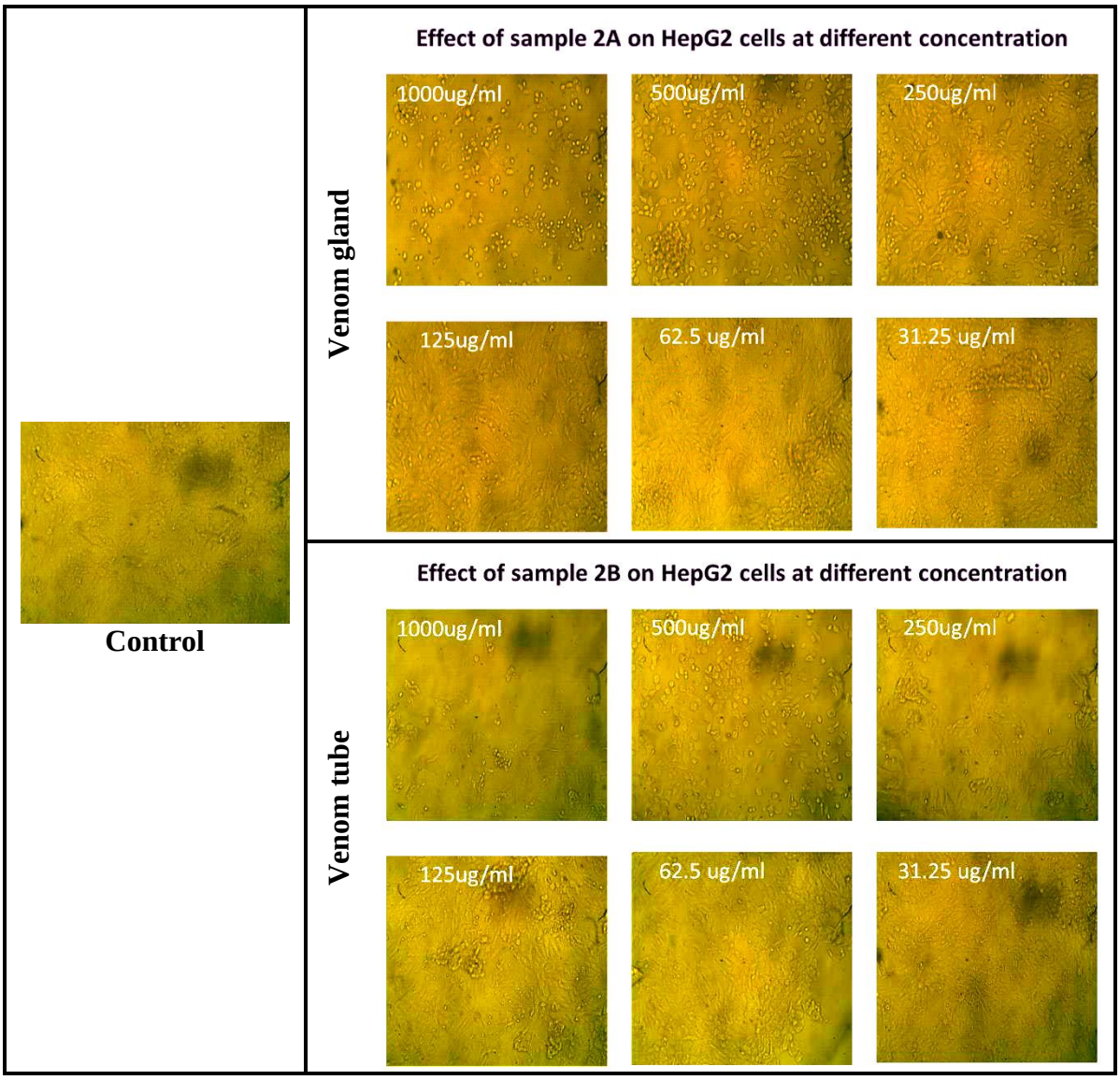

Plate II. Morphological *in-vitro* assay of venom gland and tube extracts of *C. virgo* on HepG2 cancer cell line

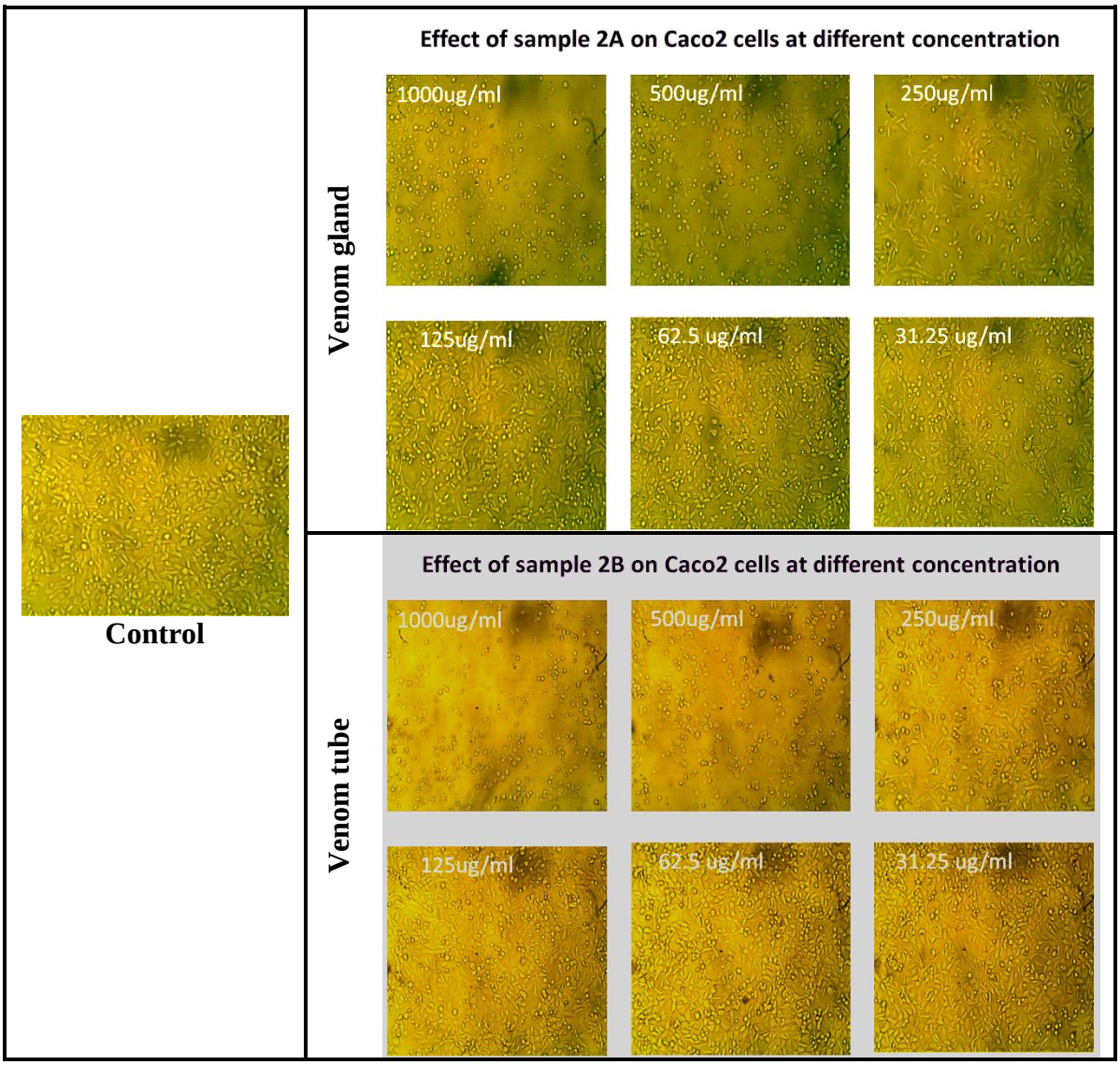

Plate III. Morphological *in-vitro* assay of venom gland and tube extracts of *C. virgo* on Caco2 cancer cell line

DISCUSSION

According to millions of years of evolution in venom, cono-peptides are an abundant source of ion channels that can be targeted by fishing strategies. These peptides bind to cell surface receptors with great affinity and selectivity, especially in neurons and other neurosecretory cells. Their high potency is used in pharmacology and neuroscience studies as a research tool. Some of them are at various stages of clinical studies for the treatment of human diseases **(Nelson, 2004)**.

Even at the highest dose (150µg/ ml), vero cells showed modest cell death or damage (80%) due to cytotoxicity. A key indicator of cytotoxicity is the loss of plasma membrane

asymmetry, an early stage of apoptosis that causes phosphatidylserine to move from the inner to the outer surface while maintaining membrane integrity **(Van Engeland *et al*., 1998)**.

To date, the cytotoxic activity of different *Conus* species' raw venom samples had been tested on different cell lines. All well-documented research had been done on conopeptides alone, and lower molecular weight conopeptides from the genus Conus have already been isolated or are currently being isolated. The larger molecular weight venom has been poorly researched, and the pharmacological properties are unknown but elucidated.

In the present work, the cytotoxic potential of *Conus* venom (gland and tube) has been studied *in vitro* against Mcf7, HepG2 and Caco2 MTT assay. It was found that, when cells incubated with *Conus* venom for 24h, it showed a significant dose dependent increase in cell death compared to the control cells. The half inhibitory concentration (IC_{50}) of venom gland and venom tube for Mcf7 were found to be at 367.26 and 232.03µg/ ml, for HepG2, it was found to be at 481.27 and 173.89µg/ ml, and for Caco2, it found to be at 243.9.27 and 243.1.89µg/ ml. These results are similar to the results found in other researches **(Jang *et al*., 2003; Ip *et al*., 2008, 2012; Park *et al*., 2009)**. The crude toxin of *Conus virgo* showed an IC_{50} value of 0.75µg/ ml on mouse glioma cells in 24 hours; the crude toxin extracted from *Conus amadis* showed an IC_{50} value of 25µg/ ml against HEp-2 cells in 48 hours, previous report stated that the venom extracted from *C. amadis* has a maximum effect at 100µg/ ml and the half inhibitory concentration (IC_{50}) was 25µg/ ml at a concentration of 45.6± 0.8 on HEp2 cell line **(Ramesh *et al*., 2014)**.

The antiproliferative properties of *C. virgo* crude venom were applied to A549 cells. The outcome unequivocally highlights a decline in cell viability at various concentrations (10-100µg/ ml). It was found that the IC_{50} was determined at 74.69µg/ ml. The impact of unrefined venom on cell structure was noted; as venom concentration increased, the cells' morphological integrity decreased. The induction of apoptotic cell death in A549 cells at 5 hours was demonstrated by the cyto-localization of nuclear morphology and DNA fragmentation test, which are in agreement with the results of **Ganesan *et al.* (2019)**.

Activity of conopeptides Cal14.1a (27µM) against lung cancer cell lines H1299, H1437, H1975 and H661 when evaluated using the MTT assay in all cell lines showed a decrease in cell viability after 24h, and when compared to untreated cells, it showed a survival rate of 30% (**Viswanathan *et al*., 2015)**.

The present results coincide with all these results, highlighting the observed cytotoxic efficacy of the venom gland and venom tube extracts of *Conus virgo* against three human cancer cell lines: Mcf7 (breast cancer), HepG2 (liver cancer), and Caco2 (colorectal cancer). The studied extracts showed significant potentials in reducing cell survival, suggesting the presence of potent bioactive compounds with anti-cancer properties. Interestingly, the study revealed a difference in the efficacy of the venom extracts, with the venom tube extract consistently showing greater efficacy compared to the gland extract across all cell lines.

CONCLUSION

In conclusion, the results of this study highlighted the observed cytotoxic efficacy of the venom gland and venom tube extracts of *Conus virgo* against three human cancer cell lines: Mcf7 (breast cancer), HepG2 (liver cancer), and Caco2 (colorectal cancer). The extracts showed significant potential in reducing cell survival, suggesting the presence of potent bioactive compounds with anti-cancer properties. Interestingly, the study revealed a difference in the efficacy of the venom extracts, with the venom tube extract consistently

showing greater efficacy compared to the gland extract across all cell lines. This suggests a difference in the composition of bioactive substances between the two types of venom, with the tube venom likely containing a higher concentration of potent cytotoxic agents. The IC_{50} values obtained provide a quantitative measure of this potency, providing a promising avenue for further exploration and potential therapeutic application. Overall, the study contributed valuable insights into the therapeutic potential of marine natural products, particularly highlighting *Conus virgo* venom as a source of novel anti-cancer agents, warranting further research into its components and mechanisms of action.

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