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Isoenzyme and Anticancer Screening of Conotoxin Proteins Extracted from Sea Snails

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Abstract

The present investigation has been reported with the cone snail *Conus inscriptus* venom proteins. MTT assay with normal and SK-BR-3 cancer cell lines evidenced anticancer properties in *Conus inscriptus* venom protein. Hemolytic, Phospholipase (isoenzyme) and hyaluronidase assays were also performed for biomedical applications.

Keywords

Conus inscriptus, Venom protein, anticancer, Phospholipase, Hyaluronidase

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1. INTRODUCTION

Marine Cone snails of genus *Conus* is a venomous carnivore. It belongs to the family of conidae. The *Conus* species carries multiplex arrays of conopeptide. Conopeptides are present in the conotoxins of conus venom (Stefan Becker & Heinrich Terlau., 2008). Conopeptides of the venomous creature presents an opportunity for bio-invention and drug design. Consequently, some studies reveal the importance of toxins and it is likely to be utilized as ascending compound for the generation of novel drugs (Bingmiao Gao et al., 2017). For investigation of conopeptides, the liquid chromatography-mass spectrometer coupled with MALDI-TOF mass analyzer arises to be most worthful analytical technique (Biass et al., 2009), which enhances quick identification, characterization, and screening of novel *Conus* marine drugs. The molecular weight of conopeptides generated can be estimated and tally to available sequences on databases, for example, ConoServer and UniProtKB (Richard et al., 2006). As the epidemic disorders are extending and cultivating resistance to available pharmaceuticals, the oceanic habitats gives a new origin for the establishment of lead drug for curing parasitic, fungal, viral and bacterial disease (Jakubowski et al., 2005) Purified conopeptides from marine origin have shown antioxidant potential and cytotoxic result on various AGS, SK-BR-3, and DLD-1 cancer cell lines (McManus et al., 1987). Anticancer drugs have been isolated from conus venom such as (ziconotide) from conus magus (Guadalupe and

Miroslava., 2012) As .broad sequences of proteins are reported in the toxin of various conus species , enzymatic activities are also reported in the conotoxins such as hyaluronidase and phospholipase activity Hyaluronidases are spreading element, which eases the tissue dispersal of venoms by abjecting hyaluronan and magnifies provincial intrinsic intoxication in the tissues of victim's The evolution of novel hyaluronidases and improvement of ability to examine their outcome can advance various therapeutic protocols, in broad because these enzymes are utilized for improving drug delivery techniques (Moller et.al., 2007). Venoms are a greatly complex concoction of several toxic constituents including phospholipases, Phospholipases hydrolyzes phospholipid bilayer. It plays an important part in the act of membrane fluidity, phospholipid turnover, maintenance, trafficking, apoptosis, and cell maturation. It has shown its application in drug delivery (Farooqui et al., 2006, Maria et al., 2007). Phospholipase activity has also been reported in marine cone snail conus toxins and its applications are observed in therapeutics (Mcintosh et.al., 1995 Marinetti.et al., 1965) Conopeptides shows strong ligand potential for a broad range of ion passages and receptors. This unique character makes conopeptides getting highly selective for pharmaceutical applications. The pharmacological potential of conotoxins, manifold peptides observed in the toxins of marine conus species cone snails, is well distinguished the therapeutically capability of the conotoxins accompanied with the welfare and efficiency reported in previous clinical protocols, guided studies on human therapeutic protocols (Cheung et al.,2015)

In the following, investigation we aimed to study. (i) Phospholipase, hemolytic and hyaluronidase activities were studied with *Conus inscriptus*. (ii) MTT assay with normal and SK-BR-3 cancer cell lines were performed to prove anticancerous properties.

In conclusion, several of the novel, bioactive conopeptide belonging to the Conus family is evaluated widely and only rare conopeptides have been examined yet. Therefore, the present investigation was done with the aim of screening *Conus inscriptus* venom for therapeutic application on life frightening diseases such as cancer.

2. MATERIALS AND METHODS

2.1 Isolation of Crude Extract

The collected venom ducts were crushed and mixed with distilled water. Crude venom extracts were obtained by dissecting the venom duct. The total protein mixtures were extracted into the homogenizing buffer solution. The homogenizing buffer solution contains 50 mM Tris hydrochloride, 5 mM potassium chloride, 120 mM sodium chloride, 2 mM calcium chloride, 1 m*M* magnesium chloride. Mortar and pestle were used for homogenizing. Fresh venom ducts were homogenized in the buffer. The mixtures were centrifuged for 10 min at 17,200 rpm/min at 4°C. The supernatant was collected as a crude extract, retained and stored at –20°C for further use (Saravanan et al., 2009)

2.2 Phospholipase activity

Assay of phospholipase was performed following the protocol from (Tan and Tan., 1988) with modifications from (Price, J.2007). The extract of (1mg/ml) of phosphatidylcholine dissolved in 5 % methanol from egg yolk was the substrate. Phospholipase assay enzyme from venom extract was diluted in chilled saline-HEPES buffer. Purified venom protein concentration was 800 ul/ml (Total protein contains 6% of phospholipase enzyme) Different aliquots of purified venom enzyme (50, 100, 200, 250, 300, 350µg/ml) were put in microtiter plate wells. Now, 200 ul of the substrate containing (3 mM TritonX-100, 2mM HEPES buffer, 8mM calcium chloride, and bromothymol blue indicator dye) was added to wells containing enzyme mixture. Buffer saline –HEPES was used as a control. Bromothymol blue indicator was prepared in water 120% (w/v) pH 7.5 at room temperature. Phospholipase activity was calculated depending on optical density at 425 nm taken after 10 minutes of incubation.

2.3. Hyaluronidase assay

Hyaluronidase activity was estimated by (Dorfman and Melvin., 1948) method with modifications from sigma Aldrich protocol (Enzymatic Assay of Hyaluronidase). Phosphate buffer, hyaluronic acid solution, enzyme diluents, acid albumin solution, an enzyme solution(venom) were prepared Phosphate buffer 200mM was prepared by using 5M sodium phosphate monobasic solution and pH 5 was adjusted by NaOH or HCL 1M each at 35°C. The hyaluronic acid (0.3mg/ml) solution was prepared in phosphate buffer utilizing hyaluronic acid and stirred heated to completely dissolve, after dissolving it was cooled at room temperature and pH 5 was adjusted by NaOH or HCl(1M) each. Now, enzyme dilutions were prepared using phosphate buffer (15mM) sodium chloride (75mM) bovine albumin serum (1mg/ml) neutral pH at room temperature was maintained Acid albumin solution was prepared using sodium acetate (15mM) acetic acid (70mM) bovine albumin serum (1mg/ml) and pH 3 at 20°C

maintained Enzyme solution (venom extract) different concentration(100, 125, 150, 250, 300, 350µg/ml) was prepared in cold enzyme diluents.(total protein contains 5.66 % of hyaluronidase) Now, 1ml from each concentration of enzyme solution was added to 1ml of the substrate that is hyaluronic acid. It was mixed and incubated for 45 minutes at 35°C After 45 minutes immediately 2 ml of acid albumin solution was added to each tube, mixed. After 10 min absorbance was read at 600nm against blank. Enzyme diluents served as blank (0.000). All chemicals were purchased from Merck. Wells with no substrate and the only enzyme was controlled.

2.4. Hemolytic activity

Hemolytic activity was carried out of crude toxins on chicken erythrocytes. The method followed was reported by (Malagoli., 2007). The hemolytic test of the crude venom extract was performed under in vitro conditions. EDTA 2.7% solution (2.7 g/100 mL) was taken as an anticoagulant. Red blood of chicken was taken from the slaughterhouse. It was washed thrice with phosphate buffer. Now, 1% erythrocyte suspension prepared was by computing 900 µl of normal saline to 100 µL of freshly taken red blood cell. Then, different. concentrations (50–250 µg/ml) of venom extracts were added to 0.85% NaCl solution and then 1% of blood suspension prepared was added. Two controls were prepared without extracts; negative control received sterile phosphate buffer saline, while positive control received 0.1% Triton X-100 The microtiter plate was lightly shaken and incubated for 1 h at 37°C. The results were observed after incubation at room temperature, by taking absorbance at 540 nm. (Zohra et al., 2014).

2.5. MTT assay for cell viability

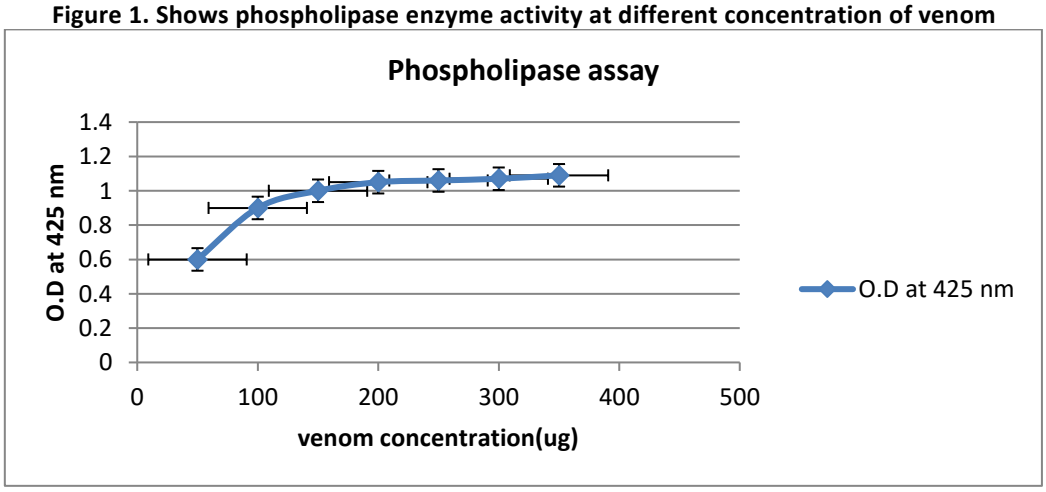
The cytotoxicity of conotoxin was observed by MTT assay (Senthilraja and Karthiresan., 2015) on Normal and SK-BR-3 cells. From NCCS, Pune, India Normal cell line and SK-BR-3 cell line were obtained. The cells were used for cytotoxicity after it reached 80% confluence. The cell sheet was not disturbed while removing the growth medium (MEM) minimum essential medium. Cells were washed for eliminating dead cell and extra fetal calf serum with MEM. The crude extracts were allayed in MEM. The control was diluted cells without the conotoxin. Different concentrations of venom were added into a microtiter well with cells. The microtiter plates were kept for incubation at 37 °C in 5% CO_2 environment. The conotoxin treated microtiter plates after incubation 48h was pulled for MTT assay. After washing each well with MEM for thrice 200 µl of MTT was added. The medium was washed out and 1 mL of DMSO was added. It was incubated for 50 seconds on a shaker to dilute the formation of crystals. The optical density of the suspension mixture was observed at 595 nm keeping DMSO as a blank. The graph was outlined by taking on x-axis conotoxin extract concentration was taken and on y-axis relative cell viability was taken.

3 RESULTS AND DISCUSSIONS

3.1. Phospholipase activity.

Phospholipase enzyme was reported earlier in *Conus.magus* (Marinetti et al., 1965). Here, from the venom of *C.inscriptus* phospholipase activity was performed. The result was shown in Figure 1. The absorbance was read after 10 minutes against blank. Optical density was observed. The assay was carried out in triplicates and the average of O.D values were calculated, and graph was plotted. And the drastic increase in observance was seen at 200 ug/ml and it was almost constant till 350 ug/ml.

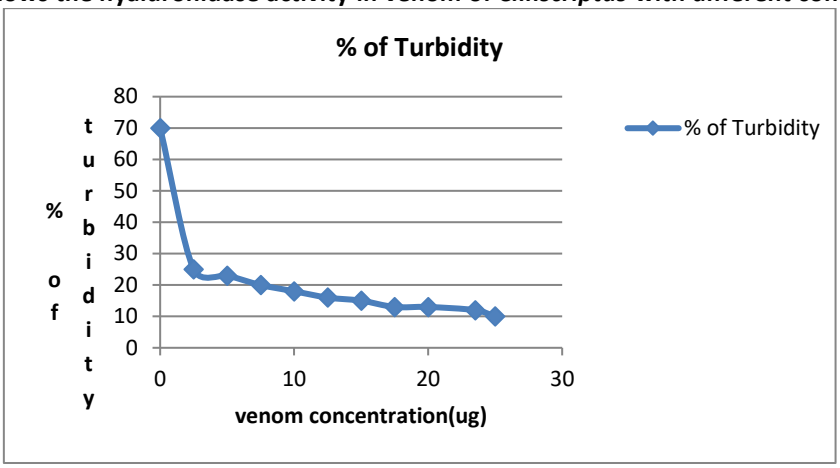
Figure 1. Shows phospholipase enzyme activity at different concentration of venom



3.2. Hyaluronidase activity

The estimation of hyaluronidase assay was turbidity dependent. Relation of turbidity was seen enzyme concentration based. The rate of reaction was also seen as changed absorbance at time intervals. Here, hyaluronidase activity was observed in the venom of *C.inscriptus*. The absorbance was taken in triplicates, mean was calculated, and the graph was plotted for standard analysis. At 5 ug/ml the turbidity was 23% calculated and as gradually concentration was increased upto 25ug/mg gradual decrease in the turbidity percentage was observed. As shown in Figure 2. *C.consors* at 0.11 ug 50% of hyaluronic acid was broken down (Violette et al., 2012). Not all conus species poses hyaluronidase activity because some earlier studies reveal that *C.victoriae* and *C.marmoreus* did not show any hyaluronidase compound. We are able to determine the presence of hyaluronidase in the venom extract of *Conus inscriptus*.

Figure 2. Shows the hyaluronidase activity in venom of *C.inscriptus* with different concentrations



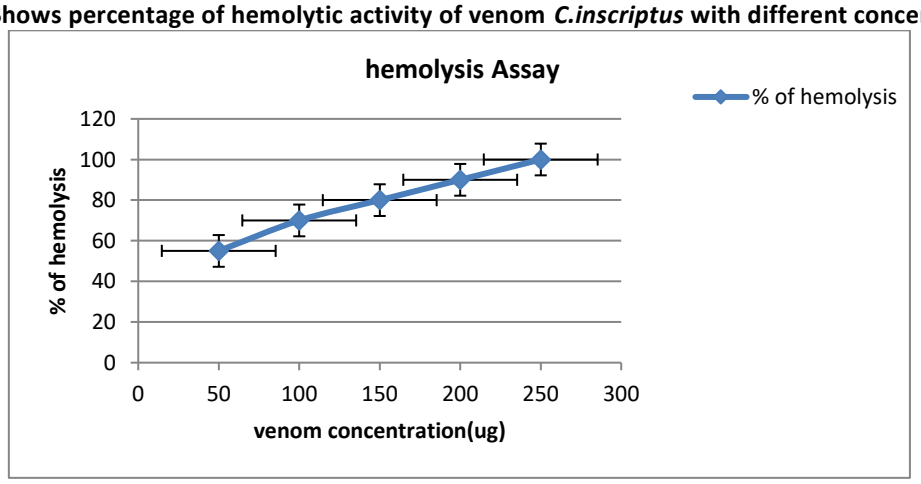
% of inhibition (turbidity)= [O.D of control-O. D of sample]/O. D of control] ×100

Note: concentration was made up to 1ml with enzyme diluents

3.3. Hemolytic activity

The uniform red color suspension in the wells considered as positive hemolysis and a button formation in the bottom of the wells were considered as lack of hemolysis. Hemolytic activity carried out on chicken blood showed that the crude toxin of *C.inscriptus* urged hemolysis of blood, At 150ug/ml 80% of hemolysis and at 50 ug/ml 55% hemolysis was observed as compared to *C.lentiginosus(*78.43 HU/mg*)* and *C.mutabilis(*18.42HU/mg*)* (Sakthivel et al., 1999) the venom of *C.inscriptus* showed higher hemolytic activity.. Similarly, *C.inscriptus* toxin extract observed greater cytolysis for chicken erythrocytes figure 3.

Figure 3. Shows percentage of hemolytic activity of venom *C.inscriptus* with different concentrations .

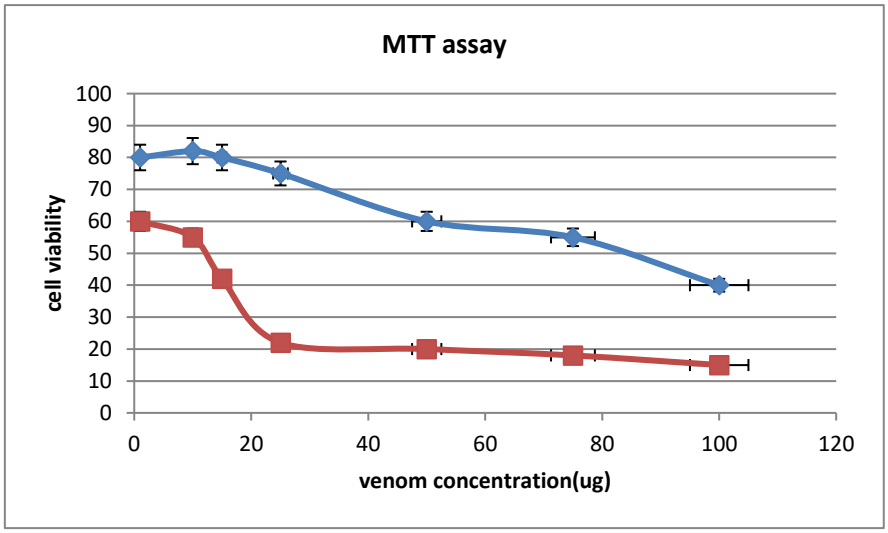


% of hemolysis= [Abs of the solution with sample-abs of control/Abs of negative control -abs of control] ×100 (Gould et al., 2000)

3.4. MTT assay for cell viability

It was found that when the concentrations of crude venom from *C.inscriptus* increased, the cell was losing its viability gradually. Lowest viability was observed at 100ug/ml. for normal (40%) and SK-BR-3 cancer cell lines (15%). Highest viability was observed at 10ug/ml normal (82%) and SK-BR-3 cancer cell lines (50%). And medium viability was observed at 25ug/ml normal (75%) and SK-BR-3 (22%) cancer cell lines. These observance shows doses depend on activity of venom against cancer cell lines. The least toxicity was seen at 1 µg/mL. In venom of *conus betulinus* highest toxicity was observed at 100ug/ml and lowest at 0.78 ug/ml against SK-BR-3 cancer cell lines (Sadhasivam et al., 2014) Maximum toxicity was also estimated at 200 µg/mL but, venom showed the least toxicity at maximum concentrations of 200 µg/mL when observed for normal cell lines. Similarly, in all the concentrations normal cell lines attended less toxicity as compared to cancer cell lines. Therefore, the extracted venom provides evidence for therapeutic application as an anticancer agent Figure 4.

Figure 4. Increasing concentrations of *C.inscriptus* venom were incubated with normal and SK-BR-3 cancer cells for 74 h at 35 °C. The estimated viability of cells by MTT was depicted as a percentage. Results were expressed in the mean±SE of experiments. (Blue indicates normal cell line, Red indicates SK-BR-3 cancer cell lines)



Cell viability % = (mean O.D/control O.D) ×100

4.CONCLUSION

The present effort was an approach to study the crude toxin from *C.inscriptus* . The eminent protein content estimated established that the *Conus inscriptus* venom contains tremendous toxicity as venom constitutes deadly proteins. Now, phospholipase enzyme also presents in the toxin mixture can assist in phospholipid bilayer degradation of the cells. It provides the route for several fatal particles into the predator's host. Thus, the conclusion is that the phospholipase and hyaluronidase can act vital part together in spreading of fatal components of venom. These enzymatic activities of Conus toxin can be considered for their anticancer efficiency, as venom constituents totally influence the living cells system. Therefore, the MTT assay concluded that *C. inscriptus* toxin observed anticancer potential screened against SK-BR-3 cells. The present investigation was approached in explore of distinguishing bioactive peptide from Conus venom for therapeutical value. Through the analysis of venom, we have recognized the presence of valuable therapeutic enzymes that can be useful in anticancer treatment and thrombolysis. Therefore, further research will flashlight on the development of new drugs from these peptides against life frightening diseases because conotoxins are found to have novel peptides for biomedical importance.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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