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Experimental Evaluation of Antitumor Effect of Russell's Viper Venom on Breast Cancer Cell Line - MDA-MB-231

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Sanchita Ahluwalia, Dr. Mrunal Ghag Sawant*, Nakul Shah, Prof. Abhay Chowdhary

Venomous Animal Unit, Haffkine Institute of Training, Research & Testing
Acharya Donde Marg, Near Wadia Hospital, Parel East, Mumbai, Maharashtra 400012

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ABSTRACT

Objective: The treatment of breast cancer needs to be changed from chemotherapy to biotherapy using biologicals, which have minimum or no adverse effects. dr-CT-1, a heat-stable protein present in *Daboia russelli* venom, has shown antineoplastic activity against HepG2. Some PLA2s isolated from Viperidae venoms are capable of inducing antitumoral activity. The objective of this study was to see the effect of Russell's Viper venom on inhibition of human breast cancer cells *in vitro*. Methods: Lyophilised snake venom was used to check its effect on breast cancer cell line MDA-MB-231. MTT assay was performed to calculate cytotoxicity and study the antiproliferative activity of the venom. Antioxidant potential was determined using TBARS assay. Immunocytochemistry was performed to understand the effect of venom on migration of cells. The protein fractions obtained using SDS-PAGE were referenced to identify the component of the venom which might act as the anticancer agent. Results: CC50 value obtained using MTT assay was found to be 0.82 µg/ml. 1 µg/ml of venom also showed effect against proliferation of the cells. With increasing concentration of venom, % TBARS also increased. 10 µg/ml of venom helped in inhibiting the tubulin fibers of the cells, thereby affecting the migration. 5 different protein bands were separated using SDS-PAGE. Conclusion: The results suggest that the snake venom might contain dr-CT-1 as well as PLA2 proteins which have shown their effect on proliferation and migration of tumour cells. Thus, the use of Russell's Viper venom may have antitumor effect against breast cancer.

INTRODUCTION

Alarming increase in breast cancer has become common in developing and developed countries and is a leading cause of death in women. The disease remains with poor diagnosis in spite of significant research in this area [1].

With over 1.1 million cases of breast cancer being diagnosed across the world each year, it is the leading cause of cancer death in females worldwide. This represents about 10% of all new cancer cases and 23% of all female cancers. There has been a tremendous change in knowledge of the genetic changes that contribute to breast cancer development & progression in the recent years [2].

How the cancer is diagnosed and the stage it is at decides the type of treatment or the combination of treatments. Breast cancer diagnosed at screening may be at an early stage, but breast cancer diagnosed when symptoms are visible may be at a later stage and require a different treatment.

The search for a cure for cancer has been vigorously pursued for over half a century, and the use of chemicals to treat cancer continues. The treatment of cancer needs to be changed from chemotherapy to biotherapy using biologicals, having minimum or no adverse effects. Monoclonal antibodies alone or coupled with ricin or other toxins and interferons are the only biologicals used until now for cancer therapy on a limited and experimental basis. Although, the fundamental understanding of cancer is progressing rapidly, there is still no academic breakthrough in therapy. There is a lot of scope for the discovery of naturally occurring biologicals that can be therapeutic and generally important to the science of oncology.

Snake Venom

Venom is the mixture of toxic as well as non toxic proteins [3]. Snake venom contains large number of biologically active proteins & peptides which are similar in structure but not identical to that of prey physiological systems. Whose effects include blood anti-coagulation, neurotoxicity, myotoxicity, nephrotoxicity, cardiotoxicity & necrotoxicity [4-9].

Snake venom has been used to develop newer drugs to combat various diseases including cancer. Calmette et al investigated the use of cobra venom in the treatment of cancer in mice. Match

showed that cobra venom, in extremely minute doses, produced analgesic effect, eg. commercially available toxins such as cobratoxin, cobrotoxin [10]. This had led to the possibility of therapeutic use of cobra venom in arthritis and cancer.

The objective of this study was to see the effect of Russell's Viper venom on inhibition of human breast cancer *in vitro* and to assess its anti-tumour activity, if any.

MATERIALS AND METHODS

Snake venom

Lyophilised Russell's Viper (*Daboia russelli*) venom was available at the Venomous Animal Unit, Department of Zoonosis, Haffkine Institute, Parel, Mumbai.

Cell culture and treatment

Human breast cancer cell lines (MDA-MB-231) were obtained from IIT, Bombay and were cultured in 4.5g/L of High Glucose Dulbeco's Modification of Eagle's Medium (DMEM) media supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. MDA-MB-231 cells were incubated overnight at 37°C in 5% CO_2 for cell adherence.

MTT Assay

A tetrazolium salt (MTT) has been used to develop a quantitative colorimetric assay for mammalian cell survival and proliferation. The cancer cells were seeded in 96-well plates at a density of 1×104 cells/well in 100 µl DMEM. After twenty-four hours of seeding, the medium was removed and then the cells were incubated for 24 hours with DMEM with the absence and/or the presence of various concentration of snake venom. Snake venom was added at various concentrations ranging from 1, 5, 10, 20, 25, 50 and 100 µg/ml. After incubation, 100 µl of MTT reagent was added into each well. These plates were incubated again for 4 hours in CO_2 incubator at 37°C. MTT reagent was decanted and 100 µl of DMSO was added as the stopping reagent. The plate was incubated again for 30 minutes in dark. The resulting MTT-products were determined by measuring the absorbance at 595 nm using ELISA reader. The cell viability was determined using the formula:

$$\text{Viability \%} = (\text{optical density of sample/optical density of control}) \times 100$$

CC50 values were calculated as the concentrations that show 50% inhibition of proliferation on any tested cell line.

Thiobarbituric Acid Reactive Species (TBARS) Assay

The TBARS Assay has provided relevant information concerning free radical activity in disease states and measurement of many compounds antioxidant characteristics. 250 µl of 1.22M orthophosphoric acid (O-H3PO3) and 250 µl TBA aqueous solution were added to 50 µl of sample solution and 450 µl distilled water. The mixture was then placed in a water bath at 95^0C for 60 min. Samples were cooled on ice followed by addition of 360 µl methanol and 40 µl of 1M sodium hydroxide (NaOH). Spectrophotometric Measurement: 150 µl of the MDA standards and samples were transferred to a 96 well microplate compatible with a spectrophotometric plate reader and absorbance was read at 532nm.

Immunocytochemistry

Immunocytochemistry is classically defined as a procedure to detect antigens in cellular contexts using antibodies. Medium were removed and washed with 1X PBS [with Ca^{++} and Mg^{++}] and then fixed with 4% PFA (Para formaldehyde) for 15 mins on ice. It was washed with PBST thrice (5 mins each) followed by wash two times with 1X PBS (with Ca^{++} and Mg^{++}) (5 mins each). Blocking solution was added for 1 hour at room temperature and then washed with PBST thrice (5 mins each). Later, 1X PBS [with Ca^{++} and Mg^{++}] was used for washing twice (5 mins each). Diluted Primary Antibody was used for incubation overnight. Washing was performed with PBST thrice (5 mins each) followed by twice with 1X PBS [with Ca^{++} and Mg^{++}] (5 mins each). Secondary Antibody and Phalloidin was used for incubation for 1 hour at room temperature. Washed with 1X PBS [with Ca^{++} and Mg^{++}] and imaging was done using immunofluoresecence microscope.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

Proteins are commonly separated by an electrophoresis technique that uses a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to denature the proteins.

Preparing the samples: 10 µl of sample and 10 µl of 4x loading buffer was combined in a microfuge tube and mixed well. The samples were heated for 10 minutes at 95°C.

Setting up, loading and running the gel: The gel was prepared and kept in the running buffer till further use. The full volume of samples (20 µl) was loaded in each subsequent lane. The run was made at 100 V. The plates were carefully separated and the gel was removed and kept into a staining dish of deionized water.

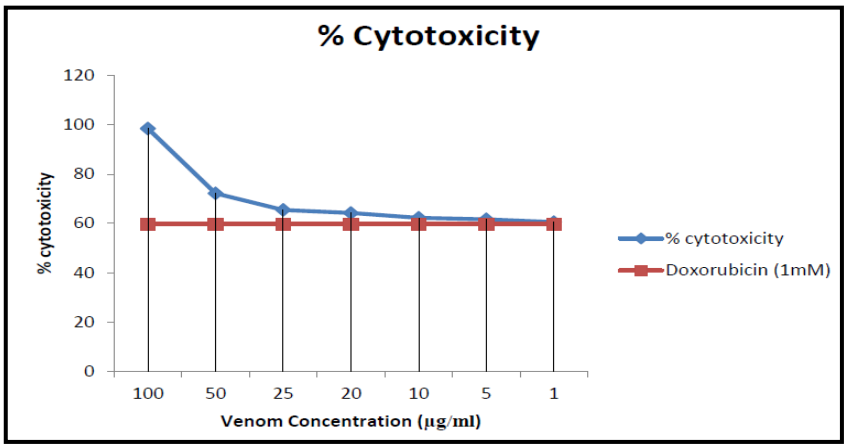
Coomasie brilliant blue staining: The gel was washed twice in fixative solution for 10 min. and then decanted. Staining of the gel was carried out in staining solution for 45 min. Staining solution was decanted and D/W wash was given. The gel was kept in destaining solution till the bands were visible.

RESULTS

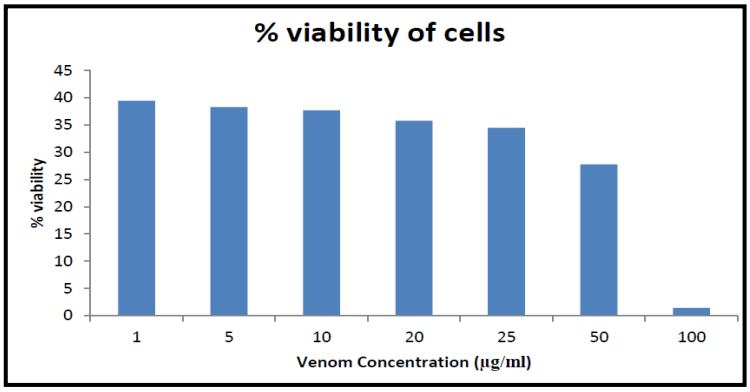
The purpose of present study was to check to the effect of lyophilised Russell's Viper venom against breast cancer using different assays which will determine the cytotoxicity, proliferation, migration and antioxidant potential of the venom.

MTT Assay

In vitro cytotoxicity and proliferation activity of venom was tested using MTT assay. The results showed that as the concentration of venom of increased, its cytotoxicity also increased. It also had an effect on the proliferation of breast cancer cells. With increasing concentration of venom, the proliferation of cells also decreased. CC50 value was found to be 0.82 µg/ml. Doxorubicin drug was used as a control wherein it was observed that the drug had similar effect on the cells. This shows that Russell's Viper venom might have cytotoxic and antiproliferative action against breast cancer cells.



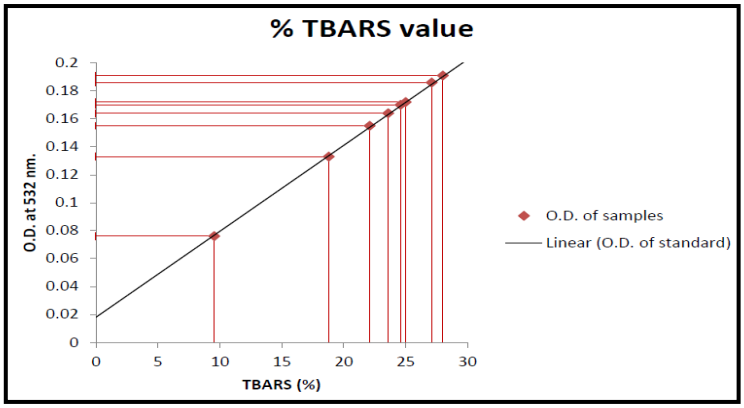
Graph 1: Percent cytotoxicity of venom



Graph 2: Anti-proliferative activity of venom

TBARS Assay

TBARS Assay was carried out to check the antioxidant potential of snake venom. Lipid oxidation has often been observed as an accompanying event of apoptosis [11, 12] and hence, it was another important parameter which was important to be studied to understand the effect of venom on cancerous cells. Lipid peroxidation refers to the oxidative degradation of lipids by stealing electrons from the lipids in cell membrane, resulting in cell damage. This may be the reason for the death of the cell. TBARS are formed as a byproduct of lipid peroxidation. Increased TBARS has its direct effect on Bax gene which accelerates programmed cell death by binding to Bcl2, by undergoing translocation of mitochondrial membrane and thus, release of cytochrome c triggering apoptosis. From the result which are seen in Graph 3, the antioxidant activity of the venom was seen to be increasing as the concentration of the venom increased, with 50 µg./ml. concentration of venom showing 25% TBARS concentration. Thus, venom contains components which help in increasing the percentage of TBARS in the cells, which might help in initiating apoptosis.



Graph 3: Anti-oxidant potential of venom

Immunocytochemistry

Tubulin, a 100 kDa αβ heterodimer, is structurally heterogeneous, with seven genes encoding α-tubulin and β-tubulin isotypes. The major differences between isotype classes reside in the last 15–20 amino acids of the carboxy-termini. Common agents currently used in treating metastatic breast cancer are the antimitotics paclitaxel and docetaxel. These drugs bind to β-tubulin, a major protein in mitotic spindles, and halt cell division at metaphase. Their effectiveness in cancer chemotherapy is thought to be due to their ability to reduce the dynamics of microtubules in mitotic spindles, thus preventing spindle assembly and interrupting the normal movement of sister chromatids toward the spindle poles [13]. Using immunocytochemistry, activity of venom on tubulin fibers was studied. The analysis has been that there is formation of more actin stress fiber due to the cytotoxic effect of venom in comparison of control condition. It could be seen that venom concentration of as low as 10 µg/ml had showed the effect on cancerous cell line and helped in reducing β-tubulin fibers, seen in Fig. 2. This clearly indicates that Russell's Viper venom might act as an anticancer agent and help in arresting the migration of cancer cells. 10 µg/ml of venom was used which showed the following effect on β-tubulin and actin fibres. β-tubulin fibers are seen in red and actin fibers are seen in green.

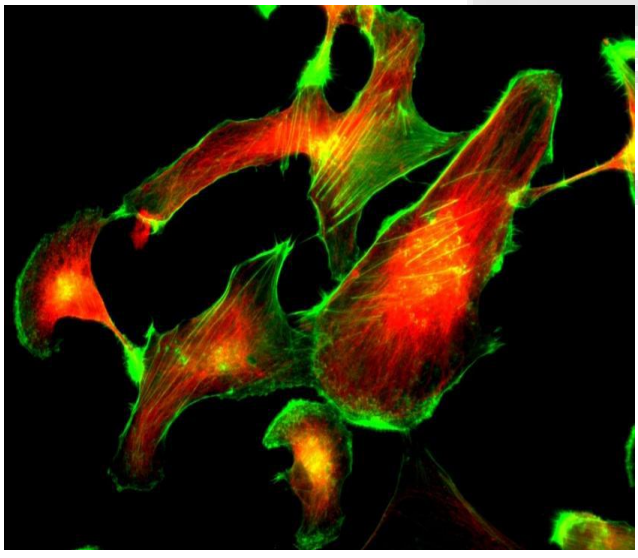


Fig.1: MDA-MB-231 cells Control-40X

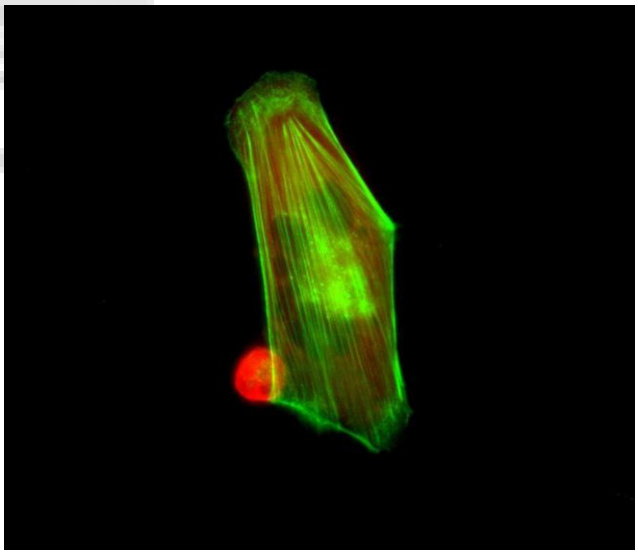


Fig. 2: MDA-MB-231 cells (cells + venom)-40X

SDS-PAGE

Protein separation was necessary to identify the component giving the anticancer activity to the venom. On carrying out SDS-PAGE of the lyophilised Viper venom, 5 different protein bands were separated as seen in Fig. 3. On comparison with the marker, it can be said that there might be presence of drCT-1 (7.2 KDa.) as well as PLA2 (14 KDa.). The ubiquitous nature of PLA2 highlights the important role they play in biological processes as cell signalling and cell growth including generation of pro-inflammatory lipid mediators such as prostaglandin and leukotrines, regulation of lipid mediators [14]. This indirectly can also be linked to antioxidant components, TBARS, present in the venom. These TBARS can also lead to cell death. At the same time, anti-neoplastic activity of venom can be due to presence of drCT-1 component of Russell's Viper venom which can be attributed to the fact that its first 20 N-terminal amino acids sequence showed homology to cytotoxins isolated from *Naja naja* venom.

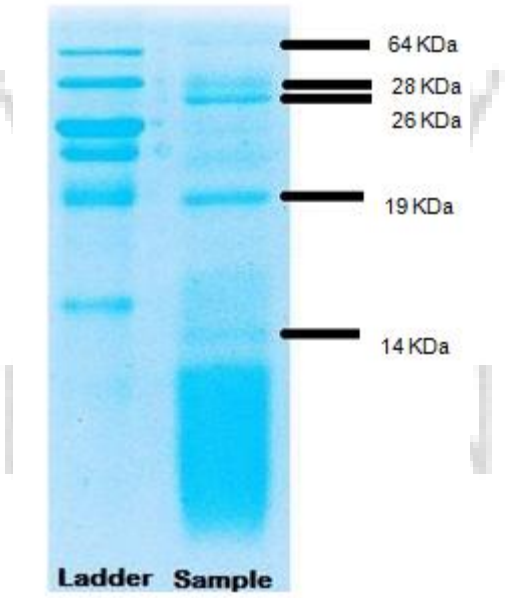


Fig. 3: SDS PAGE Results

CONCLUSION

The objective of this project was to see the effect of Russell's Viper snake venom on inhibition of human breast cancer *in vitro*. Lyophilised snake venom was used to check its effect on breast cancer cell line MDA-MB-231. First cytotoxicity and antiproliferative activity of the venom on breast cancer cells were estimated through MTT assay. To further determine its effect on

cancerous cells, antioxidant activity was carried out of the venom samples using TBARS assay. This was followed by immunocytochemistry to see the effect of venom on migration of breast cancer cells. Lastly, separation of venom was carried out using SDS-PAGE to find the presence of different proteins in the venom.

Cell cytotoxicity assay (MTT) was carried out to determine CC50 (cytotoxic dose) of the venom samples. The assay showed that the 50 percent cytotoxic dose of venom was as low as 0.82 µg/ml. Thus, less than 1 µg/ml of venom is showing cytotoxic effect on the cells. With increasing concentration of venom, the proliferation of cells also decreased, which might help us in saying that Russell's Viper venom has antiproliferative activity against breast cancer.

TBARS Assay was carried out to check the antioxidant potential of snake venom. Antioxidant activity was expressed in terms of the TBARS (%). As the concentration of venom increased, the presence of TBARS also increased, thereby, increasing its antioxidant potential and its activity to kill cancer cells.

Using immunocytochemistry, activity of venom on tubulin fibers was studied. It could be seen that venom concentration of as low as 10 µg/ml had showed the effect on cancerous cell line by increasing the actin fiber content and it might act as an anticancer agent. SDS-PAGE analysis showed the presence of 5 different protein bands in the venom which might include drCT-1 and PLA2 fraction, providing the venom with its anticancer activity.

This helps us conclude that Russell's Viper venom might have an effect against breast cancer, by acting as an antiproliferative and antimigratory agent.

Future prospects of this study include a number of steps to ascertain the findings obtained. Firstly, the individual components of the venom can separated and purified to check their effect on the cell line. At the same time, the cytotoxic effect of these components should be tested on normal breast cell line to take it one step ahead. Also, the venom can be tested as a whole along with its individual components *in vivo*. This will help us to establish a clearer picture on the effect of Russell's Viper venom against breast cancer.

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