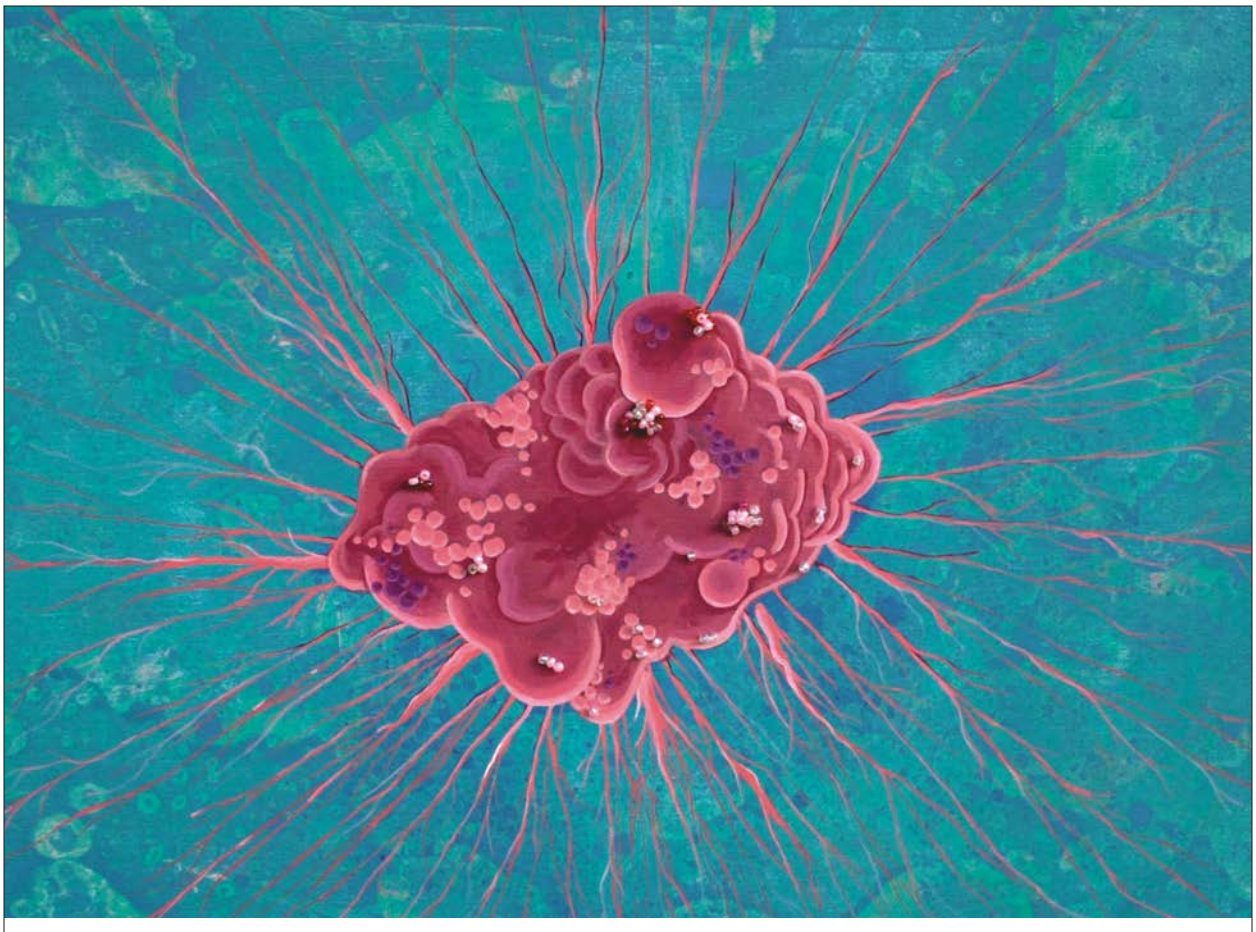

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Research Article
In vitro Biochemical Characterization and Anti-cancerous Property of Common Krait Snake Venom

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Abstract

Background and Objective: The resistance to various chemotherapy, radiotherapy by cancerous cells has made its treatment more complicated and has opened wide doors in research for biological components which might be more efficient, stable and target oriented in destroying cancer cells. Snake venom are considered as a cocktail of several biological components and research is been and being going on in identifying these components which would modify its molecular targets, thus, acting as a good therapeutic medicine. Many research papers have shown that cobra venom belonging to elapidae family has the ability to reduce nucleic acid levels in cancerous cells. The main objective of this paper is to carryout biochemical characterization of Common Krait (*Bungarus caeruleus*) venom and to check whether Common Krait (*Bungarus caeruleus*) belonging to same Elapidae family has the ability to act against cancer. **Materials and Methods:** The biochemical characterization was done by using SDS PAGE, HPLC, plasma clotting, anti-bacterial sensitivity, MTT and protein estimation by Bradford's methods. The anti-cancerous property of venom was studied on MCF 7 breast cancer cell line and its effect was studied by carrying out different assays like comet assay, cell migration assay and nanodrop spectrophotometer for measuring the effect of venom on nucleic acids of cancer cells. **Results:** Lysophospholipase enzyme was hypothesized to be one of the major protein in venom and it was observed that 1 μg mL^{-1} concentration of venom was highly toxic to cancerous cells which was mainly targeting nucleic acids. **Conclusion:** From all the assays applied for determining biochemical characterization and anti-cancerous property it was hypothesized that lysophospholipase might be one of the major protein component which might be acting as anti-bacterial or as a pro-coagulant. From all the assays used for determining anti-cancerous property it was found that venom at a concentration of 1 μg mL^{-1} is toxic to cancerous cells and least toxic to normal cells. Based on further research using various bio-informatics application these identified proteins can be designed *in vitro* and by using gene cloning recombinant DNA technology, the gene coding this protein can be transfected in cell lines which would produce recombinant protein that can be used as an anti-cancerous drug and hence there would be no need of venom extraction for production of drug.

Key words: *Bungarus caeruleus*, blood co-agulation, HPLC, lysophospholipase, nanodrop spectrophotometer

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cancer is one of the leading causes of death worldwide and because of its resistance to various treatment modalities like radiation therapy, surgery, chemotherapy, immunotherapy and hormonal therapy its treatment has become more complicated and there is an urgent need to find better treatment. Also, one of the major problems with successful cancer therapy using chemotherapeutics is that patients often do not respond or eventually develop resistance after initial treatment. This has led to the increased use of anticancer drugs developed from natural resources. The biodiversity of venoms and toxins makes them a unique source from which novel biotherapeutics may be developed. The anticancer effect of cytokine 1 from *Naja atra cantor* venom was studied and it was found that it can activate both necrotic and apoptotic cell death of cancer cells. The cobra venom has shown potential of reducing the nucleic acid concentration in breast cancer tissue cells[1,2]. Biochemical characterizations of different snakes like Indian cobra, Russell's viper is well studied[3,4]. But biochemical characteristics of Common Krait are not much known. The main objective of this project is to study the biochemical characteristics and anti-cancerous property of common Krait snake venom on cancerous cell line.

MATERIALS AND METHODS

Common Krait crude venom is taken from the Haffkine Institute. Snake venom was stored in venom vials which are freeze-dried and kept in the dark at a low temperature (either-20°C) in a well-sealed flask, precisely identified with a number, up to the time of use.

Biochemical characterization: Bradford's method was used for estimating protein concentration of venom. Stock of Bovine Serum Albumin (BSA) 5 μg mL^{-1}, Bradford's reagent (Coomassie Brilliant Blue G-250, 85% Ethanol, 95% orthophosphoric acid), 0.9% NaCl, 96 well Microtiter plate, Micropipettes, micro-centrifuge tubes, Multimode reader (Synergy HT Biotek Company with Gen 5 software) were the requirements.

The HPLC was carried out of the crude venom and the presence of protein components was confirmed by carrying out SDS PAGE.

The SDS-PAGE was performed of the 10-fold diluted crude venom sample of common Krait according to the method of Laemmli[5]. The gel was stained by using silver staining. Molecular weights of the bands were matched with standard ladder. About 30% acrylamide stock (Acrylamide-bisacrylamide), stacking buffer (p^h-6.8), resolving buffer (p^h-8.8), SDS solution (10%), APS (10%), Laemmli buffer, sample loading buffer (4x), staining solution-silver staining were the requirements.

Kirby-Bauer antibiotic testing (KB testing) method was used for carrying out AST (Anti-microbial Sensitivity Testing). One gram positive micro-organism-*Staphylococcus aureus*, 3 g negative micro-organism-*Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were used. Muller Hinton Agar, Petri plates, cork borer, positive control-amikacin, negative control-saline, crude venom were the requirements.

Pro-coagulant assay was done to determine the pro-coagulant property of Krait venom. Plasma was treated with crude venom and the calcium chloride was added to break the bonds and the micro-centrifuge tube was tilted at 45° angle. The clotting time was recorded by using stop watch.

The MTT assay was carried out for determining the cytotoxicity and proliferation activity of common krait venom. 96 well plate (flat bottom), normal (LLCMK2) and cancerous cell line (MCF7), MTT powder, DMEM medium, ELISA reader and Snake venom were the requirements. It's a colorimetric assay which measures the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to insoluble dark colored formazan product. The cells are then solubilized with an organic solvent DMSO, solubilized formazan reagent is measured spectrophotometrically at 550 nm. Since reduction of MTT can occur only in metabolically active cells the level of activity is a measure of viability and proliferation of cells.

Anti-cancerous properties of venom: Cell migration assay was done using Scratching method[6]. Cancerous Cell line (MCF7), Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum, PBS, BD Falcon 24-well tissue culture plate, Pipette tips 1 mL, Cell culture incubator 37°C and 5% CO_2 were the requirements. Doxorubicin drug was used as positive control, 1:100 and 1:1000 dilutions of crude venom were used as sample.

Nucleic acid concentration determination method, venom has the property of reducing the RNA content by 25% and DNA content by 95% of the cancerous cells. Based on this concept the concentration of the DNA of crude venom treated and untreated cancerous cell line was determined using nano drop spectrophotometer[7], which measures the DNA content at nano level. Then AGE was runned of these samples and bands were observed under UV transilluminator.

The DNA damage was studied using Comet assay. It is also known as Single cell gel electrophoresis[8] used to detect DNA damage at the level of individual eukaryotic cell. It measures DNA strand breaks. Clean slides, micropipettes, slide Tray, electrophoretic setup, heating block, reagents-PBS, lysing solution, electrophoresis buffer, neutralization buffer, staining solution, 0.5% low melting point agarose, 1% normal melting agarose, crude venom were the requirements.

RESULTS

Biochemical characterization

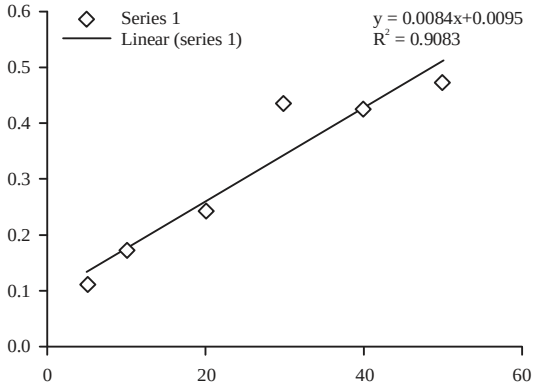
Protein estimation: The protein concentration of venom was determined by using Bradford's method. Knowing these proteins are important as, it can be used in improving therapies against snake bites and also to find the role of different proteins in different medical treatments. Protein concentration was estimated by using Bradford's method and it was found to be 5.25 µg/100 µL (Table 1, Graph 1, Fig. 1) .

HPLC: The HPLC was carried out of crude venom sample and only one major protein component was determined at a retention time of 3.95 min (Fig. 1).

SDS PAGE: The result of HPLC was confirmed by carrying out SDS PAGE. Three bands were prominently visible (Fig. 2) which were estimated to be Lysophospholipase (13-26.5 KD), Acetyl cholinesterase (100-113 KD), Phospholipase A2 (~13 KD)[9-11]. These proteins might be playing a role in cancer treatment or in plasma clotting or might be effective against bacteria.

Table 1: Bradford's method for protein analysis

Concentrations	Absorbance at 595 nm
0	0.002
5	0.123
10	0.198
20	0.305
30	0.440
40	0.456
50	0.466
Venom	0.137



Graph 1: Concentration Vs O.D at 595 nm

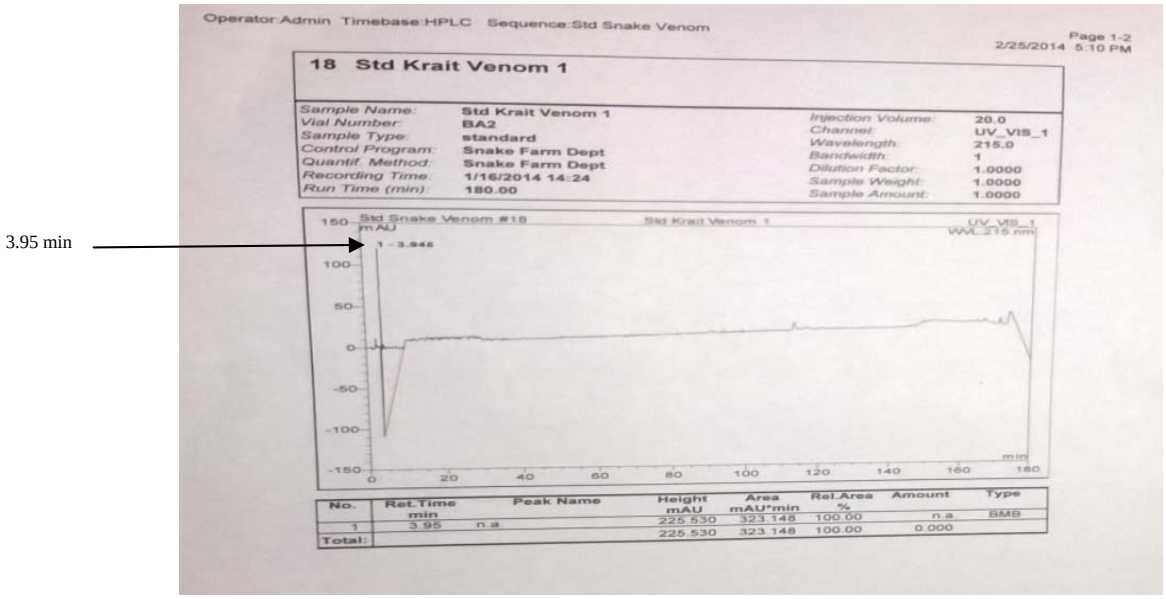


Fig. 1: HPLC analysis of common Krait (Bungarus caeruleus) crude venom

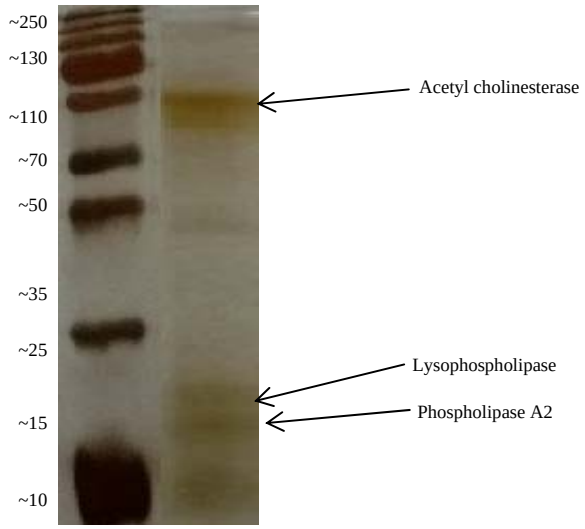


Fig. 2: SDS PAGE analysis of crude venom

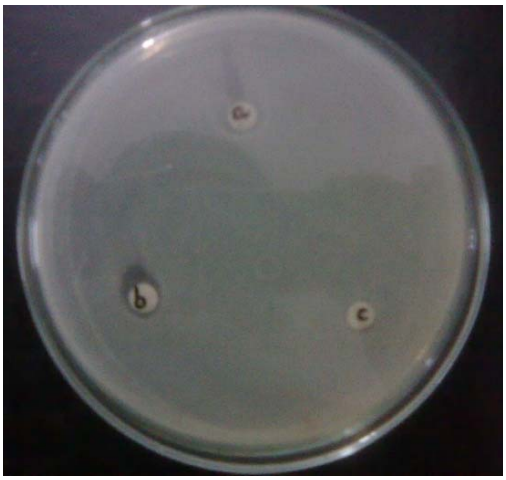


Fig. 3: *Klebsiella pneumonia* (AST assay)

Fig. 4: *Staphylococcus aureus* (AST assay)

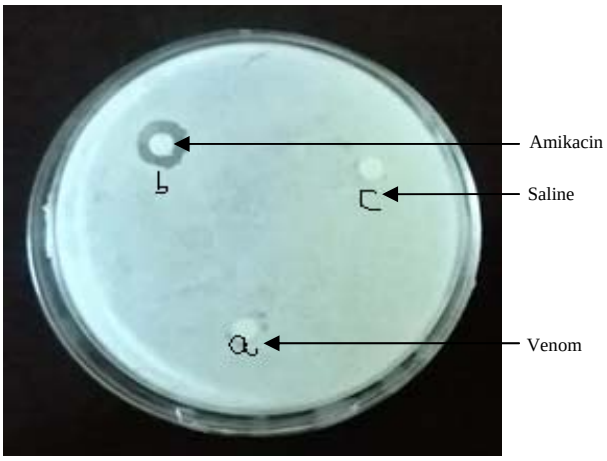


Fig. 5: *Escherichia coli* (AST assay)

Kirby-bauer antibiotic sensitivity testing: Kirby bauer disc diffusion method was used for carrying out antibacterial sensitivity testing of Common krait venom[12,13]. The results obtained showed that:

- Crude venom
- Amikacin antibiotic positive control
- Saline negative control

Figure 3 shows Gram-negative *Klebsiella pneumonia* was sensitive to venom, the zone of clearance of venom sample and control antibiotic amikacin were found to be 9 mm which indicates that both venom and antibiotic have same effect against *K. pneumonia*.

Figure 4 shows Gram-positive *Staphylococcus aureus* was sensitive to venom, the zone of clearance of venom sample is 11 mm and control antibiotic amikacin is 10 mm it indicates that compared to antibiotic, snake crude venom is more effective against *S. aureus*.

Figure 5 shows Gram-negative *Escherichia coli* was resistant to venom, the zone of clearance of 13 mm was seen in *E. coli* plate which indicates its sensitivity towards amikacin and no clearance zone of crude venom was seen on MH plate of *Escherichia coli*.

Figure 6 shows Gram-negative pseudomonas was resistant to venom and amikacin, as no clearance zone of crude venom and amikacin was seen on Pseudomonas MH agar plate.

These AST results indicated that compared to Gram-negative bacteria (*K. pneumonia*), krait venom is more effective against gram positive bacteria (*S. aureus*).

Fig. 6: *Pseudomonas aeruginosa* (AST assay)

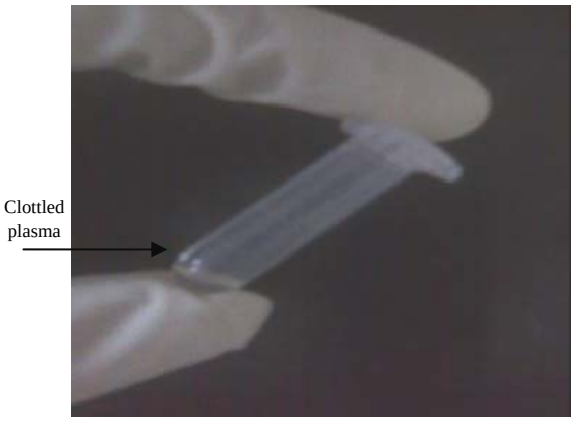


Fig. 7: Pro-coagulant assay results

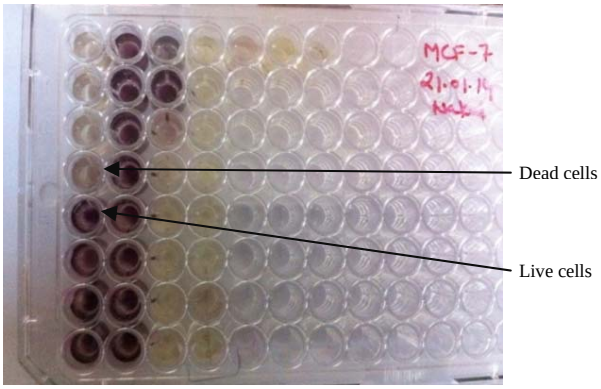


Fig. 8: MTT results analysis of venom on MCF 7 cell line

Pro-coagulant assay: Pro-coagulant assay was carried out and re-calcification time was determined of Krait venom and the results showed that clotting time of normal Human blood plasma was found to be 58 sec and the clotting time of venom treated human blood plasma was found to be 15 sec (Fig. 7). This indicates that there must be some protease enzyme in krait venom which is showing pro-coagulant activity[14].

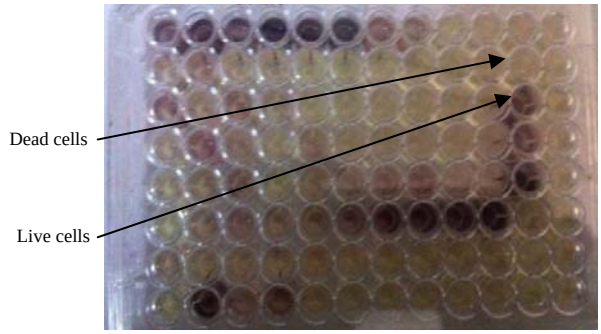


Fig. 9: MTT results analysis of venom on LLCMK 2 cell line

Table 2: MTT assay observation on MCF 7 cell line

Concentration of venom (μg mL^{-1})	O.D. at 550 nm	Cytotoxicity (%)
4	0.211	96.781
3	0.181	82.988
2	0.129	59.080
1	0.094	42.988
0.5	0.079	36.091
0.25	0.058	26.436
0.125	0.031	14.022
0.0625	0.002	0.689

Table 3: MTT assay observations of LLC MK2 cell line

Concentration of venom g mL^{-1}	O.D. at 550 nm	Cytotoxicity (%)
2.0	0.124	57.57
1.5	0.096	44.62
1.0	0.053	24.73
0.5	0.034	15.95

MTT: The MTT assay was carried out for determining the cytotoxicity and proliferation activity of common krait venom and it was found that with the decreasing concentration of venom the toxicity of venom on cells is also decreasing.

Figure 8 and 9 show yellow tetrazole color reduced to purple formazan in live cells and no color change in dead cells at a concentration of 1 μg mL^{-1} 42.98% of MCF 7 cancer cells were damaged and at a concentration 0.062 μg mL^{-1} very few cells are damaged. This determines that at low concentrations Krait venom is not toxic to MCF 7 cancer cells (Table 2, Fig. 8).

When same concentration of 1 μg mL^{-1} was used for studying cytotoxicity of normal cell line LLC MK2 very few cells (24.73%) were damaged (Table 3, Fig. 9).

Anti-cancerous properties of venom

Scratching method (cell migration assay): Cell migration assay was carried out using Scratching method[13] and following results were observed:

Figure 10 shows day 0 of MCF 7 cell monolayer treated with 1:100 venom dilutions, where scratch is created across the cells.

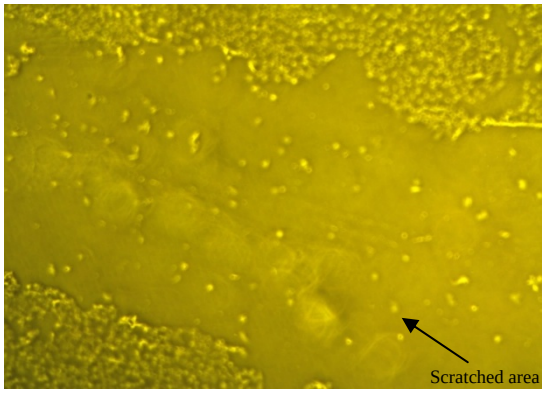


Fig. 10: Day 0 MCF 7 cell monolayer venom treated (1:100) dilution

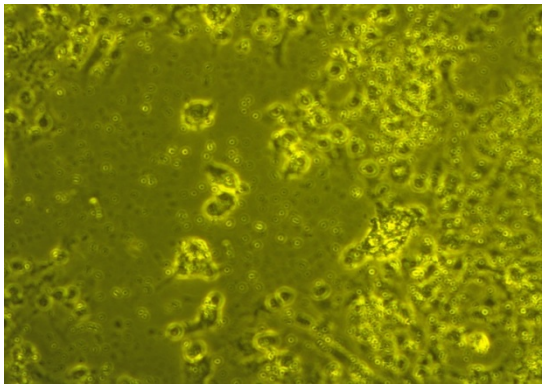


Fig. 11: After 15 h of incubation venom treated (1:100) dilution

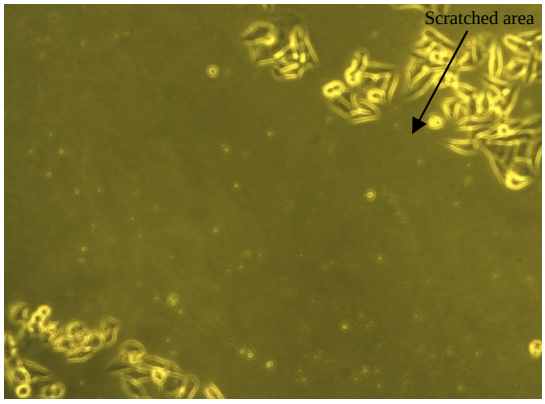


Fig. 12: Day 0 MCF cell monolayer venom treated (1:1000) dilution

Figure 11 shows after 15 h of incubation of monolayer cells treated with 1:100 venom dilutions, it was observed that all the cells were killed.

Figure 12 shows day 0 of MCF 7 cell monolayer treated with 1:1000 venom dilutions, where scratch is created across the cells.

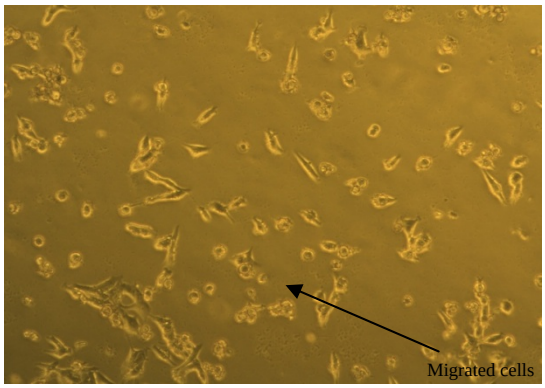


Fig. 13: After 15 h of incubation venom treated (1:1000) dilution

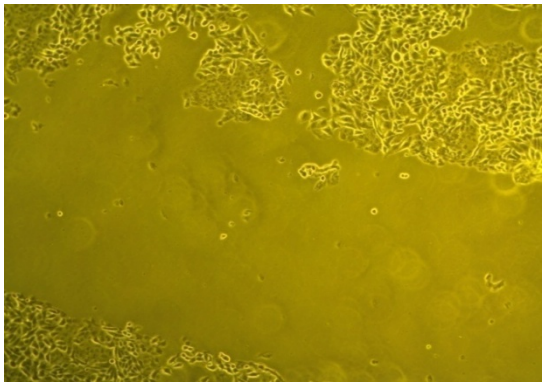


Fig. 14: Day 0 MCF 7 cell monolayer Without venom

Figure 13 shows after 15 h of incubation of monolayer cells treated with 1:1000 venom dilutions, it was observed that very few cells were migrated and few cells were dead.

Figure14 shows day 0 of MCF 7 cell monolayer without venom addition, where scratch is created across the cells.

Figure 15 shows after 15 h of incubation of monolayer cells without venom addition, it was observed that cells were migrating and recovered the scratched area.

Figure 16 shows day 0 of MCF 7 cell monolayer treated with doxorubicin drug as a positive control, where scratch is created across the cells.

Figure 17 shows after 15 h of incubation of monolayer cells treated with doxorubicin drug, it was observed that few cells were migrated and few cells were killed.

Very little migration of cells was seen in plate where 1 μg mL^{-1} (1:1000 dilution) venom was added but few cells are damaged. These two assays i.e., MTT and cell migration assay's shows that at a concentration of 1 μg mL^{-1} venom is toxic to cancer cells and below that concentration is less toxic to cancer cells and least toxic to normal cells.

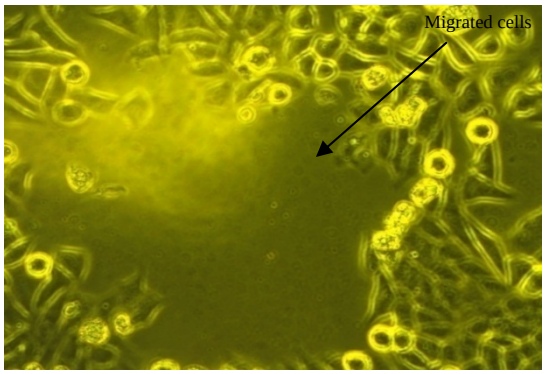


Fig. 15: After 15 h of incubation without venom

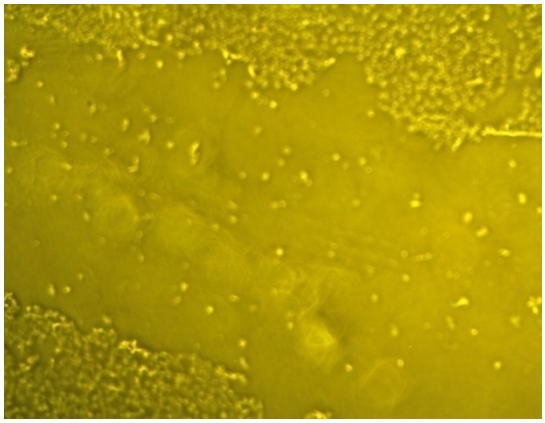


Fig. 16: Day 0 MCF 7 cell monolayer drug treated

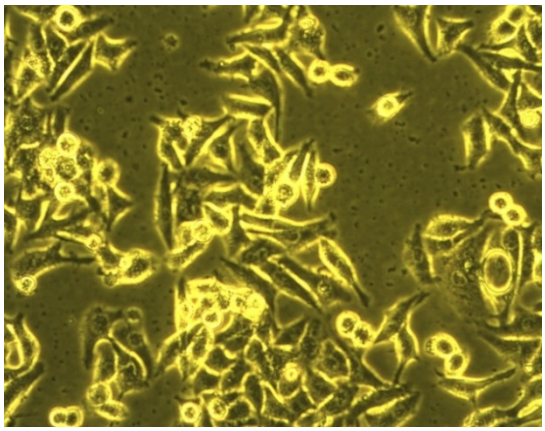


Fig. 17: After 15 h of incubation drug treated

Nanodrop spectroscopy: Nucleic acid concentration was determined in MCF 7 cancer cells before and after treating with venom. The results showed that the concentration of DNA in untreated MCF 7 cancer cells was 142.8 ng μL^{-1} and that of venom treated MCF 7 cancer cells is 41.1 ng μL^{-1}. (Table 4).

Table 4: Nucleic acid concentration analysis by nanodrop spectroscopy

Samples	Concentration of DNA (ng μL^{-1})	260/280
A	41.1	1.82
B	142.8	1.78

Sample A : Venom treated MCF7 cells

Sample B : Venom untreated MCF7 cells

Comet assay: The DNA damage was studied using Comet Assay[8]. The comet shaped DNA was not visible. This showed that DNA is damaged from the result of nanodrop spectrometer, but comet tail was not visible in comet assay, this might be because of the breakage of phosphodiester bond and the tail is usually visible when hydrogen bonds break.

DISCUSSION

From biochemical characterization assays the venom protein concentration was determined. One prominent protein peak was observed when venom sample was runned in HPLC, on carrying out SDS PAGE three prominent bands were observed and on comparing with standards it was estimated to be to be Lysophospholipase (13-26.5 KD), Acetyl cholinesterase (100-113 KD), Phospholipase A2 (~13 KD)[9-11]. Snake venom antimicrobial mechanism is complex and is affected by factors like amino acid sequence, net charge of protein, three-dimensional structure and even bacterial membrane composition[7]. Kirby bauer disc diffusion method was used for carrying out antibacterial sensitivity testing of common krait venom and it was observed that Gram-positive *Staphylococcus aureus* was more sensitive to crude venom compared to control amikacin antibiotic. Samy *et al.*[13] have done a research project in the year 2009 where they did a comparison study of snake, scorpion and bee venom and in that they found phospholipase A2 enzyme is mainly responsible for venom antibacterial property. In normal blood plasma two pathways are involved extrinsic and intrinsic pathway which merges together at a point which initiate co-agulation cascade. The intrinsic pathway is initiated by re-calcification time and extrinsic pathway by pro-thrombin time. The crude venom re-calcification time was determined by this pro-coagulant assay and it was found that the human blood plasma was clotted in 15 sec on treatment with venom. Rajan *et al.*[14] in Tamil Nadu have done the Biochemical Characterization of Krait venom and it was found that Krait venom acts as a pro-coagulant. Proteases enzymes in venom might be the

important factor in reducing this clotting time. On carrying out the MTT assay for determining the cell proliferation and cytotoxicity of venom it was found that at a concentration of 1 μg mL^{-1} 42.98% of MCF 7 cancer cells were damaged and at same concentration of 1 μg mL^{-1} very few cells of normal cell line LLC MK2 (24.73%) were damaged. Cell migration assay results determined that very few cells migrated in the MCF 7 monolayer cell line treated with 1 μg mL^{-1} venom. The MTT and cell migration assay's shows that at a concentration of 1 μg mL^{-1} venom is toxic to cancer cells and below that concentration is less toxic to cancer cells and least toxic to normal cells.

Sheikh *et al*.[2] have carried out a project in which they showed that cobra venom belonging to Elapidae family has significantly decreased 25% of RNA and 95% of DNA content in breast cancer tissue. Based on this concept, DNA extraction was carried out using traditional method and the concentration of DNA was measured before and after treating with crude venom. It was found that the concentration DNA of untreated MCF 7 cancer cells is 142.8 ng μL^{-1} and that of venom treated MCF 7 cancer cells is 41.1 ng μL^{-1}, this decrease in concentration determines that venom is acting on the DNA which is resulting in reduction in mitosis and ultimately cell death. The DNA damage studied by comet assay determined that there was no comet shape which indicated that the venom might be targeting phosphodiester bond, whereas, comet shape is observed only when there is alteration in hydrogen bond. From these two assays i.e., determining DNA concentration of nucleic acid by nano drop spectrophotometer and by carrying out comet assay it was confirmed that krait venom was targeting DNA of the cell.

These assays determined that venom carries proteins which carries a anti-cancerous, anti-bacterial or pro-coagulant properties.

CONCLUSION

From all the assays and results it is assured that common krait snake venom has some biochemical components which is showing anti-cancerous and anti-bacterial property. It is hypothesized to be some proteins like Lysophospholipase, Acetyl cholinesterase, Phospholipase A2 or protease enzymes. The significance of these experiment is that with the development in the field of bioinformatics and recombinant DNA technology, the structural mechanism of these proteins can be identified and by cloning technique efficiency duplicates can be created *in vitro* and by animal tissue culturing techniques the focused protein can be synthesized, purified and used as a chemotherapy drug in treating targeted cancerous cells.

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