Breast cancer is one of the leading cancers in the globe occurring in 12% of women suffering from various cancers. Lot of research in medical science on breast cancer is going on with researchers finding new molecules or therapies to treat this disease with minimum or negligible side effects. Traditionally, venom of venomous animals has been used to treat various diseases at minute concentrations and has been proved to be an effective therapeutic agent. Venom of viper like snakes has been reported to have therapeutic effect on breast cancer. Malabar pit viper snake, endemic to Western Ghats of India, is being evaluated for the anticancer potential of its venom in current work. Venom is a mixture of many proteins of varying molecular weight and properties. The fractionation and purification of these venom proteins has been carried out to separate the active protein with has anticancer potential. Gel permeation chromatography followed by SDS PAGE analysis indicate the presence of 5 fractions which were then subjected to cytotoxicity (IC\(_{50}\) value) using MTT assay on normal cell line (HEK293) and breast cancer cell line (MCF7), the IC\(_{50}\) values of fraction 1, 2 and 3 are lower for MCF7 than their IC\(_{50}\) values for HEK293. This shows that these fractions are more toxic to cancerous cells as compared to normal cells. As per the result obtained, not all the fractions at all concentration are lethal to cancerous cells. 10% concentration of fraction 1 and fraction 2 shows significant anticancer potential.
INTRODUCTION:
Snake venom would be best choice to treat the different types of tumors in comparisons to some of available anticancer drugs since. Natural toxins especially non-toxic dose of snake venom has been separated to reduce the solid tumor size and to block the angiogenesis. A complete cure though is not expected from present available anticancer drugs, they cause irreparable side effect that multiplies miseries. Venom research is being carried out throughout the world for more than 100 years; using snake venom either as medical research tools or directly as therapeutic/diagnostic agent. Calmette showed that Cobra venom could treat cancer in mice. Thereafter, many reports have established the anticancer potential of different species of Elapidae, Viperidae and Crotalidae snake venoms. Contortrostatin, a toxin derived from Agkistrodon contortrix, is an important component showing antineoplastic activity, which blocks several critical steps in tumor metastasis including angiogenesis. Recent studies by Park and co-workers reported a toxin from the venom of Vipera lebetina turanica that caused apoptosis of human neuroblastoma cells. Certain cardiovascular drugs from snake venom sources are already in clinical use. Batroxobin, a drug derived from Defibrase purified from Bothrops moojeni, has therapeutic application in acute cerebral infarction, non-specific angina pectoris and sudden deafness. Captopril, a drug developed from Bothrops jararaca venom, is used to treat kidney disease in diabetes, high blood pressure and heart failure. Recently a novel glycoprotein 1b-binding protein jerdonibitin has been reported from Trimeresurus jerdonii venom, which showed potent platelet inhibiting activity.

One of the earliest reports on snake venom is on its phosphodiesterase activity. In 1959 snake venom protease was isolated by a Japanese group isolated α-bungarotoxin from snake venom. To date, α-bungarotoxin is one of the most selective markers of certain subtypes of nicotinic acetylcholine receptor. Snake venom is a fused mixture of enzymes, peptides, carbohydrates, minerals and proteins. The cytotoxic effects of snake venom have been reported to have potential to degrade/destroy tumor cells. Anticancer action of various venoms and venom components has been reported earlier. Snake venom peptides isolated from Viperidae and Crotalidae species containing RGD/ECD sequences in their structures proved to be invaluable tools to recognize specific structures/receptors of platelets and some somatic cells, as well as to promote physiological and biochemical changes at cellular level. To date, these compounds are also complements for new therapeutic strategies in mutagenesis-related diseases. Venoms of Trimeresurus and Agkistrodon genera contain peptides that potentially inhibit platelet aggregation frequently induced by tumor cells.

Therapeutic uses of snake venom in various diseases have been explored by Acharya Vagbhata in 48th chapter of Uttartantra of Ashtanga Sangraha, i.e. “Vishopayogyaa Adhyaya.” Forty-seven different Formulations have been mentioned by Acharya vagbhata in which snake venom is pertinently used with other Ayurvedic herbal drugs of Indian system of medicines.

T. malbaricus (Malabar Pit Viper) is an endemic forest dwelling snake of western ghats, India. Various pharmacological activities have been reported on the venom of Malabar Pit Viper but its bioactivity as an anti-cancer agent is not yet reported. Therefore, in the current work, fractions of Malabar Pit Viper venom were purified by Gel filtration Chromatography and were evaluated for their anticancer potential.

MATERIALS AND METHODS:
Tris buffer (Merck), DMEM- (Thermo scientific - 11995065), Primocin – (Biogene India – ant-pm-2), FBS – (Thermo Fisher Scientific – 10437028), PBS – (Thermo Fisher Scientific -10010023), 0.5% Trypsin EDTA – (Thermo Fisher Scientific -15400054), Trypan Blue – (HiMedia -TC193), DMSO – (Sigma – D8418), MTT – Himedia (TC191) were procured. HEK 293 and MCF-7 cell lines were procured from National center for cell sciences (NCCS), Pune, Cytotam 20mg (Cipla) (Tamoxifen 20mg salt). Knauer make BIOLINE FPLC system, 60×0.9cm, glass column (Spectra), superdex 200 (GE healthcare), UV visible spectrophotometer,1800 (LABINDIA), Cooling centrifuge, C30BL (REMI), Mass Analyzer (Shimadzu LC-MS/MS 8020), Incubator Shaker.
(Ascension Innovation), CO₂ incubator (Skada), Inverted microscope (Evos), 96 well plate reader - (Biorad, USA), 96-well flat bottom culture plates (Nunc A/S, Denmark) were used during the current work.  

1.0: Venom collection and storage:
Venom sample from male and female snakes were collected from different location of Amboli forest, Maharashtra, India. Requisite permissions of the forest department of Maharashtra state were obtained for the study. Venom collection is by manual pressure technique on venom gland. Venom is collected in 1 ml centrifuge tube and all snakes captured for venom collection were of similar length, size and habitat. Cured venom is stored at -80°C and it is centrifuged at 4000rpm and lyophilized till further use. 

2.0: Venom fractionation using GFC:

Venom sample preparation:
Pooled snake venom sample is prepared by mixing cured male and female venom in ratio of 1:1 followed by dilution at 1:5 in 20 mM Tris buffer at pH 8.0. The prepared venom sample is loaded on GFC column packed with Superdex 200 (40 cm X 0.9 cm), previously equilibrated with 20 mM tris, pH 8.0. The flow rate is optimized to achieve good separation and resolution of fractions. The detector is set at 280 nm, and separation carried out for a total run time of 100 min at room temperature. The separated fractions are then quantitated using Bradford assay and loaded on 10% PAGE for further analysis. 

3.0: Protein quantitation and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE) analysis:
The total protein concentration of the fractions was determined by the method reported by Bradford, using BSA as a standard protein. All venom fractions are processed under reducing conditions and are loaded on 10% Polyacrylamide gel. The visualization was done by 0.25% CBB staining. The molecular weights are determined by comparison with the prestained standard molecular weight marker. 

4.0: Mass spectroscopy:
The fractions obtained from GFC are diluted to 1:1000 using mobile phase (ACN: 0.1% Formic acid v/v). The venom samples are injected into the mass analyzer. The parameters used for work is listed below: 

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass analyzer</td>
<td>Shimadzu LC-MS/MS 8020</td>
</tr>
<tr>
<td>Ion source</td>
<td>Electro spray ionization</td>
</tr>
<tr>
<td>Nebulising gas flow</td>
<td>3L/min</td>
</tr>
<tr>
<td>Drying gas flow</td>
<td>15L/min</td>
</tr>
<tr>
<td>Scan speed</td>
<td>909u/sec</td>
</tr>
<tr>
<td>Event time</td>
<td>1s</td>
</tr>
<tr>
<td>DL temperature</td>
<td>250°C</td>
</tr>
<tr>
<td>Heat block temperature</td>
<td>400°C</td>
</tr>
</tbody>
</table>

5.0: Cytotoxicity assay:
The cytotoxicity of GFC purified venom fractions on HEK 293 and MCF-7 cell lines is determined by MTT colorimetric assay. Cells in DMEM containing 10% fetal bovine serum were seeded into 96-well flat bottom culture plates (Nunc A/S, Denmark) at 1 x 10⁴ cells/well. After 24 h of incubation, at 37°C in 5% CO₂ humidified atmosphere, various concentrations of each venom fraction (final concentrations from 10% to 100%) are added to the culture and incubated for 24h at 37°C in 5% CO₂ humidified atmosphere. Then, 10 µL/well of MTT (5 mg/mL) is added to the cell and incubated for 4 h at 37°C in 5% CO₂ humidified atmosphere. The reaction is stopped by 100 µL of DMSO. The plate is then shaken in shaker for 10 min. The absorbance of each well is read at 570 nm wavelength in Elisa Reader (Biorad, USA), using wells without cells as blanks. All experiments are
performed in triplicate. The effect of venom fractions on the proliferation of human breast cancer cells is expressed as the % cytoviability, using the following formula:

\[
\% \text{ Cytoviability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \times 100\%
\]

**RESULTS AND DISCUSSION:**

**SDS PAGE Analysis of crude venom:**

The crude venom of *T. Malabaricus* shows proteins ranging from 250kDa to 15kDa. Few proteins show prominent bands on gel indicating abundance while some light bands are also be seen. All these proteins are considered in the investigation for its individual as well as synergistic effect on cancer cell line.

**Crude venom fractionation using Gel Filtration Chromatography:**

Chromatographic conditions:
- Column: Superdex 200
- Detection: 280 nm
- Mobile Phase: 20 mM Tris pH 8.0
- Temperature: RT
- Flow Rate: 0.8 mL/min
- Pressure: 1 bar
- Loading volume: 0.2 mL
**Figure 2**: Fractionation of *T. Malabaricus* pooled venom by Superdex200 Gel filtration. Soluble venom from *T. Malabaricus* is loaded onto a S200 column and eluted with 20 mM Tris buffer pH 8.0.

**Protein quantitation of post GFC fractions using Bradford assay:**

<table>
<thead>
<tr>
<th>Fraction no</th>
<th>Concentration of protein (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.00</td>
</tr>
<tr>
<td>2</td>
<td>0.33</td>
</tr>
<tr>
<td>3</td>
<td>0.46</td>
</tr>
<tr>
<td>4</td>
<td>0.51</td>
</tr>
<tr>
<td>5</td>
<td>0.63</td>
</tr>
</tbody>
</table>
SDS PAGE of post GFC fractions:

![SDS PAGE Image]

**Figure 3**: 10% PAGE, non-reducing condition, stained with 0.25% Coomassie Brilliant Blue R250, showing venom fractions obtained after GFC.

Five fractions of *Trimeresurus malabaricus* venom were separated using gel filtration chromatography (S200) and analyzed on SDS PAGE. Each fraction showed multiple bands. Fraction 2 and 4 showed two prominent bands at similar molecular weight (Fig. 3). After gel filtration chromatography, the purified fractions are subjected to Mass spectroscopy for molecular weight identification.

**Figure 4**: Represent the Mass spectra of each venom fraction.
The purified fractions showed multiple masses of proteins, the numbers of proteins reflected in fraction 1 are much more as compared to the other fractions, which is evident from SDS PAGE as well as MS spectrum. Some of the molecular weights identified by MS can be observed in SDS PAGE. From fraction 1 one can locate bands of approximately 66, 98, 47,31kDa. Similarly fraction 2 shows 97 and 19kDa proteins, fraction 3 shows 28, 96, 31 and 69kDa proteins, Fraction 4 shows 59, 85, 83kDa proteins and fraction 5 shows 43, 62, 66, 98kDa proteins.

Cytotoxicity Assay:

![Graphical representation of cytotoxic activity of venom fractions against HEK 293 cells.](image-url)
Table 3: IC\textsubscript{50} values

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>IC\textsubscript{50} (%)</th>
<th>MCF7</th>
<th>HEK293</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction 1</td>
<td>42.26</td>
<td>49.2</td>
<td></td>
</tr>
<tr>
<td>Fraction 2</td>
<td>17.37</td>
<td>44.85</td>
<td></td>
</tr>
<tr>
<td>Fraction 3</td>
<td>14.84</td>
<td>29.9</td>
<td></td>
</tr>
<tr>
<td>Fraction 4</td>
<td>Killing at 10%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction 5</td>
<td>40.86</td>
<td>37.7</td>
<td></td>
</tr>
<tr>
<td>Tamoxifen Std.</td>
<td>Killing at 10%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

From the protein quantitation using Bradford assay, these IC\textsubscript{50} values were calculated in the µg/mL format.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>IC\textsubscript{50} (µg/mL)</th>
<th>MCF7</th>
<th>HEK293</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction 1</td>
<td>1693.60</td>
<td>1897.20</td>
<td></td>
</tr>
<tr>
<td>Fraction 2</td>
<td>56.45</td>
<td>145.86</td>
<td></td>
</tr>
<tr>
<td>Fraction 3</td>
<td>67.71</td>
<td>137.82</td>
<td></td>
</tr>
<tr>
<td>Fraction 4</td>
<td>Killing at 51µg/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction 5</td>
<td>256.79</td>
<td>238.14</td>
<td></td>
</tr>
<tr>
<td>Tamoxifen Std.</td>
<td>Killing at 10 µg/mL</td>
<td></td>
<td>Not Killing at 10 µg/mL</td>
</tr>
</tbody>
</table>

The IC\textsubscript{50} values of fraction 1, 2 and 3 are lower for MCF7 than their IC\textsubscript{50} values for HEK293. This shows that these fractions are more toxic to cancer cells as compared to normal cells.

The purified fractions are subjected to cell cytotoxicity assay using HEK 293 and MCF 7 cells. The cells are treated with four different concentrations of each fraction. The IC\textsubscript{50} values are calculated for both the

Figure 6: Graphical representation of cytotoxic activity of venom fractions against MCF7 cancer cell line.
cell lines based on the data obtained from MTT assay, which are found to be 1897.20, 145.86, 137.82 and 238.14µg/mL for fraction 1, 2, 3, 5 respectively on HEK293 cell lines. For MCF-7 cell line IC\textsubscript{50} values were found to be 1693.60, 56.45, 67.71 and 256.7µg/mL for fraction 1, 2, 3, 5 respectively. Fraction 4 and Tamoxifen has IC\textsubscript{50} values below 51.8µg/mL and 10µg/mL respectively on both HEK293 and MCF-7 cell lines. As per the data obtained not all the fractions are lethal to cancerous cells. The difference between the IC\textsubscript{50} values of fraction 2 and fraction 3 for normal and cancer cell line is vast, that shows its anticancer potential at the concentration which is nontoxic to normal cells. It can also be commented based on the remaining data that other fractions might show anticancer potential even at lower percentage.
The IC\textsubscript{50} value for cancer cell line, it is very near to the IC\textsubscript{50} value of normal cell line, hence cannot be a good anticancer agent. Fraction 4 proved to be lethal for both the cell lines at its lowest concentration tested in the study, thus needs to be tested further to check its anticancer potential. Mixture of various proteins in the fraction shows cytotoxic effect on breast cancer cell lines which indicates that these proteins synergistically affect the cancer cells. In recent years remarkable progress has been made for the treatment of cancer. The major drawback of the current methods of cancer treatment is the resistant to the therapies after initial treatments. This has led to the increased interest on anticancer drugs developed from natural sources. The diversity of bioactive proteins in snake venom is a unique source from which novel therapeutics can be developed.
Snake venom is a complex mixture of several proteins, peptides, enzymes, organic and inorganic molecules. The complex mixture of snake venom can be separated in protein fractions using Gel filtration column. SDS PAGE separation provides another dimension to the analysis and could resolve all the proteins present in each fraction suggesting the heterogeneity of the fractions. In accordance to Raghavendra Gowda et al, proteins in the fractions of venom can be present in its multimeric forms. We have processed the samples under nonreducing conditions and Carried out the separation as the protein tends to aggregate in the solution under storage conditions. These multimeric forms of the proteins do show activity against cancer cells.\textsuperscript{25} In-vivo cytotoxicity results indicate that fraction 2 and 3 is toxic to the breast cancer cells, at concentrations which are nontoxic to normal HEK293 cells. The degree of toxicity differed in different fractions. The cytotoxic effect of fraction 1, 2 and 3 is more pronounced for MCF-7 than HEK 293 suggesting that MCF-7 cells are more susceptible to these 3 fractions. The mechanism behind the specific cancer cell killing ability of snake venom is yet to be elucidated. Future research on venom proteomics can justify such differentiating activity which can be attributed to variation in venom constituents.

**CONCLUSION:**
Traditionally, venoms are used to kill infections and diseases but at lowest or minimal dosage level. Safety and efficacy of venoms in therapeutics is proven in spite of its toxic action. Indian Ayurveda strongly believes in efficiency of venoms from different animals and has been reported to have formulations out of it. The current study focuses on the new paradigms of cytotoxic effects of snake venom. The work describes the purification, quantitation, analysis and studying its anticancer potential of $T$. malbaricus venom. As per the previous reported literature snake venom is concentrated mixture of many proteins, majorly metalo proteins. These proteins were fractionated, quantified, analyzed for molecular weights of the proteins present in the fraction and activity range determination against normal as well as cancer cell lines. Further purification of the venom fraction showing cytotoxicity against MCF7 cells to the homogeneity could open the doors for the in-depth studies on pathways related to cytotoxicity. The antitumorogenic, anti-metastatic or anti-angiogenetic activity of the protein or peptide can be determined by receptor binding assays using flow cytometry, real-time PCR or western blot analysis of targets. The proteomic approach would provide the most sensitive and reliable tool to generate novel biomarker in oncological research.

**ACKNOWLEDGEMENTS:**
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during on field venom collection, NFB laboratory for providing support for chromatography instrument and forest department of Maharashtra for necessary permission for venom collection.

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