WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.045
Volume 3, Issue 9, 1130-1145. Research Article ISSN 2277– 7105

ANTICANCER EFFECT OF NEMATOCYSTS VENOM FROM JELLYFISH *ACROMITUS FLAGELLATUS*, ON HUMAN BREAST CANCER CELL LINE

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Article Received on
04 September 2014,

Revised on 29 Sept 2014,
Accepted on 23 Oct 2014

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ABSTRACT

Background: Marine organisms comprise approximately half of the total biodiversity which serves as a vast source of discovered therapeutics and new anticancer drugs. **Objective:** The present investigation is made to evaluate the efficiency of the crude venom of jellyfish *Acromitus flagellates*, a cnidarian for its antiproliferative activity. **Methodology**: The nematocysts were isolated from marginal filament and oral arms of jellyfish. The crude venom of nematocyst was tested against anticancer potential on human breast cancer cell lines (MDA-MB-231) with 200 µg IC_{50}. **Results:** The protein content of the crude venom was estimated to be 1.5 mg/ml. The protein profile of crude venom protein was determined by SDS-PAGE and the molecular weight was found to be in the range of 205 kDa to 29 kDa. The isolated crude venom was tested for its antiproliferative activity against human breast cancer cell line MDA-MB-231 by MTT assay. The results revealed that the crude venom showed antiproliferative activity at an IC_{50} concentration of 200 µg/ml. Propidium iodide staining and DNA fragmentation analysis expressed the induction of apoptosis in the cancer cells. Cell cycle analysis and changes of the mitochondrial membrane potential shows the cell cycle arrest at G_1 phase which confirmed the potential anticancer activity of jellyfish venom against human breast cancer cell MDA-MB 231. **Conclusions:** *A. flagellatus* venom may have the potential to serve as a template for future anticancer drug development. Further analysis is warranted and necessary to substantiate our findings.

KEYWORDS: *Acromitus flagellates*, Nematocyst, Venom, MTT assay, DNA fragmentation.

INTRODUCTION

Cancer is a chronic degenerative disease considered to be the second most common cause of death in economically developed and developing countries [1]. According to a recent report by the international Agency for Research on Cancer (IARC), there are currently more than 10 million cases of cancer per year worldwide. In 2008 alone there were 12.7 million new cases of cancer worldwide and the WHO estimates that the diseases will cause about 13.1 million deaths by 2030 [2]. Breast carcinoma (BC) is the most common cancer among women and the second highest causes of cancer [3]. Most cancer occur during age of 45-55, it also occurs in men but is more than 100 fold less frequent that in woman [4]. Breast cancer is about 25 to 31% of all cancer among women in Indian cities [5].

The venoms are complex mixture of a number of proteins, peptides, enzymes, toxins and non-protein inclusions. Venom composition and chemistry varies among species, age, sex and different geographic regions. Toxins, occurring in venoms and poisons of venomous animals, are chemically pure toxic biological systems. The biodiversity and specificity of venoms and toxins make them a unique source of structural templates form which new therapeutic agents are being developed [6]. Medicinal application of venoms and toxins has been mentioned in Ayurvedic, Unani and Chinese Homeopathic system of medicines [7]. Venoms and toxins have found a niche in the pharmaceutical market. Several isolated toxins with a known mode of action have practical applications as pharmaceutical agents, diagnostic reagents or preparative tools [8]. For the past three decades jellyfish have been undertaken for research to find the anticancer properties of its venoms, this lead to the discovery of several promising molecule having anticancer activity, some of which are in clinical trial and may emerged to be a future drug in cancer therapy [6]. Antitumor activities were well documented with peptide from different synthetic as well as natural sources in different tumor models [9, 10, 11 and 12]. The jellyfish, *Acromitus flagellatus* (*A. flagellatus*) [13] a Cnidarian of Class Scyphozoa, Order Rhizostomea, and Family Catostylidae, is distributed widely in the east coast of Tamilnadu, India. The *A. flagellatus* have oral arms which bear numerous long filament surface has uncountable number of nematocysts [14]. Hence the present study was carried out to isolate the crude venom from nematocyst of *Acromitus flagellatus* and evaluate the efficacy on human breast cancer cell line MDA-MB-23.

MATERIALS AND METHODS

The jellyfish *Acromitus flagellates* was collected from brackish water in Muttukadu, Tamilnadu, India during the month of January and February 2014. It was identified and authenticated by an expert The Zoological Survey of India, Chennai.

Isolation of nematocyst

The nematocyst from *A. flagellatus* was isolated in the laboratory followed by the method of Feng et al., [15]. The marginal filament and oral arms were removed from the sub umbrella margin of the animal body and was stored at 20°C until further process. The frozen marginal filament and oral arms were placed in five volume of fresh sea water at 4°C and kept four days autolysis. The supernatant was decanted and the settled material was resuspended once every day. The resulting suspension was filtered through a 54 µm sieve filter. The final suspension containing nematocysts was allowed to settle. The sediments were collected separately and washed several times with 0.9 % NaCl saline by centrifugation at 3000 g with 4°C for 30 minutes. Then these washed sediments containing nematocysts were stored at -20°C until further process.

Morphological identification of nematocyst

The marginal filament and oral arms from live animal were freshly excised and washed briefly using sieve filtered seawater then centrifuged and pellet the was collected. The pellets were dissolved in PBS for observation in Light microscope to determine nematocyst distribution.

Extraction of venom from nematocyst

The jellyfish *A. flagellatus* venom was extracted from the nematocysts using a standard method of Carrette and Symour [16]. The isolated venom was homogenized with Tris-HCl buffer (10 mM, pH7.8) and centrifuged 13,000 g with 4°C for 15 minutes. Then the supernatant was collected and stored at -20°C.

Estimation of protein

Protein estimation was carried by the method of Bradford [17]. The standard protein sample was prepared at 5 mg/ml of BSA and the absorbance was measured at 595 nm in Double beam spectrophotometer (Shimadzu 160 UV-Vis) dilution protein concentration 20-100 µg/ml.

Protein profile analysis

The molecular weight of proteins from the crude venom was determined by SDS-PAGE carried out according to the Laemmli method [18] in running containing 10% polyacrylamide gel with 5% stacking gel. Sample were resuspended in SDS-PAGE sample buffer (Tris-HCl pH 6.8, 10% glycerol, 1% SDS, 0.01% bromophenol) and incubated 95°C for 2 min than keep on ice until use. The crude venom was electrophoreses for 90 minute at 70 V. The molecular size marker, 3.5 to 200 kDa (Ge NeI^{TM} BIO-SOURCE and SURGICALS; Chennai, India) Protein bands was visualized by Coommssie R-250.

Cell culture

The human breast cancer cell line MDA-MB 231was purchased from the National Centre for Cell Science (NCSS), Pune, India. Cells were cultured in Rosewell Park Memorial Institute (RPMI) 1640 (Hi-Media Laboratories, Mumbai, India) medium supplemented with 10% (v/v) FBS, penicillin 100 µg/ml, streptomycin, sodium bicarbonates solution. The cells were maintained as monolayer in 25 mm^2 plastic tissue culture flasks at 37°C in a humidified atmosphere containing 5 % CO_2 in air. Cells were grown exponentially and cells numbers of each well were counted on a hemocytometer under microscope.

Cell viability analysis

The cytotoxic effect of *A. flagellatus* crude venom was measured by the colorimetric MTT reduction assay method of [19]. Cells were cultured in 96 well plates at a density of $5x10^4$ cells per well and incubated overnight. The medium was discarded and cells were exposed to different concentration of crude venom (50, 100, 150, 200 and 250 µg/ml) following at different time intervals of 24, 48 and 72 h, incubation. Then 20µl of MTT stock solution (5 mg/ml) was added to each well. After 4 h incubation, purple coloured precipitates visible under the microscope, the supernatant was aspirated and the formazan precipitates were solubilized by the addition 100 µl per well of dimethyl sulfoxide (DMSO). Following 1 h of incubation in dark room temperature absorbance was determined at 570 nm using micro plate reader. The following formula was used to analyze the cell viability.

$$\text{Percentage of viability} = \frac{\text{OD value of the experimental sample}}{\text{OD value of the experimental control}} \text{ x } 100$$

Analysis of cell morphology

Cells were seeded in 96 well plates at density of $5x10^4$ cells per well and incubated overnight. Medium was replaced and cells were exposed to 200 µg/ml of crude venom. Morphological alterations of cells were monitored after 24 h incubation at 37° C and 5% CO_2 using in inverted microscope.

Cell cycle analysis

Cell cycle distribution and per cent of cells were performed by flowcytometery [20]. After trypsinization, approximately 10^4cells were collected by centrifugation at 1000 g for 5 minutes. cells were then washed in PBS followed by resuspension and fixation in 70% ethanol for approximately 2 h. The cells were washed with PBS and resupended in 500 µl of PBS containing 100 µg/ml RNase (Roche Molecular Biochemicals, Indianapolis, IN) followed by 30 minutes incubation period at room temperature. Cellular DNA was stained by addition of 50 µg/ml propidium iodide, and cells were analysed on a FACS can flow cytometry.

Propidium iodide staining

Propidium iodide (PI) stain was performed to determine the number of living cells as described by the method of Kume et al., [21]. The cells were incubated for 24 h in six well plates. After incubation, cells were washed with PBS and fixed in Methanol: Acetic acid (3:1, v/v) for 10 minutes and stained with 50 µg/ml of PI was added into each well and incubated at room temperature for 20 minutes in dark. The cells were detected by fluorescence inverted microscope (LSM 710, Carl Zeiss) at 40x magnification.

DNA fragmentation analysis

DNA fragmentation is used as biochemical hall mark of apoptosis for majority of cells. The extraction of DNA was carried out by the method of Bortunr et al., [22]. Cultured human breast cancer cell (MDA-MB-231) were washed twice PBS and lyed in buffer (100 mM NaCl, 100mM Tris-HCl pH 8.0, 25mM EDTA, 0.5% SDS, and 0.1mg/ml proteinase K) at 37°C for 18 h. DNA was extracted with an equal volume of Phenol/Chloroform (1:1) and precipitated at -70°C, DNA pellet were resuspended in 10 µl of 10mM Tris pH 7.8, 1 mM ETDA buffer and incubated for 1 h at 37°C with 1 µg/ml RNase to remove RNA. DNA pellet were electrophoresed for 2 to 3 h at 90 V on 1.8% agarose gel. The gel was stained with ethidium bromide, and DNA fragments were visualized under ultra violet light.

Statistical analysis

All the statistical analysis was conducted using the SPSS commercial statistical package (SPSS, version 16.0 for windows, SPSS Inc., Chicago, USA). The inhibitory 50% (IC_{50}) was calculated from the concentration/ effect regression line. In each case, an appropriate range of five concentrations was used.

RESULTS AND DISCUSSION

Many investigations have been carried out on the biological activities of molecules in various species of Cnidaria. Venom of such animals is the strategy to survive in a specific environment so that it exhibits very potent biological effects. Among the various classes of cnidarians the venom was isolated and characterized in which most of them are proteineous in nature. In this present study the jellyfish *A. flagellatus* were collected and identified by following the standard literature of Fernando and Fernando (Zoological survey of India). The isolated nematocysts from *A. flagellatus* were washed with saline to reduce the debris and other body tissues. The nematocyst suspension was observed through light microscope (Fig. 1). The amount of protein content in crude venom showed 1.5 mg/ml respectively.

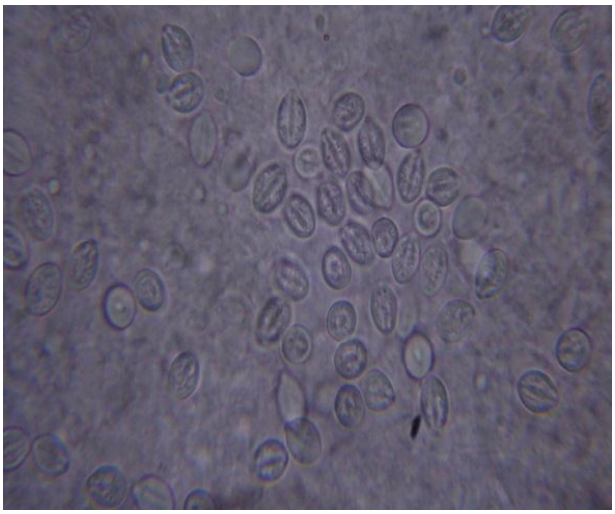

Fig. 1 Light micrograph of *A. flagellatus* nematocysts

Jellyfish venom is varying from one species to another, thus it biochemical composition and activity is differing from each other. Recently, much attention has been paid to marine organisms for the screening of biologically active compounds [23]. It was reported that a major protein component in *Chrysaora quinquecirrha* venom had a 19 kDa molecular weight, whereas the major protein component in *Chrysaora achlyos* venom was 55 kDa [24]. The venom of *Chiropsalmus quadrigatus* contains a hemolytic toxin of 44 kDa [25] and that from *Chironex fleckeri* nematocysts venom contain a 20 kDa protein [26]. The crude nematocyst

venom of *A. flagellatus* consists of several protein bands ranging from 205 kDa to 29 kDa. The molecular weight of the crude venom protein were determined by SDS PAGE on 10% polyacrylamide gel using standard protein marker and calculated to be 205, 97, 66, 43 and 29 in the same order (Fig. 2).

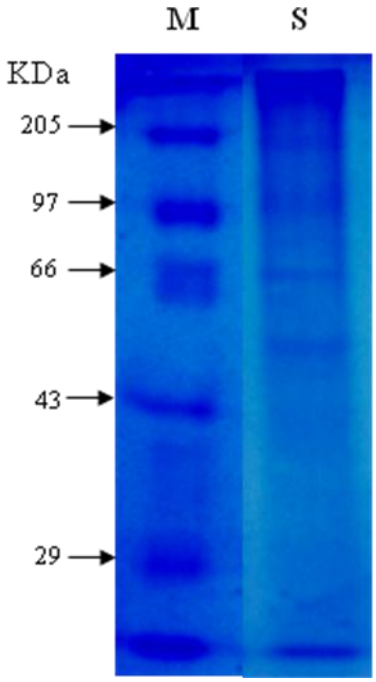

Fig. 2 SDS-PAGE analysis of Jellyfish *A. flagellatus* nematocyst crude venom

(10% Polyacrylamide gel stained with Coomassie Blue). Lane 1.Standard marked, Lane 2. Crude sample.

The toxicity associated with cancer chemotherapy arises primarily from the lack of specificity for tumor cells. To handle this problem, the focus of many studies is natural compounds obtained from marine organisms that inhibit cancer cells more selectively than normal cells. Here, we determined the cytotoxic concentration in which the jellyfish crude venom significantly inhibited growth of breast cancer cells. The cytotoxicity test has been proposed as a reliable and reproducible assay for assessing the toxic potency of jellyfish crude venom with a higher sensitivity than mouse lethality test [27]. Although the cytotoxicity test of jellyfish venom has not been widely used, there are previous literatures including the IC_{50} values of jellyfish venom from *Chrysaora quinquecirrha* on CCL-13 hepatocysts (IC_{50}< 1µg/ml) [28], from *Cyanea capillata* on HepG2 hepatoma cells for 48 hrs (IC_{50} = 20.3µg/ml) [29] on from nematocyst free tissue of *Rhizostomea pulmo* on V79 lung fibroblast for 3 hrs (IC_{50} = 37.6µg/ml) [30] and the toxicological aspects of *Nemopilema nomurai* jellyfish on

H9C2 and C2CL2 have strong cytotoxic activity against heart muscle. The analysis of the data shows that *A. flagellatus* jellyfish crude venom has moderate toxicity in comparison with other cnidarian. Our results show that *A. flagellatus* nematocyst contains at least one or more toxic components that have a strong cytotoxic activity against breast cancer cell line MDA-MB-231.

The *A. flagellatus* crude venom was evaluated for their effect on cell viability against human breast cancer cell line at different concentrations. The crude venom showed a dose dependent decrease in cell viability. Increasing the time of incubation showed a further decrease in cell viability. Approximately 50% inhibition of cell viability was seen at 200µg/ml respectively, upon 48 h exposure (Fig. 3 and 4).

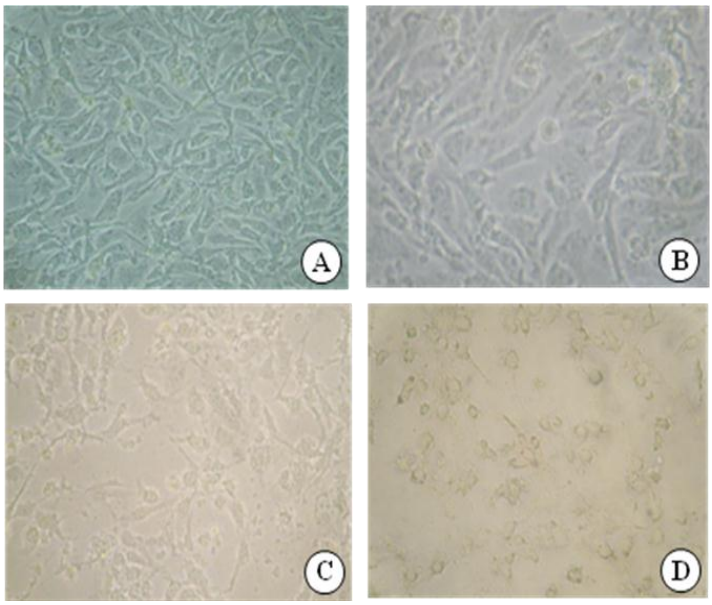

Fig. 3 Cytotoxic effect of *A. flagellatus* nematocyst crude venom showing the morphological variations of control and venom treated MDA-MB-231 cells

A- Control cells

B- 200 µg/ml venom for 24 hours

C- 200 µg/ml venom for 48 hours,

D- 200 µg/ml venom for 72 hours

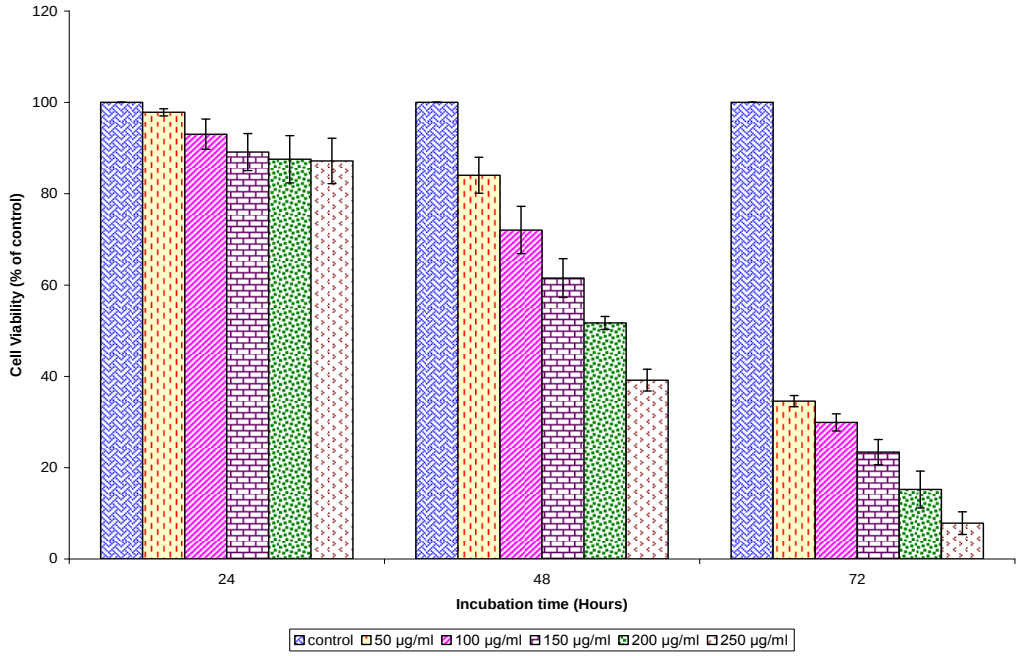

Fig. 4 Effect of different concentration of *A. flagellatus* nematocyst Crude venom on MDA-MB-231 cells

To investigate this, flow cytometry was performed to test the crude venom in cell cycle arrest. Figure five shows that incubation of crude venom with MDA-MB-231 cells for 48h significantly reduced the DNA content make them appear in the G_1 region indication of apoptosis with consequent loss of cells in the G_0 phase 98.69% of cells were in sub G_1/S phase, 1.07% cells were in the same phase in the respective cell cycle after treatment of MDA-MB-231 cells with crude venom were seen in G_1 phase. The results showed crude venom induced cell cycle arrest in G_1 phase and time dependent manner. To confirm whether the cytotoxic effect induced by crude venom involves apoptotic changes, the nuclear condensation was studied by the propidium iodide staining method. The control cell shows the decrease in propidium iodide positive cells. In the case of cells treated with 200 µg/ml of crude venom with exposure times 48 h and progressive increase in the number of propidium iodide positive cells were observed in maximum concentration (Fig. 6). DNA fragmentation analysis is a typical assay to analyze drug induced apoptotic cell death. DNA was isolated from the MDA-MB-231 cancer cells cultured in the presence of crude venom at various concentrations for 48 h. The cells were treated with 200 µg/ml of crude venom for 48 h. In the control lane the DNA was intact without any fragmentation. In the lane which received treated cells, there was DNA fragmentation which is seen as a “ladder pattern” (Fig. 7).

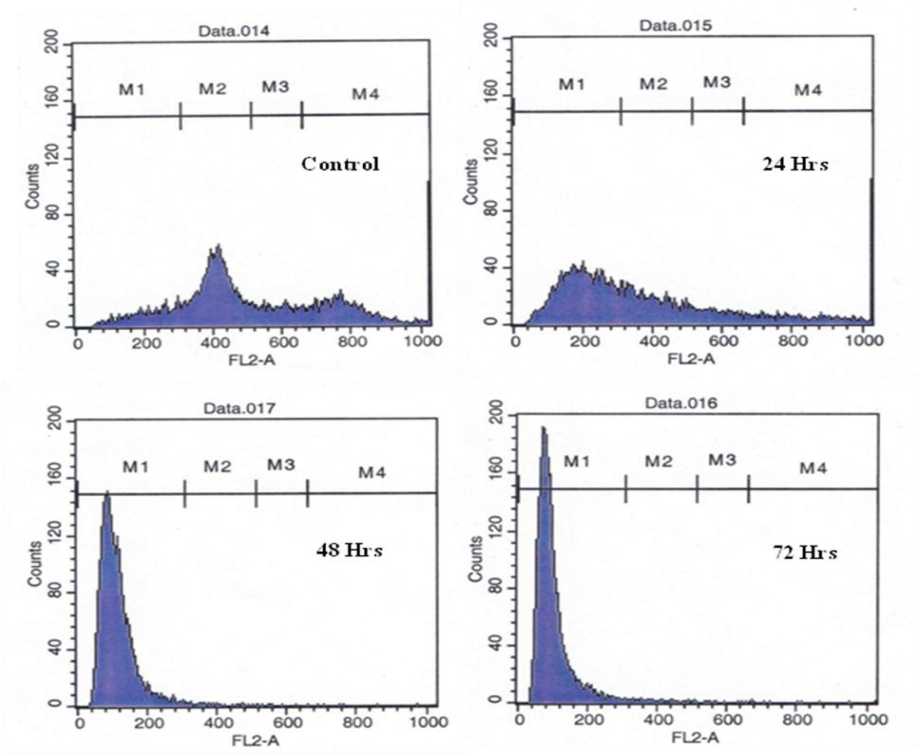

Fig. 5 Effect of *A. flagellatus* nematocyst crude venom on cell cycle transition in MDA-MB-231 cells

A- Control cells

B- 200 µg/ml venom for 24 hours

C- 200 µg/ml venom for 48 hours

D- 200 µg/ml venom for 72 hours

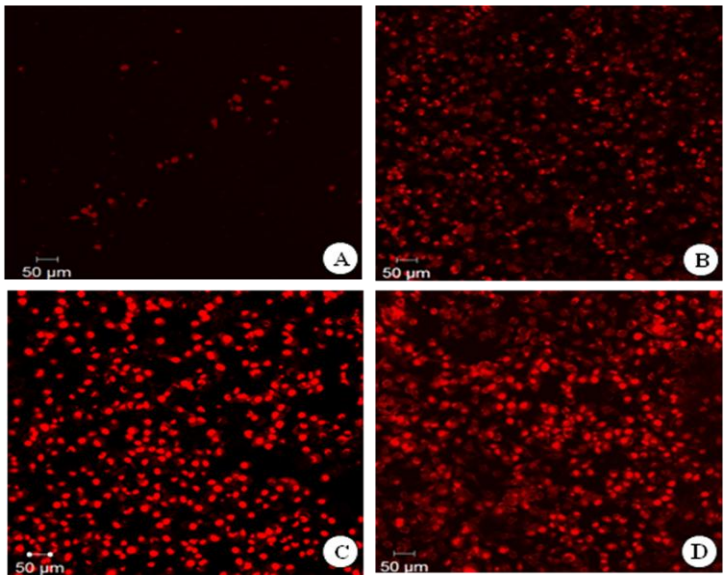

Fig. 6 Shows the Nuclear localization of MDA-MB-231 cells by propidium iodide staining with control and crude venom treated

A- Control cells

B- 200 µg/ml venom for 24 hours

C- 200 µg/ml venom for 48 hours

D- 200 µg/ml venom for 72 hours

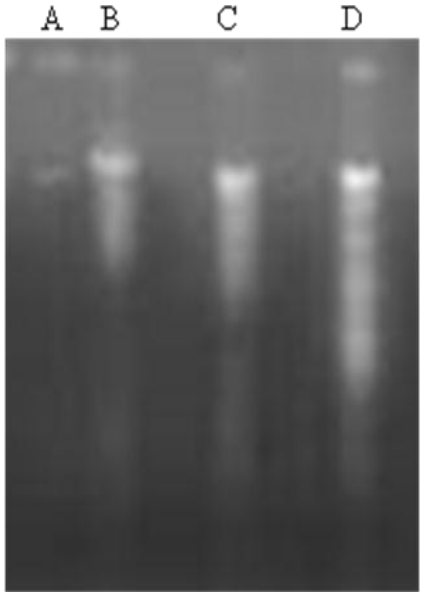

Fig. 7 DNA fragmentation analyses of *A. flagellatus* nematocyst crude Venom control and treated MDA-MB-231 cells.

A- Control cells

B- 200 µg/ml venom for 24 hours

C- 200 µg/ml venom for 48 hours

D- 200 µg/ml venom for 72 hours

Cell cultures have been used as a useful model to evaluate gene expression and to establish *in vitro* experiments to control the tumor growth [31]. One of the major defects in cancer is the lack of cells driven into the apoptotic mode, due to malfunction of molecules like ras [32], Bcl_2 [33], telomerase [34]. P^{53} [35] and caspases [36].

The crude venom produced cytotoxicity and also showed a growth inhibition activity in a dose and time dependant manner. The viability of the cells was assessed by the MTT assay and the cytotoxic nature of the cells was confirmed by changes in the morphological character of the cells. At the end of 48 h, 50% viability was seen with a concentration of 200 µg/ml for crude venom. Our, finding are in accordance with previous data from Mariottini et al., [37].

The morphological changes in an apoptotic cell indicate the activation of proteolytic enzymes which eventually mediate the cleavage of DNA. Several studies have demonstrated a positive association between DNA fragmentation and apoptosis [38]. Apoptosis has been shown to be a significant mode of cell death after cytotoxic drug treatment [39]. When cells undergo apoptosis, several classical phenomena can occur, including membrane blebbing, condensation of cytoplasm and the activation of end nuclease/ specific proteases. Later on, the genome will be fractured, and smaller apoptotic bodies will be formed [40]. Macrophages will phagocytose the apoptotic cell. Through the process of apoptosis, unwanted or dysfunctional cells can be eliminated. As an important tumor suppressor gene, p53 is involved in cell-cycle regulation [41]. One of the functions of the wild-type p53 protein is to cause G_1 arrest, thus permitting efficient repair of damaged DNA before the initiation of the cell DNA synthesis (G_1 arrest) thus avoiding the propagation of mutagenic lesions and possible neoplastic transformation. The wild-type p53 regulates cycle progression by the induction of its downstream target gene WAF1/CIP [42 and 43].

Meanwhile, we measured apoptosis using flow cytometry. Apoptosis in breast cancer cells (MDA-MB-231) was increased in the jellyfish *A. flagellatus* crude venom treated group. It is possible that apoptosis contributes to the cytotoxic effect of crude venom changes in cells associated with the early phase of apoptosis induce a loss of cell membrane. The effect of crude venom MDA-MB- 231 cells was analyzed using fluorescien-labeled PI-stained cells. The possible role of apoptosis in present study was examined with Propidium iodide staining of treated and untreated cells. The nuclear fragmentation clearly demonstrates the role of apoptosis in *A. flagellatus* crude venom treated cells. In this result it was clearly observed that DNA fragmentation shows apoptosis in MBA-MB-231 cell line.

CONCLUSION

In conclusion, we isolated crude venom from *A. flagellatus* to evaluate the cytotoxic activity using MDA-MB-231 cells. Further the purification and determination of crude protein and biochemical investigation in progress to characterize the protein nature of *A. flagellatus* nematocyst to identify the extracts of clear mechanism in action. Overall, *A. flagellatus* venom may has the potential to serve as a template for future anticancer-drug development. Further analyses are warranted and necessary to substantiate our findings.

ACKNOWLEDGEMENTS

The authors are thankful to University Grants Commission-Basic Scientific Research (UGC-BSR), Delhi, India, for providing the meritorious fellowship.

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