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ANTIPROLIFERATIVE EFFECT OF CRUDE PROTEINS EXTRACTED FROM MARINE CLAM *DONAX VARIABILIS* ON HUMAN CANCER CELL LINES

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Keywords:

Clam, Protein extract, Antiproliferative activity, Human cancer cell lines, Apoptosis

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ABSTRACT: Marine environment is a huge reservoir of various important resources for new bioactive molecule discovery from marine organisms. The aim of this investigation is to evaluate the anti-proliferative potential of crude proteins extracted from different organs of *Donax variabilis* (gills, adductor muscles, mantle and whole tissue) on human Lung (A549), oral (KB3-1), breast (MCF-7) and colon (HT-29) cancer cell lines. The crude proteins extracted from four different organs of marine clam were biochemically and qualitatively analysed using Native and SDS PAGE. These extracts were tested against normal (vero) cell line to determine the toxicity of crude proteins. Among the four organs, proteins from mantle potentially inhibited growth of lung, oral and breast cancer cell lines in a dose dependent manner and inhibition of growth in colon cancer cell line was time dependent when examined by MTT assay. Significant inhibition of cell viability on A549 cells observed at (IC_{50}) 100 µg/ml, KB 3-1, MCF-7 cells at (IC_{50}) 250 µg/ml concentration after 24 h of incubation and HT-29 at (IC_{50}) 500 µg/ml concentration after 48 h of incubation. The crude (mantle) protein treated cancer cell lines showed modifications in cell morphological features. Further investigation on nuclear morphology was done by propidium iodide staining under fluorescence microscope and DNA ladder assay showed fragmented pattern of DNA that confirms induction of apoptosis. On observation, it can be concluded that crude extract (mantle) from *D. variabilis* highly effective against human cancer *in-vitro*.

INTRODUCTION: Over past few decades cancer is one of the most lethal diseases in human being and it has become a worldwide health issue. Cancer is the generic term for a large group of disease characterized by the growth of abnormal cells and they can invade other organs beyond their usual boundaries.

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Cancer cells have some distinct features which isolate them from normal cells and make them to cause threats to life. These features are sustained proliferative signalling, evading growth suppressor, resisting apoptosis, replicative immortality including angiogenesis and metastasis [1].

Lung cancer is a prevalent cancer in both men and women and became leading cause of cancer related death globally. Much progress has been made in the treatment of lung cancer. However, is not amenable to chemotherapeutic, radio therapeutic and the survival rate is extremely poor due to the extensive metastasis. In Indian subcontinent especially in India itself oral cancer is main public

health challenge. More than 100,000 cases of oral cancer reported all over India [2]. Colon cancer is the second leading cause of cancer death in western countries [3]. Along with all other countries, India is also facing the threats of breast cancer. Breast cancer is the second most common cancer preceded by cervical cancer among women in India. Due to various genetic and epigenetic alterations which effect the regulation and gene function causes breast cancer.

There is a lots of drugs and techniques are present to combat cancer but none of these are able to cure cancer completely. So researchers are looking for anticancer drugs from natural sources. Not only for anticancer drug, anti-microbial and anti-inflammatory compounds also discovered. Sea life is harsh, compared to terrestrial environment. Marine organisms are facing extreme condition of temperature, salinity, pressure and often anoxic and hypoxic conditions. They adopt themselves through complex biochemical and biophysical process. *Donax variabilis*, a bivalve mollusc from the Donacidae family, is mainly inhibit southern part of India in Bay of Bengal. The aim of this investigation is to extract a protein from different organs of *D. variabilis* and to determine its inhibitory effect on the proliferation of human cancer cell lines.

MATERIALS AND METHODS: Tris-HCl, SDS, Coomassie brilliant blue R-250, Dimethyl sulfoxide (DMSO), MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Penicillin-Streptomycin, Dulbecco's Modified Eagle's medium (DMEM), Phosphate buffered saline (PBS), Trypsin, Fetal Bovine serum (FBS), Molecular weight markers were obtained from Himedia Chemical Co. All chemicals and reagents used were of analytical grade.

Sample Collection: The live samples of *D. variabilis* were collected from light house, Chennai and it were identified by Zoological survey of India, Chennai. The different organs were dissected, weighed and stored at -20 °C.

Preparation of Crude Extract from Experimental Animal: The different organs of *D. variabilis* (100 mg) was washed with 4 °C tap water three times, followed by washing with 4 °C distilled water three times. It was then homogenized with double volume of PBS (10 mM, pH 8.0) at 4 °C for 3 min. After centrifugation (16,000 g, 20 min) at 4 °C, the supernatant was collected and the crude extracts obtained. Then the collected crude extract was lyophilized for further use.

Estimation of Biochemical Constituents: The Biochemical composition such as protein, Lipid and carbohydrate were estimated by following standard methods. The extracted protein was estimated by the method of Lowry *et al*., (1951) using Bovine serum albumin as a standard [5]. The extraction of lipid from the experimental animal was done according to Folch *et al*., (1957) [6] and the estimation was done according to Barnes and Blackstock (1973) [7]. The Estimation of carbo-hydrate was done according to the Roe (1955) [8].

Analysis of Protein Profiles: Native poly-acrylamide gel electrophoresis (Native-PAGE) was carried out using 10% separating and 5% stacking gel [9]. The Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed with Laemmli [10] gel with an acrylamide concentration of 5% for the stacking gel and 10% for the running gel. Forty micrograms of protein sample was loaded on the gel. Protein bands were detected by the Coomassie blue staining method.

Cell Lines and Culture: Cell lines used for evaluation of the *in-vitro* cytotoxicity in this study included four cancer cell lines and one normal cell line, namely A549, HT-29, KB3-1, MCF 7, and Vero cell line. All of the cell lines were obtained from National Centre for Cell Science (NCCS), Pune and the cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 µ/ml penicillin and 100 µ/ml streptomycin cultured in an atmosphere of 5% CO_2 at 37 °C.

Evaluation of Cytotoxicity: Cultured cells were taken during their exponential growth phase. For the anticancer screening tests, crude protein extracts were dissolved in DMEM medium. The concentrations of crude protein tested was 50, 75, 100, 250, 500, 750 and 1000 µg/ml on Lung, Oral, Breast cancer cells for 24 h and the HT-29 cells were treated for 24 and 48 h with the mentioned

concentration. The toxicity assessment on normal cells (African green monkey kidney cells) were treated with the same concentration for 24 h. All the tests were performed by MTT assay [11]. Briefly, 5×10^3 cells were seeded in the wells of 96-well microtiter plates and incubated with different concentrations of crude protein at different time intervals. Ten micro litre of MTT (5 mg/mL) was added to each well and the plates were incubated for another 4 h at 37 °C. The supernatant was aspirated and formazan crystals were dissolved in 200 µL of DMSO. Absorbance was measured spectrophotometrically at 570 nm. Cell viability was evaluated by comparing the absorbance of treated and untreated cells. The percentage of cell growth inhibition was calculated as the following formula.

$$\text{Cell viability} = \frac{A^{570} \text{ of treated cell}}{A^{570} \text{ of control cells}} \times 100$$

Cell Morphological Study: The general cell morphological changes of *D. variabilis* crude protein treated lung cancer (A549) oral cancer (KB 3-1), breast Cancer (MCF-7) and colon cancer (HT-29) cells plated in 40 × 10 mm dishes with DMEM medium containing 10% FBS. The cells were incubated for 24 and 48 h under 5% CO_2, and 95% O2 at 37 ºC. The medium was removed and the control dishes received fresh medium and the treatment dishes received IC_{50} and high (µg/ml) concentrations of crude protein of *D. variabilis*. The culture plates were incubated and the cells were visualized and photographed under inverted light microscope (Nikon, Eclipse TS 100) at 20X magnification.

Nuclear Morphological Observation: Fluorescent staining of apoptotic nuclei was performed by the method of Keum *et al*., (2002). Cancer cells (5×10^4 cells/ml) were seeded in 6 well plates with DMEM medium containing 10% FBS. The cells were incubated for 24 hand 48 h under 5% CO_2, 95% O_2 at 37 ºC. The medium was removed and the control well received fresh medium and the treatment plates received IC_{50} and high (µg/ml) concentrations of potentially active crude protein extracts of *D. variabilis*. Then the culture plates were incubated and the cells were washed with PBS and fixed in methanol: acetic acid (3:1 v/v) for 10 min and stained with 50 µg/ml of propidium iodide for 20 min. After staining, the cells were visualized immediately under florescence microscope at 20X magnification.

DNA Fragmentation Analysis: DNA extraction and agarose gel electrophoresis were performed by the method of Luisa *et al*., (2006). The selected cell lines were plated in a 6 well plate at a density of 3×10^5 cells and treated with potentially active crude protein extracts of *D. variabilis* at 37 ºC for 24 and 48 h. The cells attached at the bottom were scraped off and collected together with unattached cells by centrifuging at 1500 g for 5 min at 4 ºC. The DNA was extracted from pelleted cells. The cells were lysed with lysis buffer and extracted with phenol, chloroform and isoamyl alcohol in the ratio of 25:24:1, the aqueous supernatant was precipitated with double the volume of ice cold ethanol with $1/10^{th}$ volume of 3M sodium acetate.

RESULTS:

Biochemical Analysis: Analysis of various biochemical constituent in crude extract from four different organs of *D. variabilis* were estimated **Fig. 1**. Total protein contained in whole tissue, adductor muscle, mantle and gill are 1.53 mg, 0.6 mg, 0.96 mg and 1.07 mg/100 mg respectively. Lipid content was estimated in all four organs, whole tissue contained 0.17 mg /100 mg, adductor muscle was found to be 0.19 mg in mantle and gill the amount of estimated lipid was 0.17 mg and 0.16 mg/100 mg respectively. After carbohydrate estimation carbohydrate content in whole tissue, adductor muscle, mantle and gill were 0.21, 0.03, 0.14 mg and 0.05 mg/100 mg respectively.

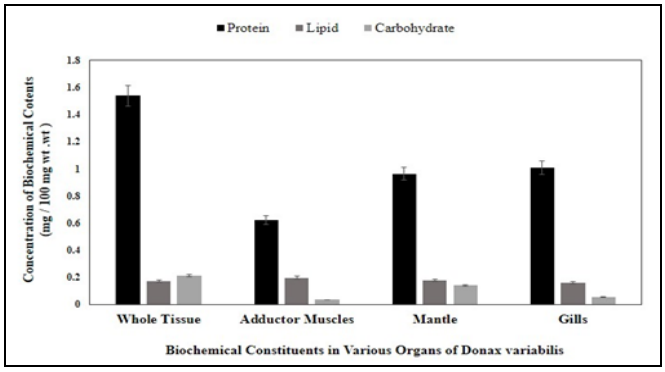

FIG. 1: BIOCHEMICAL COMPOSITIONS (PROTEIN, LIPID AND CARBOHYDRATE) (µg/100 mg WET WT.) OF MARINE CLAM *DONAX VARIABILIS*

Qualitative Analysis of Crude Protein from Marine Clam *D. variabilis*: Qualitative analysis of

crude protein extracted from *D. variabilis* was done by Native and SDS-Polyacrylamide gel electrophoresis (PAGE). Native PAGE showed different distinct bands in all four crude protein samples extracted from four organs. SDS-PAGE revealed that crude protein extracted from different organs whole tissue, adductor muscles, mantle and gills were resolved and different subunits of native protein was visualized as a bands after coomassie brilliant blue (CBB) staining which indicates the presence of polypeptides in each sample. These polypeptides range from 8 kDa -72 kDa **Fig. 2** [1].

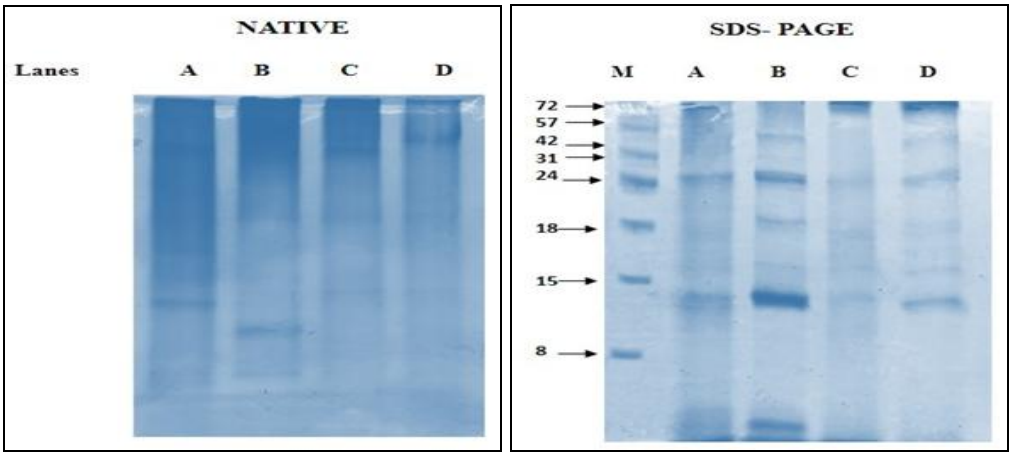

FIG. 2: QUALITATIVE ANALYSIS OF CRUDE PROTEIN EXTRACTED FROM VARIOUS ORGANS OF *DONAX VARIABILIS*. LANES: A-WHOLE TISSUE, B- ADDUCTOR MUSCLES, C-MANTLE D-GILLS, M- MARKER

Toxicity Assessment: Toxic effects on normal cells of crude proteins extracted from all four organs were tested upon vero cell line (green African monkey kidney cells) by MTT assay. Seven different concentration 50, 75, 100, 250, 500, 750 and 1000 µg/ml of crude protein from *D. variabilis* were applied on vero cell line and incubated for 24 h. In all cases results showed that even at maximum concentration (1000 µg/ml) more than 80% cells were viable **Fig. 3**.

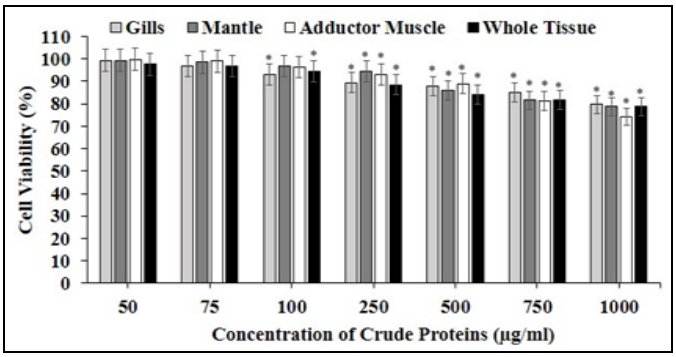

FIG. 3*: IN VITRO* CYTOTOXICITY ASSAY OF CRUDE PROTEINS FROM *DONAX VARIABILIS* TREATED AGAINST NORMAL CELL (VERO) LINE AT 24 h

Anticancer Activity: Anticancer activity of the crude proteins extracted from different organs of *D. variabilis* was investigated against A549, KB 3-1, HT-29 and MCF-7 cell lines. Among all four crude proteins from four different organs, proteins from mantle showed significant activity on A549, KB 3-1 and MCF- 7 in dose dependent manner. In Lung cancer cells (A549) it was found that crude protein extracted from mantle was the most effective and the IC_{50} value was recorded to be achieved at 100µg/ml concentration. IC_{50} value was found at 250µg/ml of mantle crude protein extract on oral cancer (KB 3-1) cells and breast cancer cells (MCF-7) after 24 h incubation. In HT-29 cells after 24 h incubation no significant cell death was noticed. Cell death was observed at 48 h incubation and it was found that 50% cell death was achieved at 500 µg/ml of mantle crude protein. Further studies were carried out using crude protein extracted from mantle for its significant anticancer activity on cell lines.

Cell Morphology: Microscopical observations of control cells and cancer cells treated with crude protein extracted from mantle was visualized using inverted microscope. All cell lines (A549, KB3-1, MCF-7 and HT-29) at IC_{50} concentration showed few shrunken cell and they acquired spherical shape. At highest concentration (1000 µg/ml) all most all cells were shrunk and found to detach from each other and their original morphology was totally disrupted. In control cells, to which no drugs were given. In control cells full confluence was observed with intact original cell morphology **Fig. 5.**

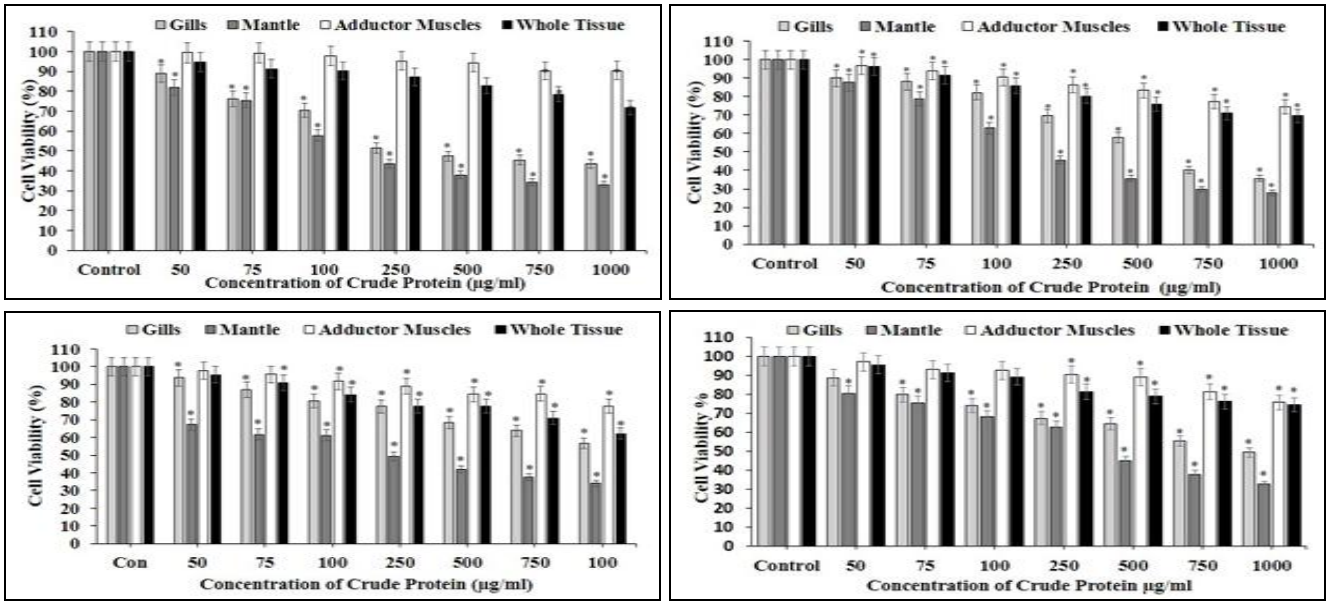

FIG. 4: ***IN-VITRO*** **CYTOTOXICITY ASSAY OF CRUDE PROTEINS FROM** ***DONAX VARIABILIS*** **TREATED AGAINST VARIOUS CANCER CELL LINES AT 24 AND 48 h (A) LUNG, (B) ORAL, (C) BREAST AND (D) COLON**

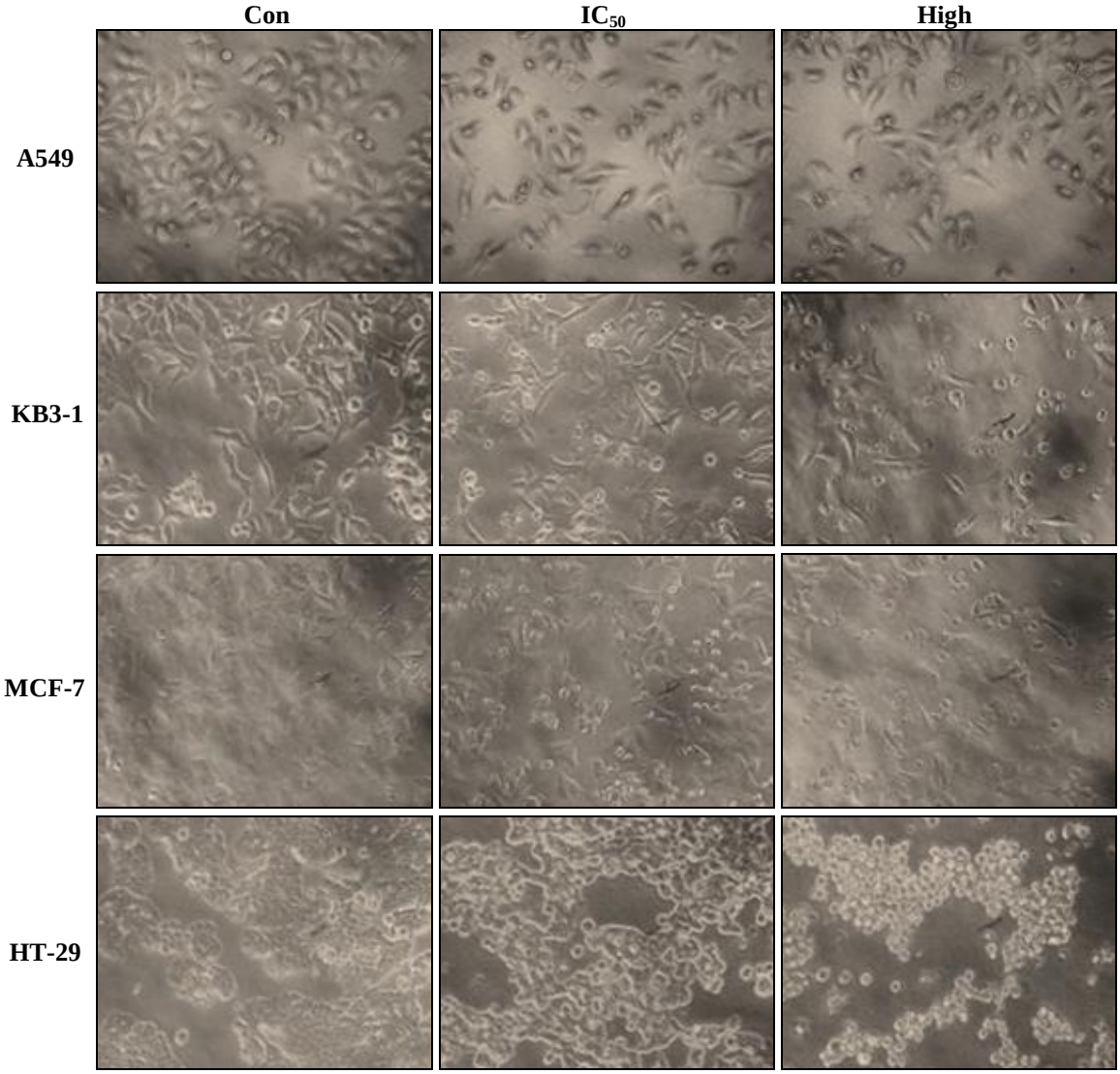

FIG. 5: MORPHOLOGICAL OBSERVATIONS (20 X) OF HUMAN LUNG, ORAL, BREAST AND COLON CANCER CELL LINES TREATED WITH CRUDE PROTEIN EXTRACTED FROM MANTLE OF ***DONAX VARIABILIS*** **AT 24 AND 48 h**

Nuclear Morphology: Nuclear morphology of the treated cells along with the control one was done by using Propidium Iodide staining method. In all cases like A549, KB3-1, MCF-7 and HT-29 cell lines control cells showed intact spherical nucleus. At IC_{50} concentration almost half of the cells showed fragmented nucleus along with intact nucleus but in case of cells treated with highest concentration revealed all cells with fragmented nucleus [13].

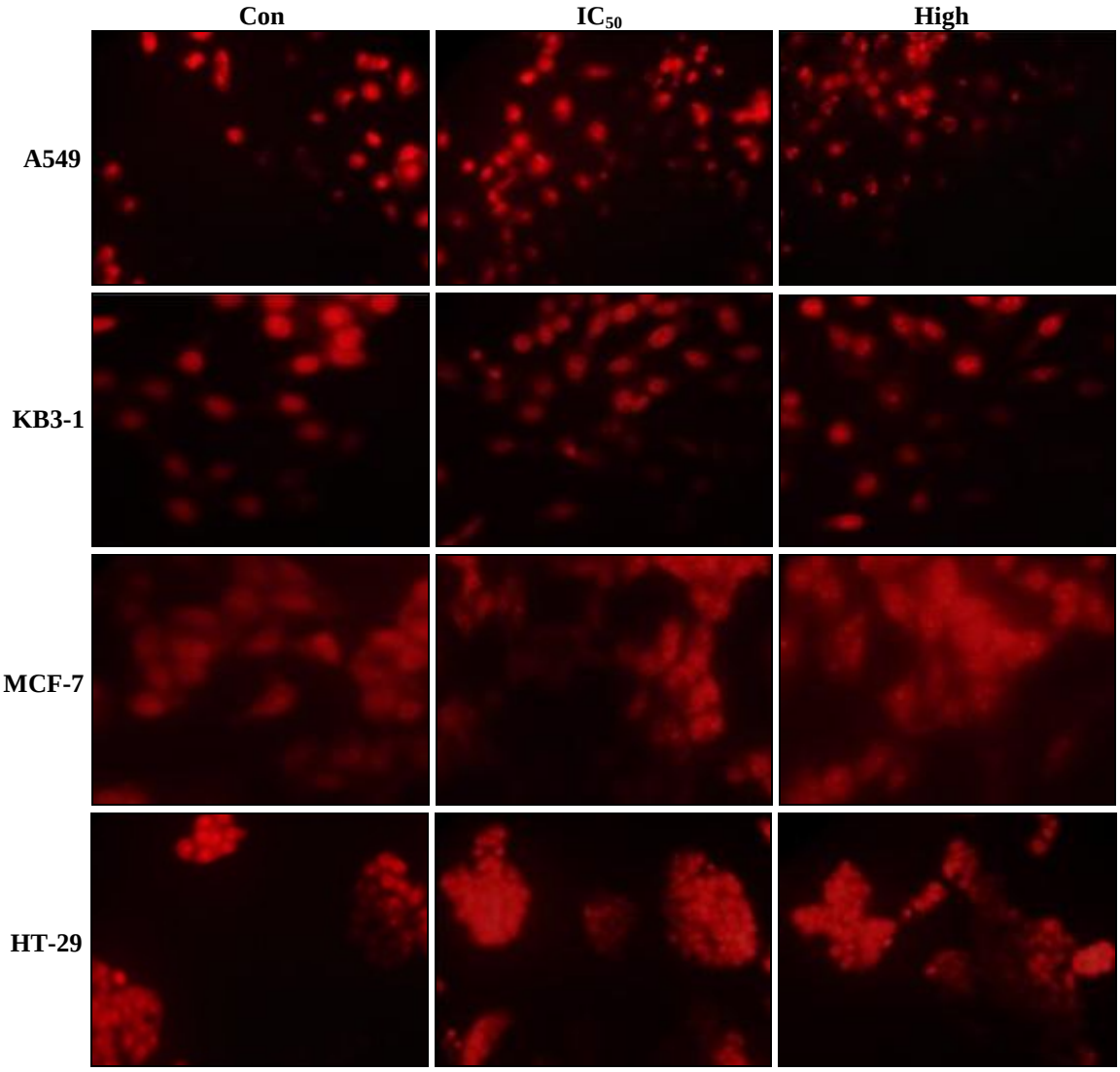

FIG. 6: NUCLEAR MORPHOLOGICAL OBSERVATION (40 X) OF HUMAN LUNG, ORAL, BREAST AND COLON CANCER CELL LINES TREATED WITH CRUDE PROTEIN EXTRACTED FROM MANTLE OF *DONAX VARIABILIS* AT 24 AND 48 h

DNA Fragmentation: For further insight on cell death and anticancer activity of crude proteins extracted from mantle of *D. variabilis* treated on different cancer cell lines was confirmed by DNA fragmentation analysis.

After treatment upon A549, KB 3-1, MCF-7 for 24 h and HT 29 for 48 h incubation with IC_{50}concentration (100 µg/ml for A549, 250 µg/ml for KB 3-1 & MCF-7 and 500 µg/ml for HT 29) and highest concentration (1000 µg/ml) were taken to agarose gel electrophoresis technique.

One single band of whole genomic DNA from control cells was found and streaks in other two lanes *i.e.* treated with IC_{50} and highest concentration which indicates the occurrence of DNA fragmentation [12].

Statistical Analysis: The experimental data were expressed as the mean ± standard deviation. Statistical analysis was carried out using statistical package for social sciences (SPSS) with 95% confidence level to find out the variation between the experimental sets and with the control.

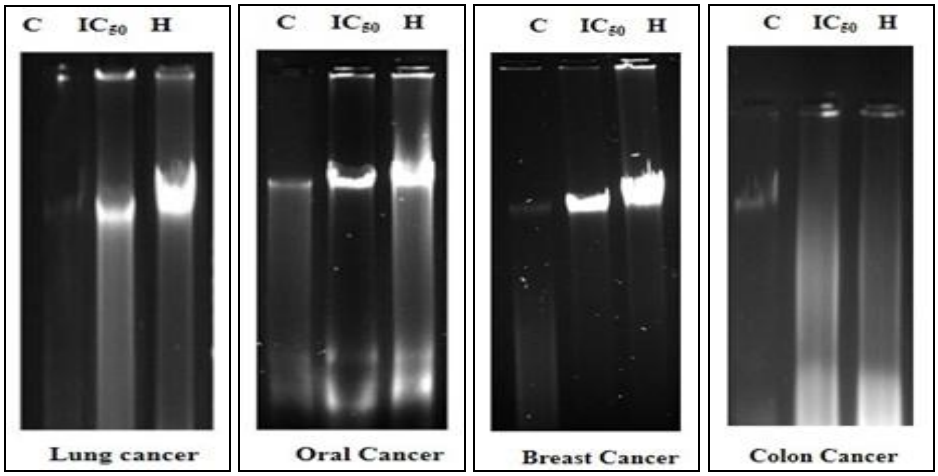

FIG. 13: DNA FRAGMENTATION ANALYSIS OF CRUDE PROTEIN EXTRACTED FROM MANTLE OF DONAX VARIABILIS TREATED ON LUNG, ORAL, BREAST AND COLON CANCER CELL LINES AT 24 AND 48 h

A) Control cells; B) IC_{50} Concentration Lung cancer (100 µg/ml), Oral cancer (250 µg/ml) Breast cancer (250 µg/ml) and Colon cancer (500 µg/ml); C) Maximum Concentration (1000 µg/ml for three cell lines)

DISCUSSION: Marine organisms are treasure of bioactive compounds due to their harsh peripheral environment which is totally differed from our terrestrial environment. Recently, lots of investigations have focused on bioactive proteins and peptides from marine sources. Many bioactive peptides with anticancer potential have been extracted from various marine animals like sponges, tunicates, soft corals, nudibranchs, sea hares, bryozoans, sea slugs and other marine organisms. These bioactive compounds are being used as a drug for cardiac diseases, hypertension, diabetes, cyst *etc*. Not only in recent time but marine bivalves have been used from the ancient time as traditional Chinese medicine. On this basis recent researches are going on to find some bioactive anticancer compound with the aim to treat cancer without producing any side effects [14].

In early reported articles it has found that Cyplisin, a 56 kDa protein isolated from *Aplysia punctate*, has shown cytotoxicity against several cancer cell lines. MML, a 40 kDa protein from *M. meretrix* also having inhibitory effects on the proliferation of human hepatoma apparently by increasing the permeability of the cell membrane and inhibiting tubulin polymerization [15]. In our current study, crude proteins were extracted from four different organs namely mantle, gills, adductor muscles and whole tissue of marine clam *Donax variabilis* with the aim of finding an anticancer protein. Extraction of crude protein followed by biochemical analysis were done using four different organs. Crude protein sample extracted from different organs were subjected to qualitative analysis by NATIVE and SDS Polyacrylamide Gel Electrophoresis (PAGE). To study the toxicity, normal (vero) cell line were selected. In this upto particular concentration there is a less amount of toxicity, which indicates once increasing the concentration which affect the normal cells also. After confirming this we further went for cytotoxicity. Crude protein from mantle showed highest activity on all cancer cell lines lung cancer, oral cancer, Breast Cancer and colon cancer cells in dose dependant and time dependant manner.

Lung cancer cells strongly inhibit the growth at 100 µg/ml at 24 h incubation. For oral cancer and breast cancer cells we got IC_{50} (at which concentration 50% cell death occurs) value at 250 µg/ml (24 h incubation) and for colon cancer IC_{50} value was achieved at 500 µg/ml at 48 h. The inhibitory activities of crude protein extracted from mantle were providing the evidence for *in-vitro* cytotoxicity. Likewise at a concentration of 52.5 µg/ml, column purified anticancer protein strongly inhibit the growth of BEL-7402 cells, MCF-7 and HCT1 [16]. Takahashi *et al*., (2008) reported a protein with antimicrobial activity of 58 kDa from clam (*Ruditapes philippinarum)* [17] and Hoarau found fractions of 20 to 28 kDa with detoxify activity [18]. Spisulosine protein (MW-285 Da) isolated from *Spisula polynyma* showed anti-proliferative activity on CVI cell line [19]. Another two peptides BEPTII, BEPTII-I from *Bullacta exarata* reported to have apoptotic effect on prostate cancer (PC3) cell line [20].

From *Arca subcrenata* two peptides P2 [21] and H3 has isolated separately. P2 found to have anticancer activity on cervical cancer (HeLa) and colon (HT 29) cancer on the other hand H3 has proved to have antioxidant activity along with antiproliferative activity on colon (HT 29) and hepatocarcinoma (HepG2) cancer [22]. Another polypeptide J2-C3 isolated from clam *Arca inflata* reported to show antitumor effect on lung (A549) cancer, colon cancer (HT 29) and hepatic carcinoma (Hep G2) [23]. Peptide (MW-3147 Da) from *Meretrix meretrix* showed anticancer activity upon BGC-823 [24].

For further investigation general morphology and nuclear morphology were observed. In general morphology the cell population were decreased based on detachment of cells from the substratum and cells become shrinked. But in control cells the original morphology were observed. In nuclear morphology mantle protein treated all four cell lines has fragmented nuclei, in the case of control cells whole intact nuclei were observed. In the present study DNA fragmentation was appeared in the crude mantle extracts treated with all four cancer cells at IC_{50} concentration. There was a no fragmentation in control cells. The DNA is degraded into oligonucleosomal fragments is a late event of apoptosis [16]. The crude protein extracted from mantle induces DNA damage in A549, KB3-1, MCF-7 and HT-29 cells and thereby causes apoptosis from this observation it is strongly inferred that crude protein extracted from mantle exert an anticancer effect through DNA damage in all four cancer cells and promote apoptosis. Likewise a novel antitumor protein from the coelomic fluid of Meretrix meretrix Linnaeus inducing apoptosis in BEL-7402 cells using VP16 as a positive control and this was characterized by fragmentation of DNA on the agarose gel [15].

CONCLUSION: The present study reveals that the crude protein extracted from mantle of *D. variabilis* having potential anticancer activity on all four human cancer cell lines (A549, KB 3-1, HT-29 and MCF-7). Even experiments showed that the crude protein extracted from mantle did not possess any toxic effect on normal (vero) cells. After considering all above observations of results we conclude that it is possible that active fraction of this crude protein extract is a potential anticancer drug in near future, before that we need to go through purification and characterization of this crude protein. For further enquiry we have to test our drug on *in-vivo* models.

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CONFLICT OF INTEREST: There is no conflict of interest.

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