International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2015): 78.96 | Impact Factor (2015): 6.391

Anticancer Activity of Protein Extract from *Perna viridis* (Green Mussel) and *Meretrix meretrix* (Great Clam)

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Abstract: ***A large proportion of the sea offers untapped sources of potential drugs with promising activities due to a large diversity of marine habitats and environmental conditions. The natural products isolated from molluscs and their structural analogues are particularly well represented in the anticancer compounds in clinical traits. The protein extract from molluscs has several advantages as a very promising material for antimicrobial and antitumor drugs without any side effects. Many bioactive components such as peptides, proteins, enzyme and enzyme inhibitors have been purified and identified from Meretrix meretix in recent years. They have their functional effects including antihypertension, hypolipidemic, antineoplastic and antioxidant effects have been proved. The species Perna viridis commonly known as the Indian green mussel is a widely distributed edible mytilid seen all along both east and west coast of India. It is been sparsely included in pharmacological studies and found to be active against all influenza, herpes and hepatitis viral strains. P.viridis had cancer preventive effects, antimicrobial, antifungal, antiviral, antihyperglycemia, anti-inflammatory and antiparasitic activities. In this present study, protein was extracted from P.viridis and M.meretrix, washed, dialyzed and observed in SDS-PAGE and assayed for anticancer activity against Human lung cancer cell lines(A549) and Cytoxocity for Vero cell lines(green monkey kidney). Protein extract from P.viridis had more bands than protein from M.meretix. The crude protein from mussel killed cancer cells (A549) upto 99.1% whereas clam killed cancer cells upto 96.2%. The extracted crude protein from mussel had the cell viability of 13.6% in 1:1 and 80.9% in 1:32. But the crude protein from clam had cell viability of 5.4% in 1:1 and 71.8% in 1:32 dilution. The result shows that crude protein from green mussel had more anticancer effects than clam. Perna viridis had less cytoxocity towards Vero cells than Meretix meretix.***

Keywords: *Perna viridis, Meretrix meretrix,* Protein extract, Anticancer

1. Introduction

Cancer has been one of the major causes of death in last couple of centuries and is the second major cause of non-communicable deaths, worldwide [29]. The varied geographical distribution of human population has never been a limiting factor for the incidence of cancer. People belonging to nations from third world, developing or the developed countries, all get victimized. This burden of cancer is increasing worldwide despite advances in diagnosis and treatment [10]. By 2020, the world population is expected to have increased to 7.5 billion; of this number, approximately 15 million new cancer cases will be diagnosed with an estimated 12 million deaths [5]. In India, cancer is the second most common cause of death, growing at 11 percent annually. There are about 2-2.5 million cancer cases in the country with 7-9 lakhs new cases added every year [25]. One in five Indian men dies between age 30 and 69 due to tobacco-related cancers [9].

In India, the most prevalent cancers- breast, cervical and oral cancers are largely detected at later stages when it is too late for effective treatment [31]. Worldwide, lung cancer kills over one million people each year. In India, it is estimated that there are approximately 2-2.5 million cases of cancer in the country at any given time and numerically it is the number one in terms of mortality amongst the Indian males. Lung carcinogenesis is a multistep and multicentre process, characterized by stepwise accumulation of genetic and molecular abnormalities after carcinogen exposure, resulting in the selection of clonal cells with uncontrolled growth capacities .The carcinomas of lung arise either from the alveolar lining cells of the pulmonary parenchyma or from the mucosa of the tracheo-bronchial tree [4]. Approximately 22,000 natural products of marine origin have been discovered so far, whereas 131,000 terrestrial natural products exist. The major sources of biomedical compounds are sponges (37%), coelenterates (21%) and microorganisms (18%) followed by algae (9%), echinoderms (6%), tunicates (6%), molluscs (2%) bryozoans (1%), etc. [22].

The first drug obtained from the sea, Ziconotide(ω-conotoxin MVIIA), is a peptide originally from a tropical marine cone snail. Molluscs are widely distributed throughout the world and have representative in the marine and estuarine ecosystem namely slugs, clams, mussels, oysters, scallops, squid and octopus etc [6].

The natural products isolated from molluscs and their structural analogues are particularly well represented in the anticancer compounds in clinical trials [30]. The use of certain molluscs, such as terrestrial pulmonates, in medicinal remedies dated to ancient Rome [1]. Molluscs also feature in a number of traditional medicines from South Africa [11], India [26] and China [12]. Several molluscan derived therapies are listed on the homoeopathic *Materia Medica* [27]. In many cultures, shelled bivalves are used as

traditional natural remedy for fever [3]. Bivalve mussels (Mytillidea) were used as therapy in ancient Crete [14] and more recently have been subjected to several patents as a source of antimicrobial and antiviral peptides [7].

M. meretrix was documented in the ancient Chinese Pharmacopoeia Compendium of material (the 16th century, by Li Shizhen) which stated that it would diminish inflammation, treat typhoid fever, hangover and relieves pain [34]. Another ancient Chinese medicinal book, Treatise on Fevers (second century, by ZHANG Zhongjing) stated that *M.meretrix* has special activities of eliminating cyst and detoxification of human body [35]. Many bioactive components such as peptides, proteins, enzyme and enzyme inhibitors have been purified and identified from *M.meretix* in recent years. The clams (*Meretrix meretrix*) have hypolipidemic, antineoplastic and antioxidant effects which have been proved [13].

The clam shells rich in calcium are used as poultry feed. The species *Perna viridis* commonly known as the Indian green mussel is a widely distributed as an edible Mytilid, which was seen all along both east and west coast of India [32]. The natural products from *Perna viridis* are nontoxic which can be used as cure and control for diabetes mellitus [28]. The extract prepared from the *P.viridis* had previously been found to be active against all influenza, herpes and hepatitis viral strains. It also found to possess not only prophylactic efficient protection from several viral diseases but it also shows a high therapeutic activity against these diseases. The alkaloids extracted from *P.viridis* have been associated with medicinal uses for centuries and one of their common biological properties is their cytotoxicity with possible interaction with cell wall and DNA.

2. Materials and Method

2.1. Collection of Specimen

The mussel *Perna viridis* and clam *Meretrix meretrix* were collected from Kasimedu market, Chennai, Tamilnadu. They were brought to the laboratory in separate plastic bags. The specimens were washed with tap water and cleaned thoroughly to get rid of the attached algae and debris.

2.2. Preparation of crude extract

The shells were removed and the whole tissue of the specimen weighed separately. The weight of the mussel was 18.66g and 14.66g and clam was 34.25g and 28.92g. 10g of each sample was homogenized with 10ml of methanol using mortar and pestle. The extract was centrifuged at 1000rpm for 30 minutes and the supernatant was collected.

2.3. Protein estimation

The protein content of methanol extract was estimated by Lowry's method [21] using Bovine Serum Albumin (BSA) as a Standard. Then the protein was dialyzed by Dialysis membrane and Phosphate Buffer Saline (PBS) which are commercially available. The molecular weight distributed in the protein was determined by SDS-PAGE, according to Lammeli [17]. SDS-PAGE was performed in 10% separating gel and 5% stacking gel.

2.4. Media Preparation

The Minimal Essential Medium (MEM) 1litre preparation
9.5g of MEM was dissolved in 900ml of pre-sterilized double distilled water, mixed well and closed tightly. 3.75g of sodium hydrogen carbonate was dissolved in 50ml of pre-sterilized double distilled water, mixed well and closed tightly. Both the contents were sterilized at 121˚C for 15 minutes. These mixtures were allowed to cool down at room temperature. 0.3g of L-glutamine was weighed and dissolved in 10ml of pre-sterilized double distilled water. 1mg of antibiotics (Streptomycin, Penicillin G and Amphotericin B) each was weighed and dissolved in 1ml of pre-sterilized double distilled water and mixed well. Foetal Calf Serum (10%) was added to media (serum media). All these were checked for pH and adjusted to 7.2-7.4. These were stored at -4˚ C. One aliquot of the prepared MEM was kept for 2 days at 37˚C and checked for sterility, pH drop and floating particles. They were then transferred to the refrigerator.

2.5. Vero Cell Line (Monkey Kidney Cells) :

2.5.1 Trypsin, Phosphate Buffered Saline, Verene Glucose [TPVG] (100ml)
84 ml of PBS, 10ml of trypsin, 10ml of 0.2% EDTA, 5ml of glucose, 1 ml of Penicillin and 1 ml of Streptomycin were taken. All these ingredients were mixed and 100µl (1 mg stock) of antibiotics Streptomycin was added. 5ml aliquot were distributed and stored at -20 ˚ C.

2.5.2 Sub culture of cells
- The medium and TPVG was adjusted to room temperature.
- The tissue culture bottles were observed for growth, cell degeneration, pH & turbidity. The bottles were selected for splitting.
- The mouth of the bottle was wiped with cotton and soaked in spirit.
- The medium was removed using a 10ml pipette.
- The cells were gently rinsed with PBS. 4ml of TVPG (pre-warmed to 37ºC) was added to the cells.
- TPVG was allowed to act for 1-2 minutes and discarded.
- 5ml of TPVG was added to 5% MEM.
- The cell clusters were broken off by gently pipetting back and forth with pipette (Passaging the cells).
- 20ml of growth medium was added to each of the Tissue Culture flask and the Cells were transferred into 96 well plates.

2.5.3 [3-(4, 5-Dimethylthiazol,-2-YL)-2, 5-Diphenyltetrazolim Bromide] MTT Assay:
The Cytotoxicity activity of samples on VERO (Green Monkey kidney cells) & A549 (Human lung cancer) cells was determined by the MTT assay [15]. Cells (1 × 105/well) were plated in 0.2 ml of medium/well in 96-well plates. It was incubated in 5 % CO2 incubator for 72 hours. Then, various concentrations were added to the samples in 0.1%

Dimethyl Sulfoxide (DMSO) for 24 & 48 hrs in 5% CO2 incubator. The Wells were viewed under inverted microscope 40X and the photos were taken. After the sample solution was removed from the well, 20µl of 5mg/mL MTT was added in Phosphate-Buffered Saline solution. After 4hrs incubation, 1ml of DMSO was added. Viable cells were determined by the absorbance at 540nm. Measurements were performed and the concentration required for a 50% inhibition of viability (IC50) was determined graphically. The effect of the samples on the proliferation of VERO & A549 cells was expressed as the % cell viability, using the following formula.

3. Calculation

$$\% \text{ Cell Viability} = \left\{ \frac{\text{O.D of test item}}{\text{O.D of control}} \text{ X } 100 \right\}$$

4. Result

4.1 Protein Estimation

The blue colour observed in the test tube indicates the presence of protein in the samples and this was done by Lowry assay.

4.2 SDS-PAGE

The proteins fractionated into bands were observed and their molecular weight was determined using standard marker. Crude Protein had more bands than the dialyzed protein samples from mussel and clam. Several bands with molecular weights between 14.3kDa to 97.4 kDa were observed in the electrophoresis gel. Previous studies had found that protein with band range from 20kda to 28 kDa had detoxification effect. 40kDa Protein had inhibitory effects on Human Hepatoma. Antimicrobial activity had band range of protein from 56kDa to 58kDa. (**Figure 1)**

4.3 Vero Cell line

The extent of cytotoxicity from every single concentration of anticancer agent was quantified as a percentage of cell viability including the absorbance values obtained. Percentages of cell viability above 80% are considered as non-cytotoxicity; 80%–60% Weak; 60%–40% moderate and below 40% strong cytotoxicity respectively. It may be seen in the histograms that these percentages were high and consequently, these substances were noxious no matter what the concentration that was used. Anticancer activity can be checked using highest percentage of activity in the cells. Mussel had killed more number of cancer cells than clam. So, it is clear that mussel had more potential agents for killing cancer cells than clams. From this study, we found that Mussel have more anticancer activity and cell viability in both Vero cell line and A549. **(Figure 2 to 5 & Table 1 to 4).**

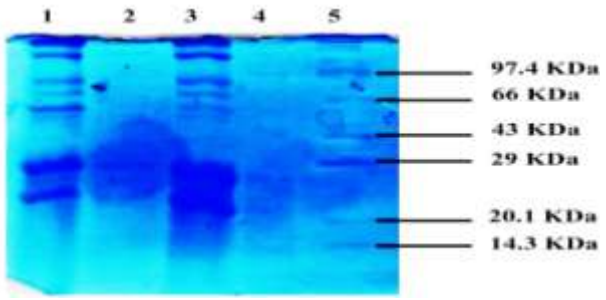

Figure: 1 SDS-PAGE

- LANE 1 – MUSSEL PROTEIN SAMPLE
- LANE 2- MUSSEL PROTEIN SAMPLE (POST DIALYSIS)
- LANE 3- CLAM PROTEIN SAMPLE
- LANE 4- CLAM PROTEIN SAMPLE (POST DIALYSIS)
- LANE 5- LOW RANGE PROTEIN MARKER (14.3 – 97.4 KDa)

5. Discussion

Cancer is still a dreaded disease, which accounts for 9% of the deaths throughout the world. It is one of the 10 leading causes of death today, in India. The magnitude of cancer problem in the Indian Sub-continent (sheer numbers) is increasing due to poor to moderate living standards [23] and inadequate medical facilities. Most frequently observed cancers in Indian population are of lungs, breast, colon, rectum, stomach and liver [33]. Marine molluscs are currently used for a range of therapeutic applications, with purified or synthesized bioactive compounds developed as pharmaceuticals and crude or semi-purified extracts as nutraceuticals [24, 20]. A number of marine molluscs are also used in traditional Chinese, Indian, South African and Middle Eastern medicines [18] as well as in homeopathic remedies. Molluscs used directly as a food source may also contribute to the prevention of disease by providing essential nutrients as well as immuno-stimulatory compounds and other secondary metabolites with direct biological activity [8, 2]. The marine animals have protein which can be used for producing drugs for various diseases. The studies carried out with marine natural products with biochemical potential, had raised the significance of many research groups towards this ecosystem as source of new drugs [16].

Marine organisms represent a large source of new compounds with biological activities. Direct extraction is one way to obtain bioactive compounds from marine organisms; this approach is widely used for isolated and purified biologically active peptides [19]. In this study, Protein extract from *Perna viridis* had more bands than protein from *Meretrix meretrix*. More band formation was seen in crude protein of Mussel and Clam. The dialysis sample had less bands and it is not visible. We found that mussel had killed cancer cells up to 99.1%,

Table1: Mussel (Post Dialysis) Protein on Human Lung Cancer Cell (A549)

S. No	Sample Volume (µl)	Dilution	Absorbance 540nm	% Cell Viability	% Anticancer Activity
1	200	1:1	0.01	0.9	99.1
2	200	1:4	0.05	4.8	95.2
3	200	1:8	0.11	10.5	89.5
4	200	1:16	0.23	22.1	77.9
5	200	1:32	0.54	51.9	48.1
6	Control cells	-	1.04	100	0

Table 2: Clam (Post Dialysis) Protein on Human Lung Cancer Cell (A549)

S. No	Sample Volume (µl)	Dilution	Absorbance 540nm	% Cell Viability	% Anticancer Activity
1	200	1:1	0.04	3.8	96.2
2	200	1:4	0.07	6.7	93.3
3	200	1:8	0.10	9.6	90.4
4	200	1:16	0.17	16.3	83.7
5	200	1:32	0.43	41.3	58.7
6	Control cells	-	1.04	100	0

Table 3: Mussel (Post Dialysis) Protein on Vero cell (Green Monkey Kidney)

S. No	Sample Volume (µl)	Dilution	Absorbance 540nm	% Cell Viability	% cytotoxicity
1	200	1:1	0.15	13.6	86.4
2	200	1:4	0.33	30.0	70
3	200	1:8	0.41	37.2	62.8
4	200	1:16	0.61	55.4	44.6
5	200	1:32	0.89	80.9	19.1
6	Control cells	-	1.10	100	0

Table 4: Clam (Post Dialysis) Protein on Vero cell (Green Monkey Kidney)

S. No	Sample Volume (µl)	Dilution	Absorbance 540nm	% cell Viability	% Cytotoxicity
1	200	1:1	0.06	5.4	94.6
2	200	1:4	0.09	8.1	91.9
3	200	1:8	0.27	24.5	75.5
4	200	1:16	0.52	47.2	52.8
5	200	1:32	0.79	71.8	28.2
6	Control cells	-	1.10	100	0

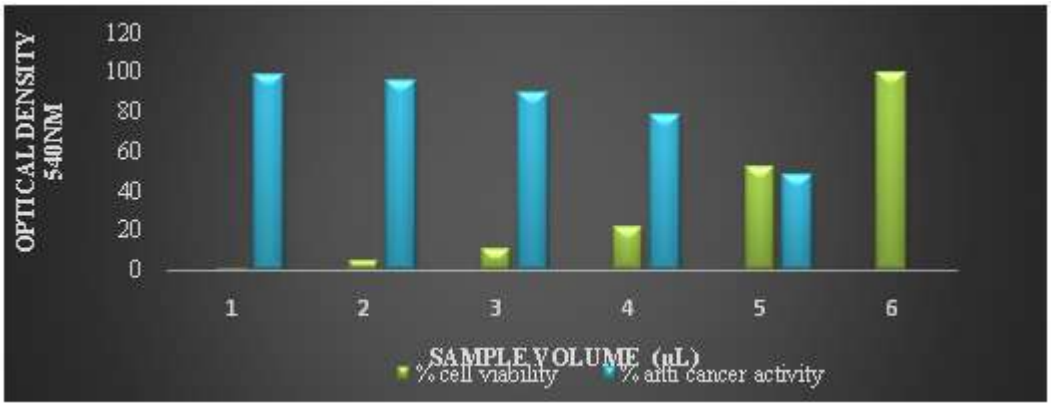

Figure 1: Mussel (Post Dialysis) Protein in Human Lung Cancer Cell (A549)

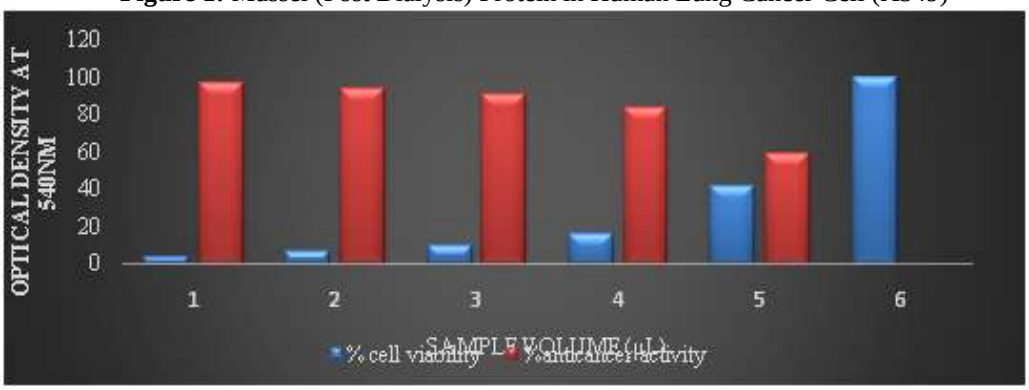

Figure 2: Clam (Post Dialysis) Protein on Human Lung Cancer (A549)

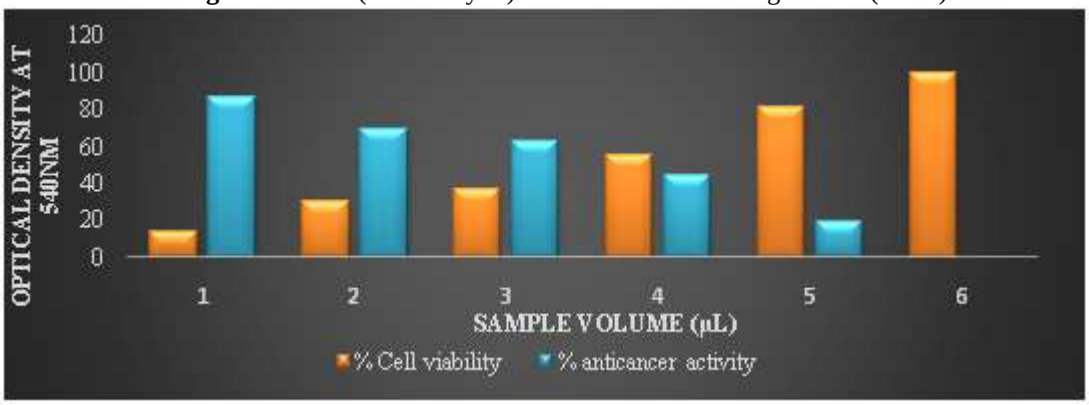

Figure 3: Mussel (Post Dialysis) Protein on Vero Cell (Green Monkey Kidney)

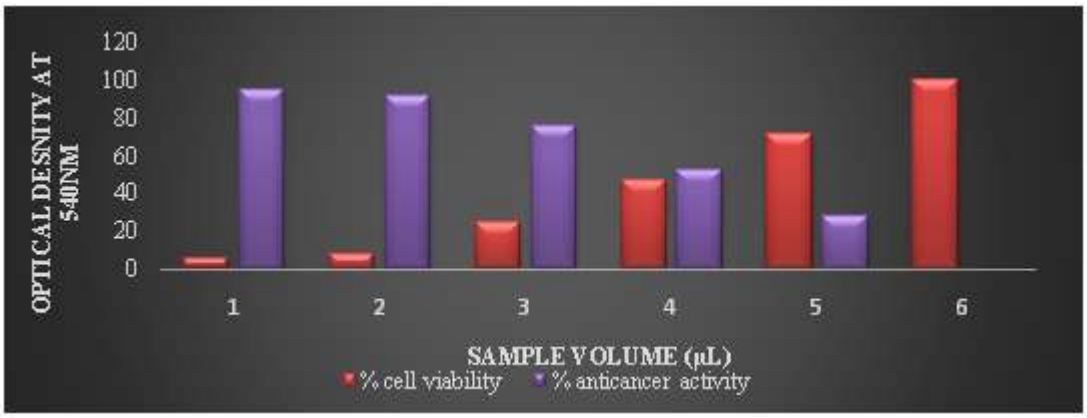

Figure 4: Clam (Post Dialysis) Protein on Vero Cell (Green Monkey Kidney)

and this percentage decreases due to the increase in the dilution of the protein. The marine animal's mussel and clam have more therapeutic uses for humans.

6. Conclusion

Present study indicates that the crude tissue extracts from *P. viridis* could be effectively used as alternative source of antimicrobial and antioxidant with subsequent health benefits. The drugs from marine animal are more effective than any other forms. It is suggested that more research should be done on these marine animals which have more therapeutic uses for humans. The bioactive compounds isolated from the marine animals have anticancer, antimicrobial, antiproliferative effects. The present study suggests that crude protein from Mussel *P.viridis* had more anticancer activity than the crude protein extract from clam *M. meretrix*. Because of partial protein purification, the mussel and clam protein are toxic to the normal cell. But mussel had less cytotoxic effective than clam.

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