4. Anticancer activity of fish *Plotosus lineatus* toxin
INTRODUCTION

Research to discover anticancer drug has been the major focus of natural product researchers worldwide. This is due to the increasing number of cancer patients, the lack of selectivity of most anticancer agents (Miot-Noirault et al., 2004), the development of resistance to drugs (Hutson et al., 2004) and the serious adverse side effects of currently available anticancer drugs (Schilder et al., 2004). Therefore, the need for new cancer drugs is very crucial.

Over the past 10 years, a number of compounds originating from marine invertebrates have entered preclinical and clinical development as cancer therapeutics (Adrian, 2007; Jimeno, 2002). C-nucleosides from the Caribbean sponge (e.g., Cryptotethya crypta) served as the chemical model for synthesis of Cytarabine, which is used in the treatment of leukemia and lymphoma (Schwartsmann et al., 2003). Likewise a new brand named Yondelis an antitumour compound for soft-tissue sarcoma has been isolated from a Caribbean and Mediterranean Sea squirt, Ecteinascidia turbinata (Marris, 2006). Compounds in clinical development include didemnins, Kahalalide F, hemiasterlin, dolastatins, cemadotin, soblidotin, bryostatins and ecteinascidin-743. Aplidine is an another anticancer drug produded from the sea squirt Aplidium albicans and its efficacy is being tested (Rawat et al., 2006; Schwartsmann et al., 2003; Marris, 2006). Many of these compounds are still being evaluated clinically and it is reported that some other compounds like
bryostatin 1 has been removed from trials because of severe side effects (Singh et al., 2008). Bonnard and colleagues (2010) recently reported the discovery of antitumor promoters in two types of Comorian soft corals; they are under investigation for potential use in cancer therapy. Sulfated fucans and galactans from marine invertebrates have also been recognized to have antimetastatic properties (Coombe et al., 1987).

Evaluation of cytotoxic activities has been performed on numerous cancer cell lines at invitro or induced on animal models. In recent years, evaluation of cytotoxic activities has been performed on V79 cells with toxins of Ceolentrates such as Rhizostoma pulmo (Allavena et al., 1998), Pelagia noctiluca (Mariottini et al., 2002), Anemonia sulcata and Aequorea aequorea (Carli et al., 1996). On the other hand, toxins of Physalia physalis have been tested on L-929 cells (Edwards et al., 2002), a mouse fibroblast cell line. Interestingly, some recent observations show that Cnidarian venom from Chiropsalmus quadrigatus can even induce apoptosis in glioma and vascular endothelial cell line (Sun et al., 2002). Moreover, Kikuchi and colleagues (Kikuchi et al., 1982; 1983) have identified clavulone, a compound from Clavularia viridis, which has been later shown to be able to inhibit tumor cell growth in leukemia HL-60 (Honda et al., 1985).

Human cervical carcinomas cell line (HeLa) has been largely used as a media to explore the cytotoxicity of numerous fish venoms (Sivan et al., 2007; Sri
Balasubashini et al., 2006c; Tsukamoto et al., 2005). The venoms of fish Scorpaena plumieri (Soprani, 2008) and Thalassophryne nattereri (Lopes-Ferreira et al., 2001) have also showed cytolytic properties against numerous cell types. Sri Balasubashini et al. (2006a) reported the anticancer effects of lionfish (Pterois volitans) venom by in vivo cancer model, and the venom induced apoptosis in Ehrlich’s ascitis carcinoma (EAC). The venoms of the scorpionfish Hypodytes rubripinnis and stonefish Synanceja verrucosa have been assayed on splenocytes and murine P388 leukemic cells (Satoh et al., 2002).

Cell death is commonly occurring in 2 types and they are differentiated as apoptosis and necrosis. It is reported that clinically, the most common and important effect of fish envenomation is local tissue necrosis (Sivan, 2009). Piscine venom induces apoptosis of cancer cells have been studied. Caspase independent apoptosis was demonstrated in rat liver cells by plancitoxin-1 of starfish Acanthaster planci venom (Ota et al., 2006). A peptide (7.6 KDa) from lionfish (Pterois volitans) venom on cultured HEp2 and HeLa cells demonstrated the role of apoptosis and this peptide selectively targets the cancer cells sparing the normal ones (Sri Balasubashini et al., 2006c).

The in vitro cytotoxicity assay has been an effective tool in the discovery process of novel anticancer agents (Cragg and Newman, 2000). This technique is less time consuming, more economical and provides quantifiable reproducible
results and lead to development of new cellular models (Fornelli et al., 2004). Jimenez et al. (2003) reported the cytotoxicity in ascidian extracts through brine shrimp lethality assay, sea urchin egg development assay, hemolysis assay and MTT assay using tumor cell lines.

There has been no study on cat fish Plotosus lineatus toxin, especially on antitumor potential along South east coast of India. So, the aim of the present study was to evaluate the cytotoxicity of *P. lineatus* by the following methods such as MTT assay, Cell cycle analysis and Propidium iodide staining.

**MATERIALS AND METHODS**

**Extraction**

Extraction of fish *P. lineatus* toxin method is given in the previous chapter (Chapter 3).

**Tumor Cell culture**

The sachet (12.0 g) was dissolved in 800 ml of sterile distilled water to which 2.5 g of sodium bicarbonate and 2.1 g of HEPES buffer was added. The beaker was covered with aluminum foil and stirred using magnetic stirrer for 10 minutes. The medium pH was adjusted to 7.2 using 0.1 M NaOH. The volume of the medium was made to 1000 ml and filtered through sterile 0.2 µ membrane filter unit. The medium quality control was checked by incubating 5 ml of filtered
medium in the CO\textsubscript{2} incubator for 2 days. The antibiotics and serum was added before it was used for cell culture.

**In vitro Cytotoxicity (MTT assay)**

The human cancer cell lines were purchased from American type culture collection (ATCC), USA. They were grown in a common medium supplemented with 10% fetal bovine serum and antibiotics as mentioned earlier.

Cytotoxicity (MTT- 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) assay was performed following the method described by Carmichael *et al.* (1987). For the MTT assay, the cells were grown in 25 cm × 25 cm × 25 cm tissue culture flasks containing DMEM/Hams F12 nutrient mix as culture medium supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin (GIBCO) and grown at 37 °C under a humidified atmosphere of 95% air and 5% CO\textsubscript{2}. Cells were regularly passaged and maintained before taken for the experiment. When a cell density in a culture flask reached 70-80% confluence, they were trypsinized and seeded in 96-well plates at varying cell numbers according to the size and shape of the cell between 5000 and 10000 cells per well in 100 µL and incubated for 24 hrs at CO\textsubscript{2} incubator. Extracts were added as 2X concentration to the cell in 100 µL volume and the concentration ranges were: 100, 10, 1, 0.1 and 0.01 µg/ml. The plates were further incubated for 48 hrs in the CO\textsubscript{2} incubator. MTT solution composed of 3-(4,5-dimethylthiazol-2-yl)-2,5-
diphenyl tetrazolium bromide (MTT) at 5 mg/ml in phosphate buffered saline (1.5 mM KH$_2$PO$_4$, 6.5 mM Na$_2$HPO$_4$, 137 mM NaCl, 2.7 mM KCl; pH 7.4); from this solution 50 µl was pipette out into each well. The plate was further incubated for 2.30 hrs and the medium was carefully decanted. The formazan crystals were air dried in dark place and dissolved in 100 µL DMSO and the plates were mildly shaken at room temperature and the Optical density (OD) was measured using Synergy H4 microplate reader at 570 nm.

From the optical densities the percentage growths were calculated using the following formula:

Percentage growth = 100 × [(T-T$_0$) / (C-T$_0$)]

if T is greater than or equal to T$_0$,

and if T is less than T$_0$,

Percentage growth = 100 × [(T-T$_0$)/T$_0$].

Where, T is optical density of test,

C is the optical density of control,

T$_0$ is the optical density at time zero.

**Cell cycle analysis by Flow Cytometry**

The cell cycle analysis was followed by the method of Sri Balasubashini *et al.* (2006c). The cancer cell line HeLa were trypsinized and seeded in 6-well plates at a density of 3 × 105 cells/well and grown for 24 hrs. Semi-logarithmic
concentrations of saline extract of fish *P. lineatus* toxin (1, 3 and 10 µg) were added to the cells and incubated for 24 and 48 hrs. DMSO was added into the vehicle control wells. After the respective experimental period, the cells were trypsinized and collected in a 15 ml falcon tube and washed twice with sterile phosphate buffered saline (PBS).

After washing the cells, pellet was fixed by gently adding ice-cold 70% ethanol (drop by drop) with simultaneous vortexing and samples were fixed overnight at 4 °C. On the day of flow cytometer analysis, samples were centrifuged for 10 minutes at 1,000 rpm. The supernatant was discarded, and the pellets were resuspended in PBS. This step was repeated twice and ethanol was completely removed from the fixed cells. Following this, the cells were then resuspended in PBS containing 0.5% Triton X-100 (Sigma-Aldrich 93443), 0.1 mg/ml RNase (Sigma-Aldrich R4642) and 40 µg/ml Propidium iodide (Sigma-Aldrich P4170) in a dark room. Triton-X and RNAase were added to permeabilize the cell membrane and to eliminate RNA. After 15 - 30 minutes incubation at 37 °C; the cells were analyzed on a flow cytometer (FACS Calibur, Becton Dickinson), equipped with an aircooled argon laser providing 15 mW at 488 nm (Blue laser) with standard filter setup. 10,000 events were collected and the percentages of each cell cycle phases were analyzed using Cellquest Pro software (Becton Dickinson, USA).
**Propidium iodide staining**

Examination of the nuclear chromatin morphology was performed following the method of Sri Balasubashini et al. (2006c). Fluorescence staining was performed with the help of Confocal Microscopy. HeLa cells were grown in 6-well plate. Cells were then treated with control, 10 and 30 µg concentrations of *P. lineatus* toxin seperately and incubated for 48 hrs. Cells were then fixed with 70% ethanol and left at 4 °C for overnight incubation. The cells were then rinsed with PBS and stained with PI (Propidium Iodide) (1 mg/ml) solution containing 0.05% Triton X and RNAase (1 mg/ml) cells and incubated for 30 min. Cell images were observed under a LSM Meta 510 Confocal Scanning Microscope (Carl Zeiss, Germany).

**RESULTS**

*In vitro Cytotoxicity (MTT assay)*

Cytotoxicity of *P. lineatus* toxin was tested with HeLa cancer cell line through MTT assay. The saline extract of *P. lineatus* toxin was tested in 10 logarithmic concentrations like 100, 10, 1, 0.1 and 0.01 µg. The Doxorubicin was used as positive control to compare the cytotoxicity with fish toxin. The growth of HeLa cell was inhibited after being treated with *P. lineatus* toxin. The degree of HeLa cells inhibition showed a dosage-dependent relationship with the fish toxin treatment (Fig 6.1).
IC$_{50}$ value (fifty percentage inhibition concentration) of fish *P. lineatus* toxin was estimated as 22.7 µg/ml for HeLa cells under the experimental condition while the lethal concentration was 100 µg. whereas in the case of Doxorubicin, IC$_{50}$ value was 0.14 µg and the lethal concentration was 7 µg. Increasing concentration of fish toxin reduced the cell viability as the positive control doxorubicin. Cell viability at 0.01 and 0.1 µg concentrations of fish toxin was 81.8 and 80.5% respectively which were found to be more or less equal to Doxorubicin in which 68.96 and 55.24% cell viability was found at same concentrations respectively. The cell viability got efficiently reduced (40.7% of viable cells) at 100 µg of *P. lineatus* toxin extract. Moderate level cytotoxicity was observed at the dose range of 10 and 1 µg with 55.2 and 64.3% of cell viability respectively.

The observed cytotoxicity of fish toxin was not much efficient when compared with Doxorubicin. It is to be noted that the fish *P. lineatus* toxin subjected to the cytotoxicity assay was in crude form and hence it exhibited lower activity than Doxorubicin. However, the present study confirms that *P. lineatus* toxin has significant cytotoxic activity against the HeLa cells.

**Cell cycle analysis by Flow cytometry**

Cell cycle of HeLa was analyzed by serial growth phases (Sub G0-G1, G0-G1, S and G2 M) by the flow cytometry. The obtained results concludes that
growth of HeLa cells was arrested at the stage of G0 - G1 after 24 hrs and S phase arrest was also observed at 48 hrs of toxin treated which describes the cell cycle arresting efficacy of fish P. lineatus toxin (Fig 6.2. A - H). Fig 6.2. I - J represents the results of dosage dependent activity.

Briefly, flow cytometry method is widely considered as a rapid technique to monitor the cell cycle status and regulation. Three phases of cell cycle is involved during the growth as an initial peak of G0/G1 cells, a valley of S phase cells and a second peak containing G2/M cells. The size and granularity of cell was measured by the Forward Angle Scatter. In the dot plot, the forward angle and side scatter (Cell size and granularity) were acquired between channels 200 and 800. A gate (R1) was drawn to show the total events. The proportion of cells in the different phases was analyzed by histogram.

In cell cycle analysis, the saline extract of fish (P. lineatus) toxin treated cells was analyzed in two different time points as 24 and 48 hrs. Marked cell cycle arrest was noticed at the S phase of HeLa cell after 48 hrs incubation. The population of apoptotic cell aggregation with DNA content increased in dose dependent manner from control to 1, 3 and 10 µg concentrations of fish toxin as 37.66, 39.43, 42.67 and 44.84% respectively which indicated the process of apoptosis induction. When compared with control, increased apoptotic population of 1.77, 5.01 and 7.18% were observed in 1, 3 and 10 µg respectively. Moderate
cell cycle arrest was noticed in G2 - M phase after 24 hrs incubation at 10 µg concentration. The fish toxin induces apoptosis with 21.95% of population aggregation with DNA content which was 4.85% higher than control value (17.10%). Obtained results of cell cycle analysis and quantification suggested the apoptosis induction potential of fish toxin at higher dose.

**Propidium iodide staining**

Monolayer of HeLa cells treated at 10 and 30 µg/ml of fish toxin contained more apoptotic cells when compared to untreated monolayer. The apoptotic cells displayed the characteristic features of reduced cell size and intense fluorescence of condensed nuclear chromatin on both concentrations (Fig 6.3).

**DISCUSSION**

Many marine organisms such as Jellyfish (Sun et al., 2002; Winter et al., 2010), sea anemone (Marino et al., 2004), ascidian (Madhavi Agrawal, 2007; Degnan et al., 1989; Fernandez et al., 2008; Williams et al., 1989), sponge (Ryu et al., 1996; Madhavi Agrawal, 2007), mollusc (Rodriguez et al., 1992; Iijima et al., 2003; Tsukamoto et al., 2005), and fishes including flatfish, soapfish, catfish, stonefish and scorpionfish (Carlson et al., 1971; Hashimoto and Oshima, 1972; Shier, 1988; Kreger, 1991; Khoo et al., 1992; Garnier et al., 1995; Carrijo et al., 2005) were reported to be producing cytotoxic activities.
Fish toxin highly inhibited the growth of HeLa cells in this study and showed 40.7% cell viability at 100 µg concentration. In a similar study, venom of oriental catfish *Plotosus lineatus* exhibited 41% cell viability in Ehrlich ascitis carcinoma cells at 1mg concentration (Fahim *et al*., 1996). Sivan *et al.* (2007) reported that 10 µg venom of fish *Scatophagus argus* collected from Cochin estuary, showed 29% cell viability against HeLa which was comparatively higher activity than present observation of 55.2% cell viability at 10 µg concentration. Extract of fish *Pollachius virens* and *Scophtalamus rhombus* collected from North Atlantic showed specific inhibition of mitochondrial activity with CC$_{50}$ values (50% cytotoxic concentration) around 500 mg/ml on the 3T3 - cell line (Hellio *et al*., 2002) which was very lower activity when compared to the present study.

The toxin of box jellyfish *Chiropsalmus quadrigatus* captured in Okinawa exhibited the IC$_{50}$ value at approximately 15.2 ± 2.6 µg/ml against rat malignant glioma cell (Sun *et al*., 2002) and which was comparable to the IC$_{50}$ value of 22.7 µg produced by *P. lineatus* toxin against HeLa cell in this study. In a similar study, *C quadrigatus* toxin produced IC$_{50}$ value with 49.8 ± 3.6 µg/ml for ECV304 (transformed vascular endothelial cell) cells and 80.3 ± 1.3 µg/ml for U251 (human malignant glioma cell) cells and difference in the activity of jellyfish was attributed to the sensitivity of cell line (Sun *et al*., 2002) which is found to be very low cytotoxic activity than this study.
It has been reported that composition of Jellyfish *Chironex fleckeri* toxin was influenced by geographical location and season of collection (Winter *et al*., 2010). Winter *et al*. (2010) reported the cytotoxicity of *C. fleckeri* venom from different geographical locations against A7r5 cell line (rat aorta smooth muscle cell line) such as IC$_{50}$ value of 0.03 and 0.04 µg/ml during 2006 and 2007 respectively from Weipa, 0.1 and 0.05 µg/ml during 2005 and 2007 respectively from Mission Beach, 0.2 µg/ml during 2007 from Karumbra and 0.7 µg/ml during 2007 from Broome (Winter *et al*., 2010) and all were higher activity if compared to the present study. Likewise, Madhavi Agrawal (2007) reported cytotoxicity of some sponges collected from Great Barrier Reef and the derived compounds such as Stelliferin A and stelliferin D riboside from *Rhabdaastrella globostellata* exhibited IC$_{50}$ value of 0.16 and 1.10 µg/ml respectively on mouse lymphoma cell line P388D1 and Spongiadiol diacetate from *Spongia* sp. with IC$_{50}$ value of 2.10 µg/ml (P388D1 cell line) and pentabrominated phenolic diphenyl ether from *Dysidea herbacea* with IC$_{50}$ value of 2.20 µg/ml against the same cell line and also Globostellatic acids A-D from *Rhabdastrella globostellata* exhibited IC$_{50}$ values of 0.01 - 0.46 µg/ml against P388D1 cell line (Ryu *et al*., 1996) which all were higher potent than the present study result of *P. lineatus* toxin.

Four active cytotoxic compounds were isolated from the sea hare, *Aplysia kurodai* of Toyama Bay, Japan. Among them potential cytotoxic activity was expressed by compound 1 and 4 with IC$_{50}$ value at 20 and 18 µg/mL against
HeLa cells which is close to this study. However, compound 2 and 3 of *A. kurodai* exerted cytotoxic activity with IC$_{50}$ value at 32 and 50 µg/mL respectively (Tsukamoto *et al.*, 2005). Cellular (EL - 4 murine lymphoma cells) viability of Dolabellanin A (DAA), an antineoplastic protein of marine mollusc *Dolabella auricularia* from Kominato, Japan was reported as 52.6% (Iijima *et al.*, 2003) which also aligns with the present study of *P. lineatus* toxin in which 10 and 100 µg/ml concentrations of toxin significantly reduced the HeLa cell viability by 40.7 and 55.2% respectively. Marine mollusc *Onchidium* sp. (Chesterfield atoll in the Mouillage Island, northwest of New Caledonia) showed antitumour activity with an IC$_{50}$ value of 20 µg/ml when tested *in vitro* against cell line P - 388 (Rodriguez *et al.*, 1992) and later cytotoxic activity of Onchidin was reported against same cell line with an IC$_{50}$ value of 8 µg/ml (Rodriguez *et al.*, 1994) which is closed to the current observation.

More than 50% of ascidians have been reported to have potent cytotoxic activity than the present observation. For example, Ulithiacyclamide and ascidiacyclamide isolated from ascidian *Lissoclinum patella* inhibited cell growth with IC$_{50}$ value of 0.25 µg/ml and 4.07 µg/ml against P388D1 cell line (Madhavi Agrawal, 2007). Rajesh (2008) screened 9 ascidians from Tuticorin coast, Gulf of Mannar for cytotoxic activity against HeLa cell line and methanol extract of *Eudistoma viride* exhibited potent activity with low cell viability of 9.2% and acetone extract of *Polyclinum madrasensis* and *Distaplia nathensis* showed
similar cell viability to the present study which was 57.68 and 58.53% respectively (Rajesh, 2008). The difference in activity may be attributed either to concentration of cancer cells used for experiment or to different sensitivity of cell lines and purity of compound (Trianto et al., 2011).

The apoptosis is defined as the programmed cell death and has been demonstrated with lots of natural products. Here, the obtained results can be compared with activity exhibited by other marine natural products. In the present study the HeLa cell’s cycle was strongly affected by the fish *P. lineatus* toxin in S-phase at 48 hrs. Similar level S phase cell cycle arrest was exhibited by venom from scorpionfish *Hypodytes rubripinnis* and stonefish *Synanceja verrucosa* which strongly affected the cell cycle of splenocytes and murine P388 leukemic cells (Satoh et al., 2002). The report of Honda et al. (1987) also coincides with the observation of S-phase of HL-60 cell cycle arrest by venom Clavulone from soft coral *Clavularia viridis*. In contrast, starfish *Acanthaster planci* venom arrested the cell cycle of rat liver epithelial cells (TRL 1215) at G2 phase. Briefly, *A. planci* venom (plancitoxin I) at 10 µg/ml concentration for 24 hrs exhibited Caspase - 3 independent apoptosis and produced CDT (cytolethal distending toxin) which induced apoptosis as well as cell cycle arrest mostly in the G2 phase (Ota et al., 2006).
In the present study 1, 3 and 10 µg/ml concentrations of *P. lineatus* toxin induced apoptosis and arrested cell cycle at G0 - G1 during 24 hrs which can be substantiated with the report of Kikuchi *et al.* (1982) who reported that tumor cell growth was inhibited by Clavulone a compound from soft coral *C. viridis* at G1-phase. Apoptotic cells can be recognized by their diminished staining ability with DNA specific fluorochromes, such as Propidium iodide, DAPI, Acridine orange (AO), or Hoechst dyes, due to DNA degradation and its subsequent leakage from the cell. Propidium iodide has been proved to be an excellent probe to distinguish live, necrotic, early and late-apoptotic cells rather than other staining (Darzynkiewicz *et al.*, 1992).

HeLa cells treated with 3 and 10 µg concentrations of fish showed apoptotic population which is characterized by reduced cell size and intense fluorescence of condensed nuclear chromatin on the both concentrations in this study. Analogously, venom of fish *Thalassophryne nattereri* showed similar level apoptotic population level on endothelial cell lines (Lopes-Ferreira *et al.*, 2002) and *Gymnapistes marmoratus, Pterois volitans* and *Synanceja trachynis* on cultured murine cortical cells (Church *et al.*, 2003). Sri Balasubashini *et al.* (2006c) demonstrated that after HEp2 and HeLa cells were treated with lion fish *Pterois volitans* venom (2 µg/ml) on propidium iodide staining, apoptosis was occurred and displayed the characteristic features of reduced size, intense fluorescence of condensed nuclear chromatin and formation of membrane blebs.
Tumour cells are distended after exposure to anticancer agent and depolarization of the cell and also ionic imbalances lead to an influx of fluid into the cytosol resulting in swelling of cells and eventually cell lysis (Church et al., 2003). The observations in this study and the results of previous studies on apoptosis suggest that propidium iodide staining method could be used as a tool to visualize apoptotic population. Moreover, it is clear that toxin of *P. lineatus* has a moderate level of cytotoxic activity which can be further analysed.

![Graph showing cytotoxicity of fish *Plotosus lineatus* toxin on cell viability of HeLa cells at different concentrations](image-url)

**Fig 6.1: Cytotoxicity of fish *Plotosus lineatus* toxin on cell viability of HeLa cells at different concentrations**
Fig 6.2.A: Flow cytometric analysis of DMSO (Control) treated HeLa cells after 24 hrs
Fig 6.2.B: Flow cytometric analysis of 1 µg concentration of fish *P. lineatus* toxin treated HeLa cells after 24 hrs
Fig 6.2.C: Flow cytometric analysis of 3 µg concentration of fish *P. lineatus* toxin treated HeLa cells after 24 hrs
Fig 6.2.D: Flow cytometric analysis of 10 µg concentration of fish *P. lineatus* toxin treated HeLa cells after 24 hrs.
Fig 6.2.E: Flow cytometric analysis of DMSO (control) treated HeLa cells after 48 hrs
Fig 6.2.F: Flow cytometric analysis of 1 µg concentration of fish *P. lineatus* toxin treated HeLa cells after 48 hrs
Fig 6.2.G: Flow cytometric analysis of 3 µg concentration of fish *P. lineatus* toxin treated HeLa cells after 48 hrs
Fig 6.2.H: Flow cytometric analysis of 10 µg concentration of fish *P. lineatus* toxin treated HeLa cells after 48 hrs
Fig 6.2.I: Flow cytometric analysis of fish *P. lineatus* toxin treated HeLa cells after 24 hrs

Fig 6.2.J: Flow cytometric analysis of fish *P. lineatus* toxin treated HeLa cells after 48 hrs
Fig 6.3: Nuclear localization of HeLa cells by propidium iodide staining
5. Isolation of associated bacteria from venom gland of fish *Plotosus lineatus* and screening for bioactivity
INTRODUCTION

Marine organisms possess unique structures, metabolic pathways, reproductive systems, sensory and defense mechanisms since they live in wide ranging environmental conditions from cold polar seas to the great pressures of the ocean floor (Faulkner, 1995). They are especially in tropical reefs, under intense competitive pressure for space, light and nutrients and so, have developed a wide range of defense mechanisms including behavioral (e.g. cryptic, nocturnal) physical (e.g. sclerites, tough external surfaces) and chemical strategies to ensure survival (Tasdemir et al., 2001).

The evolution of antibiotic-resistant bacteria has stimulated the search for potent antibacterial agents from natural sources. The department of Ocean Development has brought out a vision perspective plan for 2015 and it appears that 80% of drugs needed for human health care could be derived from marine natural sources (Pomponi, 1999). In this scenario, marine microbes have been shown to have an increasing interest as a source of new drugs which could even be utilized for future cancer therapy (Bollmann et al., 2010).

Bacteria inhabit the surface and internal spaces of marine organisms. Interaction between epibiotic marine bacteria and their host organisms are known to play a significant role in marine ecosystem (Wahl, 1995). They often form symbiotic association with host organisms. Under specific conditions, certain
bacterial communities that are ideally suited to the habitat become established on the epithelial tissue of host organisms and remain constant for a long time. In case the bacterial growth on mammals or invertebrates is removed, the animals usually fall ill. The metabolic system is disrupted and the immune system gets weakened. The animals can no longer defend themselves against infections caused by pathogenic bacteria and viruses. For example, epithelium or outer body surface of Cnidarians are colonized by a complex and dynamic community of microorganisms (Bollmann et al., 2010).

Invertebrates which produce bioactive compounds are insufficient for producing commercial quantities of metabolites of interest. In addition, the concentrations of many highly active compounds of marine invertebrates are often minute and which is considered as most significant threat to marine lives (Sabdono, 2008). From sustainability point of view, isolating bioactive-producing bacteria from their host animal is obviously offers a much better approach. Furthermore, in most cases cultivating and harvesting marine invertebrates are extremely difficult (Radjasa and Sabdono, 2009). Therefore, a solution to overcome the problem of supply is needed. Association of bacteria with many marine invertebrates, like sponges, bryozoans, bivalves, oligochaetes, sea anemone, jellyfish, ascidian and corals have been reported (Kim et al., 1995; Haygood and Davidson, 1997; Sipe et al., 2000; Dubilier et al., 2001; Webster et al., 2001; Munn, 2004; Williams et al., 2007; Moss et al., 2003). It is also reported
that some of the bioactive compounds derived from these invertebrates such as sponges and tunicates are truly of bacterial origin (Gil-Tunes and Fenical, 1992; Gerard, 1997; Proksch et al., 2002; Thiel and Imhoff, 2003; Radjassa et al., 2007b).

Marine bacteria are typically cultured from seawater, marine sediments and from the surfaces and tissues of marine algae and invertebrates. Unfortunately, it appears that many obligate marine symbionts cannot at present be cultured. Even though it is estimated that only a few percent of marine bacteria are amenable to culture and some interesting new metabolites have been reported in the past 5 years (Kothari, 2007).

Some studies have investigated bacteria from venomous marine animals and considered that associated bacteria as a potential source for toxin (Noguchi et al., 1986; Yasumoto et al., 1986; Yotsu et al., 1987). Bacterial strains were isolated first from the intestine of a xanthid crab, Atergatis floridus (Noguchi et al., 1986) and then from starfish Astropecten polyacanthus (Narita et al., 1987), the blueringed octopus (Octopus maculosus) (Hwang et al., 1989), lined moon shell Natica lineate (Hwang et al., 1994) and gastropod N. clathrata (Cheng et al., 1995). There are many debates on the origin of Tetrodotoxins in TTX bearing organisms and the best supported hypothesis is TTXs are produced by symbiotic bacteria. TTXs have been detected in at least 15 genera of bacteria from TTX-
bearing marine animals, seawater and sediment (William, 1993). Additionally, Tetrodotoxin producing bacterial isolates were identified from skin of puffer fish, *Fugu poecilonotus* (Yotsu *et al.*, 1987), intestine of *Fugu vermicularis* (Noguchi *et al.*, 1987; Lee *et al.*, 2000) and from some other organs (Yu *et al.*, 2004).

Venomous gastropod *Nassarius semiplicatus* from Lianyungang, Jiangsu were studied to probe into the relationship between bacteria and toxicity of nassariid gastropod (Wang *et al.*, 2008a). Similar studies on bacterial association in venom gland of fish are scanty in Gulf of Mannar. Hence, in the present study, an attempt has been made to isolate and evaluate the antibacterial potential of venom gland associated bacterial strains from the venomous cat fish *Plotosus lineatus*.

**MATERIALS AND METHODS**

**Isolation of bacteria from venom gland of fish *Plotosus lineatus***

The venom gland of fish *P. lineatus* was microscopically examined and aseptically excised along with basal part of venomous spine from freshly collected 10 individuals and homogenized in a sterile blender. The mixture (1 gm) was suspended in 10 ml sterile seawater and serially diluted. Isolation of bacteria was achieved by spreading 0.5 ml diluted sample on Marine agar (Zobell marine agar) plates. The plates were maintained at 20 °C (Wang *et al.*, 2008a) for 4 days. Results were expressed as colony forming unit (CFU/gm). Colonies on
each plate were further purified by streak-plate method with a new agar plate until obtaining pure culture (Wang et al., 2008a; Wahl, 1995) and stored in Zobell marine agar slants at 4 °C for further studies.

**Culture supernatant preparation**

The selected cultivable bacterial strains (5) were given designated code such as F1, F2, F3, F4 and F5. All strains were cultured in Zobell marine broth. Selected strains were inoculated into 100 ml autoclaved culture medium separately and incubated at 20 °C for 5 days on a mechanical shaker. The whole culture was then centrifuged at 5000 rpm for 15 min to obtain the culture supernatant (Wang et al., 2008a). The resultant supernatants were used for the screening purpose.

For solvent extraction, the liquid-liquid extraction method (Galliot, 1998) was employed. Immiscible polar and non polar solvents such as hexane, diethyl ether, dichloro methane and butanol were used. Equal volume of solvent was mixed with culture supernatant separately and stirred for 5 to 10 min and allowed to settle for few minutes. Separating funnel was used to separate the solvent phase from the supernatant. The evaporated and concentrated crude extracts were used for further screening (Zheng et al., 2005a).
Antibacterial activity

Antibacterial activity of culture supernatant and solvent crude extracts of associated bacteria were evaluated against ten human bacterial pathogens such as *Escherichia coli* (MTCC 50), *Shigella flexneri* (MTCC 1457), *Bacillus subtilis* (MTCC 1133), *Salmonella typhi* (MTCC 531), *Staphylococcus aureus* (MTCC 3160), *Vibrio cholerae* (MTCC 3906), *Pseudomonas aeruginosa* (MTCC 424), *Vibrio parahaemolyticus* (MTCC 451), *Streptococcus pyogenes* (MTCC 1923) and *Micrococcus luteus* (MTCC 4821) which were obtained from Microbial Type Culture Collection (MTCC) and Gene Bank, Chandigarh.

For screening culture supernatant of associated bacteria, agar well diffusion assay was followed (Barry, 1980). 100 µl of culture supernatants were added into wells (6 mm) on Muller Hinton agar plates containing 24 hours old human pathogenic bacteria. To evaluate antibacterial activity of solvent crude extract, paper disc method was performed (Becerro *et al*., 1994; Murugan and Santhana Ramasamy, 2003). Paper discs (6 mm diameter) were impregnated with 200 µg of crude extract and allowed for evaporation and placed on Muller Hinton agar plates seeded with human pathogenic bacteria and incubated at 37 °C.
RESULTS

Isolation of bacteria

The bacterial density in the venom gland of *P. lineatus* was \(5.5 \times 10^2\) CFU/gm. Five cultivable bacterial colonies with different morphology were selected for further screening.

Screening for Antibacterial activity

Culture supernatants of all the five strains showed antibacterial activity at least against 5 pathogens. The wide spectral antibacterial activity was exhibited by F3 strain with a zone range of 2 - 10 mm and inhibited growth of *Escherichia coli*, *Micrococcus luteus*, *Bacillus subtilis*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus*, *Vibrio cholera*, *Shigella flexneri* and *Salmonella typhi* considerably (Table 4.1). The highest zone of 10 mm was observed against *E. coli* followed by 6 mm against *S. flexneri* and lowest of 2 mm against *S. pyogenes*.

The remaining 4 strains produced 1 to 4 mm zone of inhibition against 50% of pathogens. Next to F3, F5 strain produced a zone of 4 mm against 2 pathogens, *E. coli* and *V. cholera* followed by 3 mm against *B. subtilis*, *S. aureus* and *V. parahaemolyticus* and 2 mm against *S. flexneri*. No activity was noticed against *M. luteus*, *S. typhi*, *P. aeruginosa* and *S. pyogenes*. Other strains did not show considerable antibacterial activity. Interestingly, it was noticed that
pathogens *S. pyogenes* and *P. aeruginosa* were resistant to the all culture supernatants except to F3 strain. Human pathogens *V. cholera* and *S. aureus* were highly susceptible to all supernatants.

**Antibacterial activity of potent strains (F3 and F5)**

Solvent crude extract of both F3 and F5 strains showed good antibacterial activity. Invariably, all the solvents inhibited at least 2 pathogens. It is noteworthy that these 2 active strains produced highest activities in butanol solvent extract followed by diethyl ether and dichloromethane (Table 4.2).

F3 strain produced higher antibacterial activity than F5 strain. Butanol extract of F3 strain produced wide spectral activity against all human pathogens except *B. subtilis*. The highest activity was exhibited against *S. typhi* with 10 mm zone followed against *S. aureus* (9 mm), *M. luteus* (5 mm), *P. aeruginosa* (4 mm) and 3 mm against *S. pyogenes* and *V. parahaemolyticus*. Next to butanol, diethyl ether extract of F3 strain produced activity (1 - 3 mm) against *S. typhi*, *S. pyogenes*, *V. cholera* and *M. luteus*. No significant activity was noticed in dichloromethane and hexane extracts.

F5 strain also exhibited moderate level antibacterial activity in butanol extract. But unlike F3, this strain produced only 2 to 4 mm zone of inhibition against 50% pathogens. Highest activity (4 mm) was observed against *V. cholera*
followed by 3 mm activity against 3 pathogens which are *E. coli*, *S. aureus* and *V. parahaemolyticus*. No significant activity was exhibited by hexane, diethyl ether and dichloromethane extracts. It was noted that pathogens *B. subtilis* and *S. flexneri* showed resistant to 90% of extracts of both strains. On the other hand, *V. cholera* and *M. luteus* were found to be very sensitive to 70% of the crude extracts.

**DISCUSSION**

The endemic habitats such as highly sulfidic, reducing environments at deep-sea hydrothermal vents and cold steeps are commonly associated with chemosynthetic endosymbiotic bacteria (Cavannaugh *et al.*, 1981; Felbeck, 1981; 1985). They support the growth and maintenance of the hosts by oxidizing the reduced sulfur compounds, abundant in hydrothermal fluid (Dando *et al.*, 1985; Powell and Somero 1986; Vetter, 1985) and the resultant energy produced by the endosymbiont is coupled to the production of carbon sources (Cavanaugh, 1985; Dando *et al.*, 1985; Rau and Hedges, 1979; Williams *et al.*, 1981). In addition, role of endosymbiont in marine animals were demonstrated as secondary simplification; briefly, related to nutrient provision or waste recycling, in particular of nitrogenous compounds (Nitrogen detoxification), supplying growth factors or chemical defense to their host (Kjeldsen *et al.*, 2010; Hay, 1986; Fenical, 1993; Hay and Fenical, 1996).
The present study explores the bacterial population in venom gland of *P. lineatus* that was $5.5 \times 10^2$ CFU/gm. Few earlier studies have investigated bacteria from venomous marine animals and have reported the associated bacteria as a potential source for toxin (Noguchi *et al.*, 1986; Yasumoto *et al.*, 1986; Yotsu *et al.*, 1987). Wu *et al.* (2005a) isolated 36 strains from the toxic puffer fish *Fugu rubripes* collected from Bohai Sea of China. Wang *et al.* (2008a) reported 45 bacterial strains from the digestive gland of the gastropod *Nassarius semiplicatus* collected from Lianyungang, Jiangsu Province (China).

Microbes have been identified as ecto and endo symbiotic organisms in many of the multicellular animals. Associated bacterial counts were high in venomous tentacle tissues than in body tissue of sea anemone *Stichodactyla haddoni* (William *et al.*, 2007). It was also reported by William *et al.* (2007) that bacterial density of *S. haddoni* collected from Tuticorin coast (South East coast of India) was about $2.5 \times 10^4$ CFU/gm which is comparable to this study. Chelossi *et al.* (2004) reported a bacterial density of $2.9 \times 10^3$ to $1.3 \times 10^4$ CFU/cm$^2$ from the sponge *Petrosia ficiformis* (coast Paraggi, Portofino Promontory, Ligurian Sea) which was lower than this study. The bacterial density of adult colonies of ascidian *Cystodytes dellechiajei* from Mediterranean Sea (Cape Palos, Murcia, Spain) was reported as $7.14 \times 10^7 \pm 0.5 \times 10^7$ CFU/cm$^3$ in the adult and up to $1.74 \times 10^9 \pm 0.8 \times 10^9$ in the larva (Martinez-Garcia *et al.*, 2007) which is substantiated to the present study. A similar result was reported by Jebasingh
(2008) who stated the gut region bacterial density of marine venomous cone snail *Conus virgo* of Tuticorin coast (Gulf of Mannar, India) as $16 \times 10^3$ CFU/ml.

Anand *et al.* (2006) reported the bacterial density of sponges *Echinodictyum* sp., *Spongia* sp., *Sigmadocia fibulatus*, *Mycale mannarensis* collected from Hare Island, Tuticorin, (South East coast of India) as $3 \times 10^5$, $6 \times 10^4$, $3 \times 10^3$ and $2 \times 10^5$ CFU/g respectively which were low when compared to the present study.

Only 1 – 10% of the microorganisms in the natural environment can be cultivated with traditional techniques (Amann *et al.*, 1995). Wang *et al.* (2008a) stated that obtaining all symbiotic bacteria from venomous gastropod was impossible by conventional cultivation and isolation procedure. Some highly toxic strains, therefore, might be missed. Likewise, in this study only 5 strains were found as cultivable.

Several isolates, associated to marine plants and animals, have been reported to express antibacterial, antifungal and antitumor activities (Zheng *et al.*, 2000). The associated bacterial density in barnacle *Balanus amphitrite* (Tuticorin coast, South East coast of India) was reported to be $41 \times 10^{-1}$ CFU/cm$^2$ and 14.3% of the 28 isolated associated bacteria exhibited antibacterial activity (Jebasingh and Murugan, 2011). Similarly in this study, 2 out of 5 strains of
venom gland associated bacteria exhibited good antibacterial activity. Zheng et al. (2005) reported that 28% (8/29) of the total bacterial strains associated with the marine sponge *Hymeniacidon perleve* showed antimicrobial activity.

All the cultivable strains (5) were identified and typed as Gram positive (F1, F2 and F3) and Gram negative (F4 and F5) bacteria (refer Chapter 7) in the present study which can be correlated with Kanagasabhapathy et al. (2005) who reported four active strains of Gram positive bacteria (PS2, PS9, PS11 and PS79) from the sponge *Pseudoceratina pupurea* from Mandapam coast (Gulf of Mannar coast, India).

Gram negative bacteria identified from the venom gland of fish *Plotosus lineatus* strengthen the data presented by many authors (Vacelet and Donadey, 1977; Wilkinson, 1978; Sochard et al., 1979; Santavy and Colwell, 1990; Ward-Rainey et al., 1996). *Vibrio* and *Bacillus* were reported as common invertebrate and venomous fish associated bacterial genera in numerous researches. Oclarit et al. (1994) reported *Vibrio* strains associated with the sponge *Hyatella* sp. were found to produce antibiotics.

Among the other genera isolated, *Bacillus* and *Micrococcus* were numerically well represented (Stierle et al., 1988). Stierle et al. (1988) stated that Diketopiperazines produced by a *Micrococcus* sp. associated with the sponge
*Tedania ignis* were found to have potent antimicrobial activity and can be comparable to this study. Another isolate of present study, *Bacillus* sp. have also been well reported from marine organisms. Strengthen this study, Hwang *et al.* (1989) reported *Bacillus* from the blueringed octopus (*Octopus maculosus*) and the strains were found to be the source of tetrodotoxin production.

Limited reports are available on *Staphylococcus* isolation from marine organisms. However, *Staphylococcus* isolated from *P. lineatus* in this study can be correlated with few recent reports. Lu *et al.* (2011) reported 395 pure strains from East China Sea including 10 species of *Staphylococcus* from marine environment and they reported that 11.4% strains were having antibacterial activity. Antimicrobial activity of *Staphylococcus equorum* from marine sponge of Tuticorin coast (Gulf of Mannar, India) was reported by Anand *et al.* (2006). Zhang *et al.* (2009) also reported *Staphylococcus* sp. (A75 strain) with antibacterial potential from south china sponges.

Antibacterial activity of culture supernatant of isolated strains in the present study was observed against bacterial pathogens. All the strains inhibited at least 50% pathogens which are comparable to Radjasa *et al.* (2007c) who screened 56 marine bacteria associated with the sponge *Haliclona* sp. and described 8 isolates as capable of inhibiting the growth of at least 1 tested indicator. Venomous fish *P. lineatus* associated strains F3, F5 and F2 exhibited 1
to 10 mm zone of inhibition against 50 to 90% of pathogenic bacteria at 100 µl culture supernatant and highest activity was (10 mm) recorded against *E. coli* in the present study.

Radjasa *et al.* (2007a) reported 5 marine bacteria associated with the softcoral *Sinularia polydactyla* (Bandengan water, Jepara, North Java, Indonesia) which are capable of inhibiting the growth of *Streptococcus* sp. In the present study F3 and F5 exhibited moderate (3 mm) activity against *Bacillus subtilis* and *Staphylococcus aureus*. Similarly, Kanagasabhapathy *et al.* (2005) reported 4 associated bacterial strains from marine sponge *Pseudoceratina purpurea* which produced antibacterial activity (3 to 12 mm) against at least 1 pathogen at 60 µl culture supernatant concentration. Sponge *Mycale microsigmatosa* from Rio de Janeiro associated bacterial strain (Mm3) produced wide spectral activity against bacteria including 6 drug resistant strains with 10 - 12 mm zone of inhibition (Marinho *et al*., 2009) and it aligns with the present study.

In the present study, among the 8 extracts (hexane, diethyl ether, dichloromethane and butanol) of 2 strains (F3 and F5), butanol extract of F3 strain produced highest and wide spectral antibacterial activity which is comparable to Kalinovskaya *et al.* (2004) who observed that butanol extract of marine isolate *Pseudoaltromonas citrea* showed antibacterial activity. Distinguishable zone of inhibition (10 mm) noticed in the butanol extract of F3
strain which aligns with Jebasingh (2008) and Romanenko et al. (2008). High antimicrobial activity of eight mollusc associated bacterial strain in butanol extract (10 - 22 mm) against pathogens was reported by Romanenko et al. (2008). Jebasingh (2008) reported that butanol extract of barnacle associated bacteria *Bacillus megaterium* strain showed wide spectral activity (2 - 12 mm). Similar activity was reported also by Thakur et al. (2005) who found 4 to 9 mm activity in butanol extract of Sponge *Suberites domuncula* Primmorph (3 - dimensional aggregates containing proliferating cells) associated bacteria (PB2) against human pathogens.

Antibacterial activity of diethyl ether extract of F3 strain (1 - 3 mm) can be correlated with Zheng et al. (2005b) who reported moderate activity (1 - 5 mm) in a similar polarity solvent ethyl acetate extract of two sponge associated bacteria against pathogenic bacteria. Butanol extract of sea anemone *S. haddoni* associated strains *Renibacteria, Aeromonas, Corynebacterium* and *Carnobacterium* - 1 produced 7 to 9 mm activity against pathogens (William et al., 2007) which is similar to the present study in which higher activity was observed in the butanol extract F3 strain.

Higher activity of diethyl ether extract of F3 strain aligns with the study of William et al. (2007) who observed 7 mm activity of sea anemone isolate *Carnobacterium* - 1 against *Salmonella typhi*. F3 (*Staphylococcus* sp.) strain
produced no significant antibacterial activity against *Bacillus subtilis* which is comparable with the study of Anand *et al.* (2006) who reported that *Staphylococcus equorum* isolated from sponge *Spongia* sp. showed trace activity against the same pathogen.

Reports regarding antibacterial activity of the bacteria associated with the venomous fish from the coastline of India are few and to the best of our knowledge this is the first study from the Gulf of Mannar. Antibacterial activity of the fish venom gland associated bacteria in the present study suggests that butanol extract of F3 and F5 strains may lead to the development of new drug after some extended research. Hence, these butanol crude extracts were taken to the further study to explore the compound characterization with structure.
Table 4.1: Antibacterial activity of fish venom gland associated bacteria

<table>
<thead>
<tr>
<th>Human pathogens</th>
<th>Zone of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100 µl/well</td>
</tr>
<tr>
<td></td>
<td>F1</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>-</td>
</tr>
<tr>
<td><em>Micrococcus luteus</em></td>
<td>-</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>2</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>-</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>3</td>
</tr>
<tr>
<td><em>Vibrio cholera</em></td>
<td>3</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>-</td>
</tr>
<tr>
<td><em>Vibrio parahaemolyticus</em></td>
<td>-</td>
</tr>
<tr>
<td><em>Shigella flexneri</em></td>
<td>3</td>
</tr>
<tr>
<td><em>Salmonella typhi</em></td>
<td>2</td>
</tr>
</tbody>
</table>

(F1 to F5 - strains isolated from fish venom gland; - No activity)
<table>
<thead>
<tr>
<th>Human pathogens</th>
<th>Zone of inhibition (mm)</th>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>200 µg/disc</td>
<td>F3</td>
<td>F5</td>
<td>F3</td>
<td>F5</td>
<td>F3</td>
<td>F5</td>
<td>F3</td>
<td>F5</td>
</tr>
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<td></td>
<td></td>
<td>H</td>
<td>DEE</td>
<td>DCM</td>
<td>B</td>
<td>H</td>
<td>DEE</td>
<td>DCM</td>
<td>B</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td><em>Micrococcus luteus</em></td>
<td></td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em></td>
<td></td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>3</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>9</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td><em>Vibrio cholera</em></td>
<td></td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>2</td>
<td>2</td>
<td>2</td>
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<td>4</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td></td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>-</td>
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</tr>
<tr>
<td><em>Vibrio parahaemolyticus</em></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td><em>Shigella flexneri</em></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Salmonella typhi</em></td>
<td></td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(F3 and F5 – strains isolated from fish venom gland; H - Hexane; DEE - Diethyl ether; DCM - Dichloro methane; B - Butanol; - No activity)