6. IN VITRO ANTICANCER ASSAY

6.1. INTRODUCTION

Marine chemotherapy is well recognized nowadays and profound development has been achieved by researchers to deal with different molecular pathways of tumors. However, the marine environment has been less explored for the production of safe and novel antitumor compounds. The chemical and biological diversity of the marine environment is immeasurable and therefore is an extraordinary resource for the discovery of new anticancer drugs (Pomponi, 1999). An exciting “marine pipeline” of new anticancer clinical and preclinical agents has emerged from intense efforts over the past decade to more effectively explore the rich chemical diversity offered by marine life.

There are plenty of works related to antitumor (Natarajan, 2010a) and antimicrobial activity (Natarajan, 2010b) carried out in ascidians, which made the chemist to isolate the active principle responsible for the actions as they are the viable source for drug discovery process (Arutuso, 1997). Bryostatin-1 has demonstrated significant antitumor activity in preclinical models against a wide spectrum of cell lines (Kraft, 1986) and, in addition, has been shown to enhance the antitumor effects of various chemotherapeutic agents, such as cytosine arabinoside, gemcitabine, vincristine, cisplatin and paclitaxel (Wender, 1999). Probably the best known of the compounds with potential as anticancer drugs are the macrolides known as bryostatins, isolated primarily from the bryozoan, Bugula neritina, although some have been extracted from sponges and tunicates (Schmitz et al., 1993). From the literature cited it is undoubtedly revealed that the sponge crab D. dehaani has not been explored for its biomedical especially in vitro and in vivo anti-
cancer potential. Hence this present research effort made an attempt to evaluate the anti-cancer properties of the hemolymph of the crab *D. dehaani* against the cell lines viz., HepG2, HT-29, Rhabdomyosarcoma and A549.

### 6.2. MATERIALS AND METHODS

#### 6.2.1. Animal and hemolymph collection:

Sponge crabs *D. dehaani* (200-250g) were collected from the Pazhayar landing center. They were cultured in 500L recirculating seawater (20-30%) tanks at the laboratory and fed twice daily with the control diet and 50% of the water was exchanged daily thrice a week to maintain the water quality. Hemolymph was collected by cutting walking legs of the crab *D. dehaani* with a fine sterile scissor. To avoid hemocyte degranulation and coagulation, the hemolymph was collected in the presence of sodium citrate buffer, pH 4.6 (2:1, V/V). Equal volume of physiological saline (0.85%, NaCl, w/v) was added to it. To remove hemocytes from the hemolymph it was centrifuged at 2000rpm for 15min at 4°C. Supernatant were collected by aspirating and stored at 4°C until use.

#### 6.2.3. Cell line and culture:

HepG2, HT-29, Rhabdomyosarcoma and A549 cell lines purchased from the National Center for Cell Science (NCCS, Pune) were grown as monolayer in minimal essential medium (MEM) (Himedia) supplement with 10% FCS, 3% glutamine Penicillin (100 U/mL) and Streptomycin (100 μg/mL) at 37°C for 5% CO₂ atmosphere. Stocks were maintained in 25 cm² tissue culture flasks. The *in vitro* cytotoxicity’s of the sample on cell lines were examined using a modified MTT assay (Plumb *et al.*, 1989).
6.2.4 Reagents:

MEM was purchased from Hi Media Laboratories. Fetal bovine serum (FBS) was purchased from Cistron laboratories. Trypsin, methylthiazolyl diphenyl-tetrazolium bromide (MTT), and Dimethyl sulfoxide (DMSO) were purchased from (Sisco research laboratory chemicals Mumbai). All the other chemicals and reagents were obtained from Sigma Aldrich Mumbai.

6.2.5 MTT assay:

The anticancer activity of samples was determined by the MTT assay (Mosmann, 1983). Cells (1 × 10^5/well) were plated in 1ml of medium/well in 24-well plates (Costar Corning, Rochester, NY). After 48 hours incubation the cell reaches the confluence. Then, cells were incubated in the presence of various concentrations of the samples in 0.1% DMSO for 48hrs at 37°C. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 200µl/well (5mg/ml) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-tetrazolium bromide cells (MTT) solution was added. After 4hrs incubation, 0.04M HCl/ isopropanol were added. Viable cells were determined by the absorbance at 570nm. Measurements were performed and the concentration required for a 50% inhibition of viability (IC\textsubscript{50}) was determined graphically. The absorbance at 570 nm was measured with a UV- Spectrophotometer using wells without sample containing cells as blanks. Cytotoxicity was expressed as the concentration of extracts or fractions inhibiting cell growth by 50% (IC\textsubscript{50}). All tests and analyses were run in triplicate. The effect of the samples on the proliferation was expressed as the % cell viability, using the following formula:
% cell viability = Treated cells / Control cells × 100%.

6.2.6 Cell viability test

The trypan blue exclusion test was used for detection of cell viability (Morgan and Darling, 1992).

6.2.7 DNA fragmentation assay:

To confirm morphological changes in the nuclei, cells were seeded in 16-mm cover slips placed in 6-well plates at $2 \times 10^6$ cells. Cells were treated 1 day after seeding with hemolymph at different concentrations for 24 hrs. HOECHST 33258 solutions was added and the cells were incubated for 30 min before being examined by fluorescence microscopy.

6.2.8 Data analysis

All data were analyzed by the software of SPSS (SPSS Science Inc.).

6.3 RESULTS

The crab’s hemolymph was tested to evaluate their cytotoxic potential against the cancer cells. Cell viability and cytotoxicity assays are used for drug screening and cytotoxicity tests of chemicals. Cytotoxicity was assessed by the morphological characteristics of the cells such as rounding of the cells, shrinkage, aggregation and cell death was observed through Phase Contrast Microscope. The sample was screened using MTT assay. This sample showed remarkable cytotoxicity against almost all the tested cells in a dose dependent manner.

6.3.1. Cytotoxicity of Vero Cell Line

The cytotoxicity of the hemolymph in Vero Cell Line is represented graphically in Fig 4. The tested samples showed high cytotoxicity on vero
cells at concentrations viz. 600 µg/mL, 500 µg/mL, 400 µg/mL, 300 µg/mL, 200 µg/mL, 100 µg/mL, 50 µg/mL to 10 µg/mL. The 50% of cytotoxicity (IC<sub>50</sub>) was observed at the concentration of 200 µg/mL. The photographic confirmation of the Vero cell line has presented in Fig 5.

**Fig: 4** Cytotoxicity of Vero cells after treating with various concentration of hemolymph

**Fig: 5** In vitro Cytotoxicity assay of *D. dehaani* in Vero Cell Line (A- Vero Cells Control; B- Vero Cells 24 Hours; C-Vero Cells 48 Hours; D-Vero Cells 72 Hours)
6.3.2. HepG2 Cell lines

The observations of the Cell Viability Count to determine anticancer activity against HepG2 cell line is represented graphically in fig 6. The hemolymph showed high cytotoxicity on HepG2 cells at concentrations viz. 195 µg/mL, 175 µg/mL, 150 µg/mL, 125 µg/mL, 100 µg/mL, 95 µg/mL, 75 µg/mL, 50 µg/mL, 25 µg/mL, 10 µg/mL to 5 µg/mL. HepG2 cells displayed dose dependent decrease in viability visible as early as 24 hrs. The hemolymph that produced the maximal effect was 195 µg/mL, and the half inhibitory concentration (IC₅₀) was 75 µg/mL. The Morphological changes on HepG2 Cell Line after treating with various concentrations of hemolymph has presented in Fig 7.

![Graph showing the effect of various concentrations of hemolymph on HepG2 cell viability.]

**Fig : 6 Anticancer Activity of HepG2 Cell Line after treating with various concentrations of hemolymph**
Fig : 7 Morphological changes on HepG2 Cell Line after treating with various concentrations of hemolymph. (A- Cell control; B-195 µg/mL; C-125 µg/mL; D-95 µg/mL; E- 75 µg/mL; F-10 µg/mL)

6.3.3. HT-29 Cell lines

The observations of the cell viability count against HT-29 cell line are represented in fig 8. The hemolymph revealed high activity on HT-29 cells at different concentrations viz., 195 µg/mL, 175 µg/mL, 150 µg/mL, 125 µg/mL, 100 µg/mL, 95 µg/mL, 75 µg/mL, 50 µg/mL, 25 µg/mL, 10 µg/mL to 5 µg/mL. HT-29 cells displayed dose dependent decrease in viability visible as early as 24 hrs. The hemolymph that produced the maximal effect was 195 µg/mL, and the half inhibitory concentration was 95 µg/mL. The Morphological changes on HT-29 Cell Line after treating with various concentrations of hemolymph has presented in Fig. 9.
Fig: 8 Anti-cancer activity of HT-29 Cell line after treating with various concentration of hemolymph

Fig: 9 Morphological changes on HT-29 Cell Line after treating with various concentrations of hemolymph. (A- Cell control; B-195 µg/mL; C-125 µg/mL; D-95 µg/mL; E- 75 µg/mL; F-10 µg/mL)
6.3.4. Rhabdomyosarcoma Cell lines

Fig. 10 represented the observations of the cell viability count against Rhabdomyosarcoma cell line. The tested hemolymph showed high activity on Rhabdomyosarcoma cells at various concentrations viz. 195 µg/mL, 175 µg/mL, 150 µg/mL, 125 µg/mL, 100 µg/mL, 95 µg/mL, 75 µg/mL, 50 µg/mL, 25 µg/mL, 10 µg/mL to 5 µg/mL. RD cells displayed dose dependent decrease in viability visible as early as 24 hrs. The hemolymph that produced the maximal effect was 195 µg/mL, and the half inhibitory concentration (IC\textsubscript{50}) was 75 µg/mL. The morphological changes on RD Cell Line after treating with various concentrations of hemolymph has displayed in Fig. 11.

![Graph showing cell viability count against various concentrations of hemolymph](image)

**Fig: 10 Anti-cancer activity of Rhabdomyosarcoma Cell line after treating with various concentration of hemolymph**
Fig: 11 Morphological changes on Rhabdomyosarcoma Cell Line after treating with various concentrations of hemolymph. (A- Cell control; B-195 µg/mL; C-125 µg/mL; D-95 µg/mL; E- 75 µg/mL; F-10 µg/mL)

6.3.5. A549 Cell lines

The observations of the cell viability count against A549 cell line are denoted in Fig 12. The hemolymph showed high cytotoxicity on A549 cells at various concentrations viz., 195 µg/mL, 175 µg/mL, 150 µg/mL, 125 µg/mL, 100 µg/mL, 95 µg/mL, 75 µg/mL, 50 µg/mL, 25 µg/mL, 10 µg/mL to 5 µg/mL. A549 cells displayed dose dependent decrease in viability visible as early as 24 hrs. The hemolymph that produced the maximal effect was 195 µg/mL, and the half inhibitory concentration (IC₅₀) was 75 µg/mL. The morphological changes on A549 Cell Line after treating with various concentrations of hemolymph has shown in Fig. 13.
Fig: 12 Anti-cancer activity of A549 Cell line after treating with various concentration of hemolymph

Fig: 13 Morphological changes on A549 Cell Line after treating with various concentrations of hemolymph. (A- Cell control; B-195 µg/mL; C-125 µg/mL; D-95 µg/mL; E- 75 µg/mL; F-10 µg/mL)
6.3.6. DNA fragmentation

Among the four cell line tested HepG2 showed pronounced activity hence it has been taken further for the DNA fragmentation studies. DNA was isolated from HepG2 cell line treated with different concentration of hemolymph from normal cell line for control. The result of the DNA fragmentation test is presented in Fig 14. The results indicate a clear DNA damage to HepG2 cells after the treatment of hemolymph. These results suggest that the crab’s hemolymph had a dose dependent deleterious effect on HepG2 cell viability.

Lane 1: Control,
Lane 2: Concentration : 62.5µg/ml,
Lane 3: Concentration : 125µg/ml,
Lane 4: Concentration : 31.6µg/ml,
M- Marker- 100 bp DNA Ladder

Fig: 14 DNA fragmentation of HepG2 Cell Line after treating with various concentrations of hemolymph
6.4. DISCUSSION

The current promises for treating human cancer are limited to excision surgery, general chemotherapy, radiation therapy and, in a minority of breast cancers that rely on estrogen for their growth, antiestrogen therapy. Although there has been considerable improvement in the treatment of cancer, the overall prognosis remains not good. Therefore, investigators continue to search for new therapeutic strategies. The present study was undertaken to investigate the anticancer mechanisms of the marine crab *D. dehaani* against several human cancer lines.

A number of researchers have focused on identifying novel marine natural product as anticancer drugs (Simmons *et al*., 2005). Anticancer compounds have characteristics of multi-function, high sensitivity, stability and so on (Leng *et al*., 2005). In the present study the hemolymph of the crabs were tested to evaluate their cytotoxic and anticancer potential. Cytotoxic compounds are one of the most important classes of drugs used for cancer treatment. There have been several researches to get new cytotoxic agents. In this regard antimicrobial compounds isolated from marine organisms showed considerable promises.

Wu *et al*., (2001) observed an enhanced scavenging effect at the low concentration of combined selenium and Ge-132. Antitumor mechanisms of carboxyethyl-germanium sesquioxide (Ge-132) in mice bearing Ehrlich ascites tumors were reported. Ge-132 showed its *in vivo* antitumor effect partly due to its inducing the antitumor immunity of the host (Zhang *et al*., 2009). The cell viability was measured by the MTT method using HaCaT cells (Lim *et al*., 2010). In the present study the results showed remarkable cytotoxicity against the cells in a dose
dependent manner. In the cytotoxicity of Vero Cell Line the IC\textsubscript{50} value was found in the concentrations of 200\(\mu\)g/ml.

In the current investigation anticancer activity half inhibitory concentration (IC\textsubscript{50}) was 75\(\mu\)g/mL against HepG2 cell lines. Similarly Erythrazole B from \textit{Erythrobacter sp.} was found to be highly toxic against H1395, H2122 and HCC366 cell lines, with IC\textsubscript{50} values of 1.5, 2.5 and 6.8\(\mu\)M, respectively (Hu \textit{et al.}, 2011). The chemical structure of Bostrycin contains a quinone moiety that is responsible for its cytotoxicity. Additionally, the phenolic hydroxyl groups enhance its effect through the reduction of ROS levels (Zhang \textit{et al.}, 2010).

In the HT-29 cell lines the IC\textsubscript{50} was 95\(\mu\)g/mL. In the Rhabdomyosarcoma cell lines the IC\textsubscript{50} was 75\(\mu\)g/mL. Methyl spongoate displays potent toxicity against six hepatocellular carcinoma cell lines, with IC\textsubscript{50} values ranging from 1.7 to 9\(\mu\)M. It is known that advanced hepatocellular carcinomas are generally resistant to anticancer drugs because of the multidrug resistant (MDR) phenomena (Thomas, 2009; Papatheodoridis \textit{et al.}, 2010). In the A549 cell lines the IC\textsubscript{50} was 75\(\mu\)g/mL. Similar results were found with Keenamide A which exhibited significant activity against the P-388, A-549, MEL-20, and HT-29 tumor cell lines (Wesson and Hamann, 1996).

An anticancer glycol peptides was reported from \textit{Meretrix meretrix} and its inhibitory rate affecting the KB (human Caucasian/epidermal carcinoma) cell line was 69\% at 200\(\mu\)g.mL\textsuperscript{-1} (Zhang and Wu, 2006). Relatively strong anticancer peptides were also found from \textit{M. meretrix} with IC\textsubscript{50} of 10\(\mu\)g.mL\textsuperscript{-1} (Liu \textit{et al.}, 2004). In addition, dalastatin-10, extracted from the sea hare \textit{D. auricularia}, has entered into clinical trials. Dolastatin-10 is a penta peptide with four of the residues being structurally unique. It is the most potent anti-proliferative agent.
known with an ED50 of $4.6 \times 10^{-5}$ $\mu$g.mL$^{-1}$ against murine PS leukemia cells (Pettit et al., 1987).

It was reported that the denbinobin obtained from *Ephemerantha lonchophylla* was found to reduce the cell viability of human colorectal cancer HCT-116 and HT-29 cells in a concentration-dependent manner as measured by MTT assay (Chen et al., 2008). The IC$_{50}$ with *Curcuma longa* hot water extract was found between 50 and 150 $\mu$g/mL on HepG2 cell line (Waiyaput et al., 2012). In the present study the half inhibitory concentration (IC$_{50}$) was 75 $\mu$g/mL. Similarly it was also reported that *Piper sarmentosum* ethanolic extract shows a profound effect on a human hepatoma cell line (HepG2) with IC$_{50}$ value at 12.5 $\mu$g/mL. In contrast, the ethanolic extract did not induce cytotoxicity in a nonmalignant cell line (Chang’s liver cell line) (Ariffin et al., 2009).

Aplidine (dehydrodidemnin B) is a second-generation didemnin that was isolated from the Mediterranean tunicate, *Aplidium albicans* (Sakai et al., 1996). In the present study the hemolymph showed pronounced activity against HepG2 cell lines. Likewise the dolastatins isolated from *Dolabella auricularia*, inhibit cell proliferation and induce apoptosis in numerous malignant cell lines. These actions are mediated through interactions with tubulin, resulting in the alteration of microtubule function (Pettit et al., 1998a, b). The dolastatins also induce apoptosis in cancer cell lines (Haldar et al., 1998). Dolastatins exerted profound cytotoxic effects in animals bearing intraperitoneal tumors; in addition, they exhibited synergistic antitumor activity with vinca alkaloids and bryostatin-1 (Mohammad et al., 1995, 1998). Dolastatin 10 has been evaluated in various phase I clinical trials (McElroy et al., 1997; Tran et al., 1997; Bagniewski et al., 1997; Garteiz et al., 1998). Extensive in vivo
work demonstrated that the agent had activity in breast and colon cancer (Garcia et al., 1996).

Programmed cell death is primarily mediated by Fas receptor signaling and shows DNA fragmentation by BMAP-27. The BMAP-28-treated U937 cell line revealed that some AMPs are endowed with cytotoxic mechanisms also involving triggering of cancer cell suicide by apoptosis (Risso et al., 1998). Cytotoxic compounds are one of the most important classes of drugs used for cancer treatment. In this regard anticancer compound isolated from marine organisms showed considerable promises. In the present study, MTT assay was used for evaluation of cytotoxic activity of the crab hemolymph and showed cytotoxic effects. The findings of the present showed that the hemolymph of the crab D. dehani might be a good source to inhibit the HepG2 cells and it would be a great source for anticancer compounds which can be useful for human welfare. To confirm the effect of hemolymph the in vivo studies has been done in the next chapter.