Chapter 2: Screening of crude extracts of Indian marine bivalves for anti-HIV-1 activity
2.1 Introduction

2.1.1 Bioactive compounds from marine organisms

The oceans represent an important resource for the discovery of novel bioactive compounds. The oceans cover more than 70% of the Earth’s surface and contain more than 3,00,000 species of plants and animals (Donia et al, 2003). Almost every class of marine organisms contains variety of molecules with unique structural features due to the physical and chemical conditions of the marine environment (Chin et al, 2006). The bioactive compounds are involved in biological functions of marine organisms such as communication, infection, reproduction and self-defense. More than 12,000 natural products have been isolated from marine algae, sponges, coelenterates, ascidians, molluscs, echinoderms and bryozoans (Matthee et al, 1999; Costantino et al, 2004). In 1950s, Spongothymidine and spongouridine were identified from Caribbean sponge *Cryptotheca crypta*, which are among the first compounds isolated from marine organisms. Their analogues Ara-A and Ara-C have potent antiviral activities and are in clinical trials (Chin et al, 2006). Identification of antiviral activity of nucleoside analogues spongothymidine and spongouridine provided a platform to screen nucleoside analogues for antiviral activity against HIV-1 virus and lead to the identification of several of the nucleoside analogues with anti-HIV-1 activity. Recently, Ziconotide, isolated from the cone snail *Conus magus* venom was approved to treat chronic pain. Ziconotide shows its effect by blocking N-type voltage gated calcium channels (Schroeder et al, 2004). Several marine organism derived compounds and their analogues are under clinical trials for different diseases, some of which are listed in Table-2.1.

Molecules with promising level of antiviral activity have been isolated from marine organisms following the bioassay-guided protocols, some of which are listed in Table-2.2. Cyanovirin-N, an antiviral lectin was isolated from a blue green alga *Nostoc ellipsosporum*. It inhibits the replication of many viruses including Ebola virus, HIV-1, Influenza Virus, Hepatitis C, Human Herpes Virus 6 and Measles Virus etc (Ziolkowska et al, 2006). Cyanovirin N binds to HIV-1
Table 2.1: Some molecules isolated from marine organisms that are in clinical trials.

<table>
<thead>
<tr>
<th>Marine organism</th>
<th>Biological activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Agelas mauritianus</em></td>
<td>It shows antitumor activity by binding to antigen receptors of Natural killer cells</td>
<td>Hayakawa et al, 2003.</td>
</tr>
<tr>
<td><em>Bulgula neritina</em></td>
<td>It binds to the phorbol ester binding cellular receptor and down modulates protein kinase C isoforms in tumor cells, leading to the inhibition of growth, alteration of differentiation and cell death</td>
<td>Newman et al, 2004.</td>
</tr>
<tr>
<td><em>Spisula polynyma</em></td>
<td>It inhibits the growth of human renal tumors, melanoma and prostate tumors in mouse models</td>
<td>Cuadros et al, 2000.</td>
</tr>
<tr>
<td><em>Squalus acanthias</em></td>
<td>It inhibits growth factor mediated endothelial cell proliferation, migration and angiogenesis</td>
<td>Moore et al, 1993</td>
</tr>
</tbody>
</table>
Table 2.2: Antiviral compounds identified from marine organisms.

<table>
<thead>
<tr>
<th>Name of the compound</th>
<th>Nature of the compound</th>
<th>Source</th>
<th>Activity against</th>
<th>Reference</th>
</tr>
</thead>
</table>

Surface protein gp120 and inhibits the viral entry. Papuamides A, Avarone and Microspinosa mide were isolated from different species of marine sponges and showed anti-HIV-1 activity (Table-2.2). Marine bivalves belong to the class Bivalvia of Phylum Mollusca and are widely distributed in oceans. They are good source of protein food and novel chemical structures. They are cultured to large numbers in coastal waters as a source of food and for identification of novel molecules. Myticin and Aplysia mytilus inhibitory peptides (AMIP), isolated from *Mytilus galloprovincialis* show antimicrobial activity (Mitta et al, 1999). Bioactivity of some of the novel proteins such as Class I metallothionein proteins isolated from *Crassostrea virginica* and Pernin isolated from *Perna canaliculus* have not yet been characterized (Roesijadi et al, 1989; Scotti et al, 2001). Sulphated beta-galactans were isolated from clams, which inhibit HIV-1 replication in T cell lines (Amornrut et al, 1999).
2.1.2 Bioactivity in Indian marine bivalves

In India, majority of bivalve species are distributed along the coastal and estuarine waters. Mussels and clams are commonly distributed along the coastal waters of Goa, Cochin, Malvan and Mangalore. Several species of marine bivalves are rich source of proteins and bioactive compounds (Chatterji et al, 2002). Antiviral activity was identified in the extracts of green mussel (*Perna viridis*), that inhibited the replication of Influenza, Herpes simplex virus and Respiratory syncytial viruses (Bichurina *et al.*, 1994; Chatterji et al, 2002). The extract of Russian Blue mussel *Mytilus edulis* is available in Russian markets under the trade name ‘Midel’ as a formulation to cure several viral infections. This extract shows antiviral activity towards Influenza, Herpes simplex virus, Hepatitis and HIV-1. But till date, no molecule with anti-HIV-1 activity has been identified from Indian marine bivalves. In the present investigation, I have screened marine bivalves available in the Goa coastline of India for anti-HIV-1 activity.

2.2 Materials and methods

2.2.1 Cell culture

“CEM-GFP”, a human CD4⁺ reporter T cell line having integrated copies of HIV-1 LTR regulated Green Fluorescent Protein (GFP) reporter gene (Corbeil et al, 1997) and P4, a CD4 and CXCR4 expressing Hela cell line, having integrated copies of LTR-β-galactosidase reporter gene (Kimpton et al, 1992) were obtained from NIH AIDS reagent program, USA. Jurkat, a human CD4⁺ T cell line was obtained from NCCS cell repository, USA. CEM-GFP and Jurkat cell lines were maintained in RPMI 1640 (Invitrogen, USA) with 10% fetal bovine serum (FBS) (Invitrogen, USA). P4 cell line was maintained in DMEM with 10% FBS. In the culture media, 100 U/ml of penicillin and 100 µg/ml of streptomycin (Invitrogen, USA) was added to avoid bacterial contamination. G418 sulphate (500 µg/ml) (Invitrogen) was added to the medium of CEM-GFP stable cell line and puromycin (0.5 µg/ml) (Sigma) was added to the medium of P4 cells as a selection antibiotic.
2.2.2 Preparation of crude extracts of Indian marine bivalves

Nine species of marine bivalves were collected from different coastal habitats in Goa as listed in Table-2.3. Two of the Indian marine bivalves are shown in Figure-2.1. Bivalves were collected from the sea using National Institute of Oceanography (NIO) marine vessel and were identified by zoologists at NIO, Goa. The crude extract of bivalves was prepared by enzyme-acid hydrolysis process as previously described (Chatterji et al, 2000). Briefly, the bivalves were collected, cleaned with freshwater and deshelled. The meat (300 g) and mantle fluid (50 ml) were collected from each bivalve. The meat of each of the marine bivalve was finely chopped and was treated with protosubtilisin enzyme isolated from \textit{Bacillus subtilis} (60 mg/ml in distilled water) and was fermented at 40 °C for 3 hrs. Into the resultant solution, 50 ml of distilled water and 150 ml of concentrated hydrochloric acid were added and boiled at 100 ± 2 °C for 18 hours.

**Fig 2.1:** \textit{Indian marine bivalves: Black clam and Green mussel.}

**Table 2.3:** Indian marine bivalves tested for anti-HIV-1 screening.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Common name</th>
<th>Scientific name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Green mussel</td>
<td>\textit{Perna viridis}</td>
</tr>
<tr>
<td>2</td>
<td>Tisri clam</td>
<td>\textit{Meretrix casta}</td>
</tr>
<tr>
<td>3</td>
<td>Mud clam</td>
<td>\textit{Polymesoda erosa}</td>
</tr>
<tr>
<td>4</td>
<td>Giant oyster</td>
<td>\textit{Crassostria gryphoides}</td>
</tr>
<tr>
<td>5</td>
<td>Oyster</td>
<td>\textit{Saccostrea cucullata}</td>
</tr>
<tr>
<td>6</td>
<td>Rock oyster</td>
<td>\textit{Crassostrea crosostrea}</td>
</tr>
<tr>
<td>7</td>
<td>Brown mussel</td>
<td>\textit{Perna indica}</td>
</tr>
<tr>
<td>8</td>
<td>Clams</td>
<td>\textit{Paphia maalabaricus}</td>
</tr>
<tr>
<td>9</td>
<td>Black clam</td>
<td>\textit{Villorita cyprinoids}</td>
</tr>
</tbody>
</table>
Then mix was allowed to cool at room temperature. It was then neutralized with sodium hydroxide solution to obtain a pH in the range of 5.2 to 6.2 and was kept in a separating funnel for 10 days. Out of three layers formed in the separating funnel, middle layer was carefully removed and pH was adjusted to 7.2 with PBS. This extract was lyophilized, 100 mg/ml of stock solution was made in PBS and was then used as the crude extract (Chatterji et al, 2000).

2.2.3 MTT cytotoxicity assay
Cytotoxicity of crude extracts was assayed using MTT cell proliferation kit (Roche, Germany) in CEM-GFP and Jurkat T cell lines according to the manufacturer’s protocol. Briefly, 2 X 10^4 cells/well were seeded in 96-well plate and were treated with the extracts at different concentration, untreated or vehicle treated cells were used as control. After 48-72 hours post incubation, 10 μl of MTT reagent (5 mg/ml) was added to the cells to allow the catalysis of MTT substrate to form formazan crystals, which were then solubilized in solubilization buffer (10% SDS + 0.01 N HCl). After 12 to 16 hours of solubilization, color development was read at 540 nm. The concentration of the extracts at which more than 95% of cells were viable was calculated for each extract.

2.2.4 Virus isolates and generation of viral stocks
HIV-1NL4-3 and HIV-1IIIb molecular clones were obtained from NIH AIDS reagent programme, USA (Popovic et al, 1984; Adachi et al, 1986). Viral stocks were generated by transfection of molecular clones into 293T cells. Briefly, around 2.5 X 10^6 293T cells were seeded in 90 mm plates and were incubated for 12-16 hours for adherence. After the incubation period, cells were transfected with plasmid DNA using calcium phosphate transfection. In this, Calcium-DNA complexes were prepared by mixing 15 to 20 μg of plasmid DNA in 125 mM CaCl₂ (Sigma, USA) with HEPES buffer. This reaction mixture was incubated for 20 to 30 minutes at room temperature and was added on top of the monolayer of 293T cells. After 12 hours post transfection, media was removed and fresh media was added. Then 24 to 36 hours post transfection, culture supernatant was collected.
and was stored at –70 °C. Virus was pelleted from culture supernatants at 28000 rpm for 2 hours 30 minutes using ultra centrifugation in SW29 rotor. The pellet was resuspended in serum free RPMI with 100 mM HEPES, pH 7.3, filtered, aliquoted and was stored at –70 °C.

2.2.5 Determination of infectious virus concentration of viral stocks

The total number of infectious virions (infectious virus concentration) in the viral stock was calculated according to the standard protocol using P4 cell line (Kimpton et al, 1992). In this, 5 X 10^5 cells/well were seeded in 6 well plate and were incubated over night. After the incubation, viral stock was serially diluted in 1 ml of complete media. The media from cells was removed and the diluted virus was used to infect the monolayer of P4 cells for 4 hours. Uninfected cells were used as negative control. The infected cells were incubated for 48 hours and then media was removed and washed with 1X PBS. Then cells were fixed with 0.5% glutaraldehyde at room temperature for 10 min. After fixation, cells were washed with PBS. Then β-galactosidase staining solution [Potassium ferriferrocyanide (12.5 mM Potassium ferricyanide and 12.5 mM Potassium ferrocyanide); 1:50 dilution of 50 mg/ml 5-bromo-4-chloro-3-indoly1, β-D-galactoside (X-gal) in Dimethyl formamide and 1 mM MgCl2 in 1X PBS] was added on top of the monolayer of fixed cells. The infected cells stained blue with β-gal staining solution. The total number of blue cells was counted under the microscope at 10X magnification in five to eight fields and average of all the fields was calculated. The total number of infectious virions/ml of the viral stock was calculated using the following equation.

\[
\text{Average of total number of blue cells X Dilution of the virus X 300} \\
\text{1 ml of viral stock}
\]

The MOI can be calculated as the ratio between the number of infectious virions to the total number of cells used in the infection assay.
2.2.6 HIV-1 infection and anti-HIV-1 screening assay

For infection, typically 5 million CEM-GFP or Jurkat cells were washed in polybrene (2 µg/ml) (Sigma, USA) containing medium and were resuspended in 1 ml of 1 µg/ml polybrene containing medium and were infected with 0.1 MOI of HIV-1\textsubscript{NL4-3} for 4 hours as described earlier (Liu et al, 1997). Cells were washed twice with serum free RPMI and were resuspended in 25 ml of complete medium. Infected cells were seeded at 2 X 10\textsuperscript{5} cells/ml in each well of the 24-well plate. The selected non-cytotoxic concentration of each of the sample was added to the cells in duplicate wells. Untreated or vehicle treated cells were used as controls. The green fluorescence emitted by cells during their exposure to UV light was used in following days to monitor the progress of HIV-1 infection. On day 7-10 post infection, the viral supernatant was collected from each well without disturbing the infected cells and was stored in –70 ºC freezer for analysis of virus production by p24 antigen capture ELISA.

2.2.7 HIV-1 p24 antigen capture enzyme linked immuno sorbent assay (p24 ELISA)

HIV-1 p24 antigen ELISA Kit (Perkin Elmer, USA) was used to detect the p24 antigen in culture supernatants, which is a direct measure of virus production. Briefly, viral supernatants were lysed in 0.5% Triton X 100, diluted in serum free RPMI and 200 µl of diluted culture supernatant was loaded into the p24 antibody coated wells. Then assay was performed to detect p24 core antigen according to the manufacturer’s protocol. The percentage of HIV-1 inhibition was calculated using following equation.

\[
\% \text{ of HIV-1 inhibition} = 100 - \left( \frac{\text{Conc. of p24 in treated culture supernatant}}{\text{Conc. of p24 in untreated culture supernatant}} \times 100 \right)
\]
2.3 Results

2.3.1 Identification of non-cytotoxic concentration of the crude extracts in T cell lines

The crude extracts of Indian marine bivalves were prepared according to the protocol described in section 2.2.2. Around $2 \times 10^5$ CEM-GFP or Jurkat T cells were treated with different concentrations of crude extract ranging from 0.1 mg/ml to 1.5 mg/ml. Untreated cells were used as control and after 48-72 hours post treatment, MTT cytotoxicity assay was performed to analyze the non-cytotoxic concentration of each extract on both T cell lines as described in section 2.2.3. All the crude extracts were non-cytotoxic till 0.55 mg/ml concentration in Jurkat cells and till 0.6 mg/ml in CEM-GFP cells. Few of them were non-cytotoxic even at 1 mg/ml concentration such as Rock oyster and clam. The highest non-cytotoxic concentration at which more than 95% cells were viable was calculated for each of the crude extracts in CEM-GFP and Jurkat T cells. The results are tabulated in Table-2.4.

Table 2.4: Highest non-cytotoxic concentrations of crude extracts of Indian marine bivalves in CEM-GFP and Jurkat cells as analysed by MTT assay.

<table>
<thead>
<tr>
<th>Name of the marine bivalve</th>
<th>Highest non-cytotoxic Conc. in mg/ml in CEM-GFP T cells</th>
<th>Highest non-cytotoxic Conc. in mg/ml in Jurkat T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green mussel</td>
<td>0.600</td>
<td>0.550</td>
</tr>
<tr>
<td>Tisri clam</td>
<td>0.800</td>
<td>0.750</td>
</tr>
<tr>
<td>Mud clam</td>
<td>0.800</td>
<td>0.850</td>
</tr>
<tr>
<td>Giant oyster</td>
<td>0.850</td>
<td>0.800</td>
</tr>
<tr>
<td>Oyster</td>
<td>1.000</td>
<td>0.900</td>
</tr>
<tr>
<td>Rock oyster</td>
<td>1.100</td>
<td>1.050</td>
</tr>
<tr>
<td>Brown mussel</td>
<td>0.950</td>
<td>0.750</td>
</tr>
<tr>
<td>Clams</td>
<td>1.100</td>
<td>0.950</td>
</tr>
<tr>
<td>Black clam</td>
<td>0.950</td>
<td>0.900</td>
</tr>
</tbody>
</table>
2.3.2 Screening of extracts for anti-HIV-1 activity in HIV-1_{NL4-3} infected CEM-GFP cells

CEM-GFP, a reporter T cell line was used as a model cell system in anti-viral-screening experiments. It is a well-established cell line for high-throughput screening of antiviral compounds (Corbeil et al, 1997). CEM-GFP has integrated copies of LTR-EGFP cassettes and upon HIV-1 infection, these cells produce large amount of GFP protein in the infected cells (Figure-2.2). Due to the increased expression of EGFP, infected cells are easily visualized under the fluorescence microscope (Figure-2.3). The GFP fluorescence of infected CEM-GFP cells can also be measured by microfluorimetry. In an antiviral screening assay, CEM-GFP cells were infected with HIV-1_{NL4-3} according to the protocol described in section 2.2.4 and were treated with crude extract of bivalves at non-cytotoxic concentration of 0.6 mg/ml. This was to enable the comparison of HIV-1 inhibitory effect of different extracts at similar concentration in the screening assay and also to avoid cytotoxicity in long term cultures, if any. Untreated infected cells were used as negative control and Azidothymidine as a positive control. Progress of the infection in infected cells was visually monitored by increased expression of green fluorescence under the fluorescence microscope for 7 to 10 days following the day of infection. Cell supernatants were collected when

Fig 2.2: A cartoon depicting the enhanced expression of green fluorescence upon HIV-1 infection in CEM-GFP reporter T cell line.
Fig 2.3: GFP expression in uninfected (upper panel) and HIV-1\textsubscript{NL4-3} infected (lower panel) CEM-GFP cells. BF, Bright field; FL, fluorescence image; UI, Uninfected; INF, Infected.

Fig 2.4: Analysis of GFP expression in HIV-1\textsubscript{NL4-3} infected CEM-GFP cells treated with crude extracts of Indian marine bivalves (0.6 mg/ml). More than 80 to 90% of the cells were green in the untreated wells under the fluorescence microscope. An aliquot of cells were used to quantitate GFP fluorescence using microplate Fluorimeter (Thermo labsystems, Finland).
A reduction in GFP fluorescence was observed in several of the bivalve extract treated CEM-GFP cells (Figure-2.4). More than 75% reduction in GFP fluorescence was observed in Black clam, Green mussel and Tisri clam crude extract treated CEM-GFP cells (Figure-2.4). Culture supernatants collected from each well were then assayed for viral core antigen using p24 antigen ELISA. The p24 ELISA results show that HIV-1 production was decreased in cells treated with several bivalve extracts. More than 70% inhibition of p24 production was observed in Black clam, Green mussel and Tisri clam crude extract treatments as shown in Figure-2.5. This indicates that crude extracts of some Indian marine bivalves, specifically of Black clam, Green mussel and Tisri clam potently inhibit HIV-1 replication.

2.3.3 Anti-HIV-1 activity of extracts in HIV-1$_{NL4-3}$ infected Jurkat cells

The antiviral property of each extract of Indian marine bivalves was further tested in Jurkat cells, which is a widely used T cell line, to confirm our results obtained
with CEM-GFP cells. Jurkat cells were infected with HIV-1_{NL4-3} as described in section 2.2.4 and were incubated for 7 to 10 days in the presence of 0.55 mg/ml concentration of crude extracts. Untreated cells were used as control. After incubation period, culture supernatants were collected and p24 antigen was quantified by p24 ELISA. Treatment with some of the bivalve crude extracts inhibited HIV-1 production in Jurkat cells. In the presence of Black clam, Green mussel and Tisri clam extracts, virus production was reduced to 75% or more, in culture supernatants as shown in Figure-2.6. Our screening data thus indicates that extracts of Black clam, Green mussel and Tisri clam have potential anti-HIV-1 activity in T cells.

![Bar graph showing % HIV-1 inhibition (p24 ELISA) for different marine bivalve extracts and AZT.]

**Fig 2.6:** *Anti-HIV activity of crude extracts of Indian marine bivalves (0.55 mg/ml) in HIV-1_{NL4-3} infected Jurkat T cells.*

### 2.3.4 Crude extracts of Indian marine bivalves also inhibit HIV-1_{IIIB} replication in CEM-GFP cells

After experiments of antiviral screening with crude extracts using HIV-1_{NL4-3} viral isolate in two different T cell lines, they were also tested for antiviral activity against a different viral isolate, HIV-1_{IIIB}, which is a frequently used isolate in
Fig 2.7: Analysis of GFP expression in HIV-1<sub>IIIb</sub> infected CEM-GFP cells treated with crude extracts of Indian marine bivalves (0.6 mg/ml).

Fig 2.8: Anti-HIV-1 activity of Indian marine bivalve crude extracts (0.6 mg/ml) in HIV-1<sub>IIIb</sub> infected CEM-GFP cells.
HIV research. CEM-GFP cells were infected with HIV-1_{IIIb} as described in section 2.2.4 and were treated with 0.6 mg/ml of crude extract of bivalves. Green fluorescence was observed following the day of infection to observe the progress of HIV-1 infection. On the day of cell harvesting, GFP fluorescence was quantified using microfluorimeter. Significant reduction in green fluorescence was observed in Black clam, Green mussel and Tisri clam crude extract treated CEM-GFP cells (Figure-2.7). Culture supernatants were collected and assayed for p24 antigen detection to analyze the differences in virus production of crude extract treated cells as compared to untreated controls. p24 ELISA shows similar results as it was observed with HIV-1_{NL4-3} viral isolate. The extracts of Black clam, Green mussel and Tisri clam inhibit 70% or more virus production in HIV-1_{IIIb} infected CEM-GFP cells (Figure-2.8). This confirmed that crude extracts of several Indian marine bivalves inhibit replication of HIV-1 viruses in T cells.

2.4 Discussion

Several novel molecules with antiviral activity have been identified from marine organisms till date (Table-2.2). Some molecules showing potent anti-HIV activity have been also isolated from marine bivalves. The crude extracts of green mussel (*Perna viridis*), prepared by the enzyme-acid hydrolysis was previously shown to inhibit the replication of Influenza, Herpes simplex virus and Respiratory syncytial viruses (Chatterji et al, 2002). In this study, we have prepared the crude extracts of Indian marine bivalves using enzyme-acid hydrolysis process (Chatterji et al, 2000). In this process, larger biomolecules like proteins, carbohydrates etc. present in the meat of the animals are processed to generate a large number of small molecules. The hydrolysate containing novel chemical structures can be tested for biological activity and the active molecules can be purified by bioactivity-guided fractionation methods to identify the active molecule. In our study, the crude extracts of Indian marine bivalves were initially tested for their cytotoxicity in T cell lines. The highest non-cytotoxic concentrations of crude extracts in CEM-GFP and Jurkat T cells were determined by MTT cytotoxicity assay. Each of the crude extracts of bivalves were screened...
for anti-HIV-1 activity at 0.6 mg/ml concentration in CEM-GFP and 0.55 mg/ml concentration in Jurkat T cell lines using HIV-1\textsubscript{NL4-3} and HIV-1\textsubscript{IIIB} viral isolates. Our screening data obtained from the analysis of GFP expression in HIV-1 infected CEM-GFP reporter cells and quantitation of p24 core antigen (p24 ELISA) in the supernatants of HIV-1 infected CEM-GFP and Jurkat cells indicate that crude extracts of several of the Indian marine bivalves inhibit HIV-1 virus replication in T cells. Treatment of infected T cells with crude extracts of Black clam, Green mussel and Tisri clam potently inhibit HIV-1 replication. More than 80% inhibition of HIV-1 replication was observed with Black clam crude extracts in both the T cell lines infected with HIV-1\textsubscript{NL4-3} and HIV-1\textsubscript{IIIB} viral isolates. So Black clam was selected for further analysis and identification of the active molecule as described in detail in the next chapter of the thesis.

2.4 References


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