CHAPTER V

Antiviral activity and characterization of bioactive compounds from Sponge *Sigmadocia pumila* and *Holothuria atra* extracts

5.1. INTRODUCTION

The screening of natural products derived from marine organisms for antiviral activity has yielded a number of active organic solvent extracts. At present, about 40 compounds are commercially available in pharmacological markets, including alternative antiviral medicines. Many more are being tested as potential antiviral drugs at the preclinical and clinical stages. The growing interest in marine-derived antiviral compounds along with the development of new technology in marine cultures and extraction are expected to provide promising strategies and set new trends for modern medicine (Jarred and Yuanan, 2010).

Herpes Simplex Virus types 1 and 2 (HSV-1 and HSV-2) are recognized as world wide occurring human pathogens. There is an urgent need to discover and develop new alternative agents for the management of HSV infection. They are frequently responsible for infections on skin and mucosa of different locations including oral and genital regions. Both types 1 and 2 were the first human herpes viruses to be discovered and are among the most intensively investigated ones due to their ability to cause a variety of infections, the capacity to remain latent in their host for life, and the possibility to reactivate, causing lesions at or near the site of initial infection (Roizman et al., 2007). Currently, there is no cure for the chronic infection and prolonged therapy with the available anti herpes drugs which has resulted in some undesirable effects and induced the emergence of drug-resistant virus strains. Moreover, HSV has been described as a risk factor for HIV infection. This has triggered the search for new anti herpetic agents (Van et al., 2008).

Marine sponges are also a rich source of compounds with antiviral properties. The high number of HIV-inhibiting compounds isolated from sponges reflect an increased potential to fight AIDS in comparison to other viral diseases. The strong focus on screening had led to the discovery of compounds such as the Papuamides Cand D and haplosamates A and B which has also been patented as an antipsoriasis drug, and HIV inhibiting compound (Ford et al., 1999).
Lira et al. (2007) discovered that the new esculetin-4-carboxylic acid ethyl ester from the Brazilian marine sponge *Axinella sp* inhibited the SARS 3CL protease at the rate of $IC_{50}=46 \, \mu M$. This was a potentially significant finding because the 3CL protease was a “high profile target” in SARS drug development as it appeared to be involved in the release of replicative viral proteins as well as the RNA polymerase. Plaza et al. (2007) described three new depsipeptides (mirabamides A, C and D) isolated from the sponge *Siliquariaspongia mirabilis* that potently inhibited both HIV-1 in neutralization and HIV-1 envelope-mediated cell fusion suggesting these compounds act at an early stage of HIV-1 cell infection, “presumably through interactions with HIV-1 envelope proteins”.

New steroid glycosides were isolated from sponges. There are several well known structural groups, including sarasinosides, ulososides and some of erylosides, which have di or mono methylsterol derivatives as aglycons. The study on the Indonesian sponge *Melophlus sarasinorum* has led to the isolation of new sarasinosides, including J, K, L and M (Dai et al., 2005). Kristina et al. (2010) have tested the sponge species from the Caribbean Sea (Curaçao) and from the Great Barrier Reef (Lizard Island) for their ability to inhibit or activate cell protein phosphatase 1 (PP1). The most interesting activities including hemolytic, hemagglutinating, antibacterial and anti-acetylcholinesterase (AChE) were obtained from the organic extracts of *Ircinia felix*, *Pandaros acanthifolium*, *Topsentia ophiraphidites*, *Verongula rigida* and *Neofibularia nolitangere*. Goud et al. (2003) revealed the inhibition of HIV by two bis-quinolizidine alkaloids petrosins isolated from the Indian marine sponge *Petrosia similis*. The extensive investigation determined that both petrosins inhibited HIV-1 replication, formation of giant cells and recombinant reverse transcriptase *in vitro*. Similarly, two new bromotyrosine derived metabolites from the sponge *Psammaphyllsina purpurea* showed significant effects of antiviral effects (Jaroslaw et al., 1993). Batzelladine A & B, novel polycyclic guanidine alkaloids from the Carribean sponge *Batzella sp.*, exhibited potent inhibition to the binding of HIV glycoprotein, on CD4 receptors of T cells (Carte, 1996). Different classes of virus inhibitors have
been found in many different sponges. For example 2, 5 oligoadenylates which are reported to be involved in the interferon mediated response against a wide range of viruses in mammals. The antiviral action is based on the activation of a latent endoribonuclease that prevents viral replication by degradation of its mRNA as well as cellular RNA (Kelve et al., 2003). Similarly, marine derived natural product brominated polyacetylenic acids from the sponge *Xestospongia muta* inhibited HIV-1 protease (Patil et al., 1992). The *in vitro* antiviral evaluation of different marine sponges viz *Cliona* sp, *Agelas* sp, *Tethya* sp, *Axinella* sp, *Polymastia* sp and *Protosuberites* sp collected from the Brazilian coastline revealed potential cytotoxicity and antiviral activity (Cordeiro et al., 2006).

The bioactive metabolites oligoglycosides obtained from the holothurians belong to the triterpene series. Xylosides of cholestanol and 7-sterols; the predominant constituents in the steryl glycoside fraction from the sea cucumber *E. fraudatrix* were also found in other sea cucumbers viz., *H. nobilis, B. tenuissima*, and *A. mauritiana* from Lakshadweep, Andaman and Nicobar Islands (Radhika et al., 2002). The sea cucumber *Telenata ananas* derived bioactive compounds were reported to act as the chemokine receptor subtype-5 (CCR5) with possible anti-HIV activity (Hedge et al., 2002). *S. japonicas* found in Korea commonly known as the marine jinseng classified into 3 groups blue, red and black sea cucumber possessed antitumor and antiviral properties. Ethyl acetate fractions of *S. japonicas* inhibited melanogenesis in murine B16 melanoma cells (Woon et al., 2010). Potential use of sea cucumber *S. liouvillei* isolated compound chondroitin sulfate (the polysaccharides) are reported to exhibit antiviral activity to inhibit human immunodeficiency virus (HIV) infection (Chen, 2003). Sea cucumber such as the *Thelenata sp* derived fucosylated chondroitin sulfates (FCS), recognized as a type of sulfated polysaccharides showed effective anticoagulant activity and inhibited human immunodeficiency virus (HIV) infection. It was suggested as the potential compound for natural therapy against HIV disorders (Wu et al., 2010). In this Chapter of the thesis the detection of antiviral activity from the extracts of *S. pumila* and *H. atra* as well as identification of active fractions are presented.
5.2. MATERIALS AND METHODS

5.2.1. Cell lines and viruses

HSV-1 and HSV-2 strains were obtained from the Department of Virology, Christian Medical College (CMC), Vellore. Virus stocks were propagated in Vero cells. The virus stocks were prepared from the supernatants of infected cells and stored at -80°C. African green monkey kidney cell line (Vero) were obtained from the King Institute of Preventive Medicine, Chennai. They were maintained in Eagles minimum essential medium (EMEM) with high glucose and glutamine (HiMedia) supplemented with 5% heat inactivated FBS and 1.0% penicillin/streptomycin, at 37°C in a humidified atmosphere containing 5.0% CO₂.

5.2.2 Hemagglutination assay

Hemagglutination assay was carried out to test the effect of *Sigmadocia pumila* and *Holothuria atra* extracts in virus adsorption to target cells against HSV-1 and HSV-2. It was measured in 96 well microtitre plates. In the first row of plates 3% hRBC suspension in 100ml of distilled water at 100 µl was added as positive control and an equal amount of PBS (pH 7.4) at 100 µl was used as negative control. A 25 µl aliquot of herpes virus 1 and 2 solution with two fold serial dilution of PBS (pH 7.4) was used. The viruses were diluted at 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 concentrations and maintained in triplicates. Each dilution was added in six wells of each row. Extracts at the concentrations of 25 and 50µl were added to each dilution and maintained in triplicates. Human blood (B group) was maintained in 8% of sodium citrate. The RBCs were washed thrice with 0.01 M PBS (pH 7.4), and resuspended in the same buffer solution to get a 1% cell suspension. 50µl hRBC suspension was added in all the wells added with extracts and the samples were incubated for 1h at room temperature. The hemagglutination titer, which was defined as the reciprocal of the highest dilution exhibiting hemagglutination, was recorded.

5.2.3. Plaque reduction assay
Vero monolayer cells grown in 24 well tissue culture plates were infected with HSV-1 and HSV-2. Virus dilutions were made from $10^1$ to $10^7$ using 0.1ml of viral suspension. Virus adsorption was carried out for 1h at 37ºC in the presence of test extract. Virus dilutions were prepared in Eagles minimum essential medium. Prior to incubation, an overlay medium comprising of 0.8% carboxy methyl cellulose with 2% FBS was added. It was done to avoid formation of secondary plaques. Infected cell cultures were incubated at 37ºC at 5.0% CO$_2$ incubator for 2 to 3 days. The infected cells were stained and observed for plaque reduction. The infectivity titers were expressed as the number of plaque forming units per ml (pfu ml$^{-1}$). After incubation, cultures were stained with 1% (w/v) crystal violet solution. The plaques were counted by visual examination and the percentage of plaque inhibition was calculated as per the method of Rovozzo and Burke, (1973).

The Pfu = Plaque number x reciprocal of dilution  x reciprocal of volume in ml

The antiviral activity was defined as the percentage of plaque inhibition as follows:

% Plaque inhibition = $[1 – (\text{Number of plaque in test}/\text{Number of plaque in control}) \times 100]$

5.2.4. Neuraminidase inhibition assay

Neuraminidase inhibition assay was employed to test the effect of the *Sigmadocia pumila* and *Holothuria atra* in neuraminidase activity in virus. Extracts were taken at 25, 50,75,100µg concentrations. Along with this 25 µl PBS were added. It was then mixed with an equal volume of HSV-1 and HSV-2 virus solution (25 µl). Equal volume (50 µl) of the substrate solution (4-MU-NANA; 2- (4-ethylumbelliferyl)-A-D-N acetyl neuraminic acid sodium, Sigma) was added and the mixture was further incubated at 37ºC for 2 h, protected from light. Optical density was measured at the wavelength of 550 nm.

Relative activities were calculated by the following formula:

Relative activities (%) = \frac{\text{NA activites with extracts}}{\text{NA activites without extracts}} \times 100

5.2.5. NMR Analysis
The active fractions obtained from column chromatography were analysed for Nuclear magnetic resonance spectroscopy (NMR) analysis. Optical rotations were measured on a Perkin-Elmer Model 341 LC polarimeter. $^1$H NMR and $^{13}$C NMR experiments were performed on Bruker Unity 400 and 600 MHz spectrometers. NMR spectra were referenced to the CD3OD solvent signals at δ 3.30 (1H) and 49.00 (13C), respectively. The spectra were obtained using the standard Bruker software. The samples were dissolved in different solvents (i.e. DMSO-$d_6$, CDCl3, and CD3OD), the choice of which was dependent on the solubility of the samples. The observed chemical shift (δ) values were given in ppm and the coupling constants (J) in Hz.
5.3. RESULTS

Hemagglutination assay

To determine whether the extracts can block the Herpes virus adsorption or cell entry, the hemagglutination titer of the Herpes simplex virus 1 and 2 was measured in 96-well microplates with U-shaped bottom. Results indicated that the Herpes simplex virus had the ability to adsorb to the human red blood cells resulting in hemagglutination. Here, it was noted that the extracts could interfere with the viral adsorption to RBC resulting in hemagglutination. The minimum concentration of extracts could be detected based on the fact that if the extracts could interfere with the viral adsorption to RBC resulting in hemagglutination. The titer was expressed as the reciprocal of the highest dilution of the virus showing complete hemagglutination.

The hemagglutination is brought by the interaction of specific virus glycoproteins with surface receptors present on the plasma membrane of RBCs. The extracts of *Sigmadocia pumila* had showed strong haemagglutination activity at the increase in dilution ranging from 1:2 to 1:64 in HSV-1 and HSV-2. It was also noted that the extracts of *Holothuria atra* also had the ability to inhibit the HSV-1 and HSV-2 only at higher dilution of 1:32 and 1:64. The presence of lattice or pellet showed that 50% of the agglutination took place on both HSV-1 and HSV-2. The higher incidence of hemagglutination was noted more for the sponge extract than the sea cucumber (Fig 5.1 and 5.2). This screening clearly indicated that the sponges and holothurians extract acted as antiviral agents.

Plaque reduction assay

Results presented in Fig 5.3 and 5.4 on the evaluation of antiviral activities in terms of plaque formation towards *Sigmadocia pumila* and *Holothuria atra* provide evidence of antiviral effect on post attachment stages of HSV-1 and HSV-2 to the Vero Cells. The *S. pumila* showed comparatively higher inhibitory action against the Herpes simplex virus 1 (HSV-1). The *H. atra* had less inhibition against the HSV-1. In this assay, it was also observed that the viral inhibition rate was high in *S. pumila* on Herpes simplex virus 2 (HSV-2) when compared to that of *H. atra*. 
The concentration of extracts for both *Sigmadocia pumila* and *Holothuria atra* was from 10\(\mu\)g/ml to 70 \(\mu\)g/ml respectively. The dilution of the viruses HSV-1 and HSV-2 were made from \(10^1\) to \(10^7\). The tested viruses were affected with the increase in concentration of extracts. The *S. pumila* and *H. atra* exhibited significant antiviral activity, and suggested the potential role of extracts.

The effect of inhibition in plaque formation was evaluated based on the \(10^1\) to \(10^7\) dilutions of HSV-1. The results showed that methanolic extracts of *S. pumila* and *H. atra* had antitherpetic compounds. Based on the Plaque forming units for each dilution at the concentration from 10\(\mu\)g to 70 \(\mu\)g of extracts, the plaque inhibition has been identified. The *S. pumila* extract showed maximum plaque inhibition of 83% with the plaque formation units of at \(1.7 \times 10^9\) pfu ml\(^{-1}\) and a minimum plaque inhibition of 42% with the plaque formation units at \(5.0 \times 10^3\). Similarly in *H. atra* the highest plaque inhibition rate was as at 75% with \(2.4 \times 10^3\) pfu ml\(^{-1}\). Less inhibition rate was observed at 33% for \(6.0 \times 10^9\) pfu ml\(^{-1}\) (Table 5.1).

The results from Table 5.2 suggest the effects of *S. pumila* and *H. atra* crude extracts on the inhibition of virus replication after attachment of HSV-2 on vero cells. Here also the *S. pumila* showed maximum effect against HSV-2 when compared to that of *H. atra*. The values of plaque inhibition were maximum at 86% with the \(1.2 \times 10^9\) pfu ml\(^{-1}\) in *S. pumila* and minimum at 31% with \(5.8 \times 10^3\) pfu ml\(^{-1}\). In *H. atra*, the plaque inhibition obtained was high at 74% with \(2.3 \times 10^9\) pfu ml\(^{-1}\) whereas less effect was seen in *H. atra* was 27% with \(6.4 \times 10^3\) pfu ml\(^{-1}\) units.

**Neuraminidase activity**

Results of the neuraminidase activity indicated that the activity of neuraminidase decreased significantly at low concentrations. The highest level of relative activity was seen in HSV-1 is *Sigmadocia pumila* as 92% and the *Holothuria arta* was 78%. Similarly the effect of the activity on HSV-2 was seen at moderate level of 60% in *Sigmadocia pumila* and 42% in *Holothuria arta* (Table 5.3 and 5.4). The Fig 5.5 shows the comparative study of *Sigmadocia pumila* and *Holothuria arta*. The inhibitory effect of neuraminidase activity was high in sponge *S. pumila* whereas the activity was less in *H. atra*. 
NMR analysis

The structure of bioactive compounds was elucidated by using NMR spectra. The 1H and 13C NMR data of sponge *Sigmadocia pumila* are summarized in Fig 5.6 (a&b) and Fig 5.7. In addition, the methyl groups were observed in the 1H NMR spectra including singlets and doublets which were integrated relatively for olefinic proton at δ position. The 13C NMR spectrum showed the presence of a carbon–carbon double and indicated the presence of two conjugated carbonyls. It also showed the appearance of two carbon signals. The identified compounds with the molecular formula and structures are:

1) 1, 2- Benzisoxazole \((C_7H_5ON)\) Mol wt: 119

2) Isobenzofuran-4,7 imine-1,3-Dione,Hexahydro-8-4M \((C_{15}H_{15}O_3NS)\) Mol wt: 321

3) 1,2,3,4 Tetrahydro-1,7-Dimethyl(6H) 1,2,4 Triazino 4,3 \((C_6H_{10}ON_6)\) Mol wt: 182

4) Benzamidines \((C_7H_8N_2)\) Mol wt: 120

5) 2 (2-Hydroxyphenyl) Benzimidazole phenol 2-1HB \((C_{13}H_{10}ON_2)\) Mol wt : 210

6) N-(Trifluoroacetyl) Prolylephedrine \((C_{17}H_{21}O_3N_2F_3)\) Mol wt: 358

7) Cinnamaldehyde \((C_9H_8O)\) Mol wt: 172

Fig 5.8 (a&b) and Fig 5.9 represent the 1H and 13C NMR data of *Holothuria atra*. Some of the bioactive compounds identified with their structures are given below:

1) 1,2-Benzenedicarbozylicacid,diethy ester \((C_{12}H_{14}O_4)\) Mol wt: 222

2) Propylparaben \((C_{21}H_{23}O_6N)\) Mol wt: 385

3) Tetrahydrodeoxycorticoster \((C_{21}H_{34}O_3)\) Mol wt: 334

4) Trans-ocimene 1,3,7-octatriene,3,7- dimethyl \((C_{10}H_{16})\) Mol wt :136

5) Cyclopropane,\{(1-propenylxoy)methyl}\- \((C_7H_{12}O)\) Mol wt: 112

6) N-(Trifluoroacetyl) Prolylmethamphetamine \((C_{17}H_{21}O_2N_2F_3)\) Mol wt: 342

7) Transcaryophyllene \((C_{15}H_{24})\) Mol wt : 204
Benzamidines and Isobenzofuran bioactive compounds found in *S. pumila* could exhibit antitumor activity. Cinnamaldehyde noted in *S. pumila* exhibited antiviral and antitumor activity. The chemical structures for bioactive compounds found in *Sigmadocia pumila* and *Holothuria atra* are given below:

1) 1, 2- Benzisoxazole

2) Isobenzofuran-4,7 imine-1,3-Dione, Hexahydro-8-4M

3) 1,2,3,4 Tetrahydro-1,7-Dimethyl(6H) 1,2,4 Triazino 4,3

4) Benzamidines

5) 2 (2-Hydroxyphenyl) Benzimidazole phenol 2-1HB

6) N-(Trifluoroacetyl) Prolylephehrine
7) Cinnamaldehyde
1) 1,2-Benzenedicarboxylic acid, diethylester

2) Propylparaben
3) Tetrahydrodeoxycorticosterone

4) Adamantane
5) Cycloheptanone

6) N-(Trifluoroacetyl) Prolylparaben
7) Transcaryophyllene (Sesquiterpene)
Fig 5.1. Hemagglutination assay of HSV-1 using *Sigmadocia pumila* and *Holothuria atra* extracts.

Fig 5.2. Hemagglutination assay of HSV-2 using *Sigmadocia pumila* and *Holothuria atra* extracts.
Table 5.1. *In vitro* antiviral activity of *Sigmadocia pumila* and *Holothuria atra* extracts against HSV-1 using plaque reduction assay

<table>
<thead>
<tr>
<th>Dilution of virus HSV-1</th>
<th>Concentration of extracts</th>
<th>Plaque forming units pfu/ml</th>
<th>% Plaque inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>S. pumila</em></td>
<td><em>H. atra</em></td>
</tr>
<tr>
<td>Control</td>
<td>Nil</td>
<td>8.5 x 10⁷</td>
<td>9.2 x 10⁷</td>
</tr>
<tr>
<td>10⁻¹</td>
<td>10 µg</td>
<td>5.0 x 10⁶</td>
<td>6.0 x 10⁹</td>
</tr>
<tr>
<td>10⁻²</td>
<td>20 µg</td>
<td>4.5 x 10⁵</td>
<td>5.6 x 10⁹</td>
</tr>
<tr>
<td>10⁻³</td>
<td>30 µg</td>
<td>3.8 x 10⁴</td>
<td>5.2 x 10⁵</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>40 µg</td>
<td>3.3 x 10³</td>
<td>4.4 x 10⁴</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>50 µg</td>
<td>2.8 x 10²</td>
<td>3.9 x 10⁴</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>60 µg</td>
<td>2.2 x 10¹</td>
<td>3.3 x 10⁴</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>70 µg</td>
<td>1.7 x 10⁰</td>
<td>2.4 x 10⁴</td>
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Table 5.2. *In vitro* antiviral activity of *Sigmadocia pumila* and *Holothuria atra* extracts against HSV-2 using plaque reduction assays

<table>
<thead>
<tr>
<th>Dilution of virus HSV-2</th>
<th>Concentration of extracts</th>
<th>Plaque forming units pfu/ml</th>
<th>% Plaque inhibition</th>
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<tr>
<td></td>
<td></td>
<td><em>S. pumila</em></td>
<td><em>H. atra</em></td>
</tr>
<tr>
<td>control</td>
<td>Nil</td>
<td>8.3 x 10¹</td>
<td>8.7 x 10¹</td>
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<tr>
<td>10⁻¹</td>
<td>10µg</td>
<td>5.8 x 10³</td>
<td>6.4 x 10³</td>
</tr>
<tr>
<td>10⁻²</td>
<td>20µg</td>
<td>4.4 x 10⁴</td>
<td>5.6 x 10⁴</td>
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<td>10⁻³</td>
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<td>10⁻⁵</td>
<td>50µg</td>
<td>2.7 x 10⁷</td>
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<tr>
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<td>60µg</td>
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</tr>
<tr>
<td>10⁻⁷</td>
<td>70µg</td>
<td>1.2 x 10⁹</td>
<td>2.3 x 10⁹</td>
</tr>
</tbody>
</table>
Fig 5.3. Antiviral activity of Herpes simplex virus (HSV-1) in plaque reduction assay using A1) *Sigmadocia pumila* and A2) *Holothuria atra*

Fig 5.4. Antiviral activity of Herpes simplex virus (HSV-2) in plaque reduction assay using B1) *Sigmadocia pumila* and B2) *Holothuria atra*
Table 5.3. Inhibitory effects of the viral neuraminidase activity of HSV-1 by *Sigmadocia pumila* and *Holothuria atra* extracts

<table>
<thead>
<tr>
<th>Concentration of extracts</th>
<th>Percentage of Relative activity (%) <em>Sigmadocia pumila</em></th>
<th>Percentage of Relative activity (%) <em>Holothuria atra</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>25µg</td>
<td>45.30</td>
<td>35.50</td>
</tr>
<tr>
<td>50 µg</td>
<td>68.75</td>
<td>55.12</td>
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<td>75 µg</td>
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<tr>
<td>100 µg</td>
<td>92.22</td>
<td>78.60</td>
</tr>
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</table>

Table 5.4. Inhibitory effects of the viral neuraminidase activity of HSV-2 by *Sigmadocia pumila* and *Holothuria atra* extracts

<table>
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<tr>
<th>Concentration of extracts</th>
<th>Percentage of Relative activity (%) <em>Sigmadocia pumila</em></th>
<th>Percentage of Relative activity (%) <em>Holothuria atra</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>25µg</td>
<td>25.45</td>
<td>20.25</td>
</tr>
<tr>
<td>50 µg</td>
<td>36.20</td>
<td>30.00</td>
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<tr>
<td>75 µg</td>
<td>45.15</td>
<td>40.25</td>
</tr>
<tr>
<td>100 µg</td>
<td>60.00</td>
<td>42.55</td>
</tr>
</tbody>
</table>
Fig 5.5. Comparative analysis for the neuraminidase activity on *Sigmadocia pumila* and *Holothuria atra*.
Fig 5.6. (a) $^1$H NMR Spectrum of *Sigmadocia pumila*
Fig 5.6. (b) $^1$H NMR Spectrum of *Sigmadocia pumila*
Fig 5.7. $^{13}$C NMR Spectrum of *Sigmadocia pumila*
Fig 5.8 (a) $^1$H NMR Spectrum of *Holothuria atra*
Fig 5.8 (b) $^1$H NMR Spectrum of Holothuria atra
Fig 5.9. $^{13}$C NMR Spectrum of *Holothuria atra*
5.4. DISCUSSION

The present study was carried out to test the antiviral activity of *Sigmadocia pumila* and *Holothuria atra* extracts against HSV-1 and HSV-2 viruses including purified fractions. Hemagglutination assay and plaque reduction assay were performed for detecting the antiviral activity. Results revealed that extracts of *Sigmadocia pumila* and *Holothuria atra* exhibited hemagglutination. The *Sigmadocia pumila* methanolic extracts showed hemagglutination activity against the HSV-1 and HSV-2 viruses at the concentration of 25µl and 50µl.

Several marine invertebrates such star fishes, brittle star, sea cucumbers and sea urchin found in Philippines act as the biosources for lectins and agglutinins. They were involved in hemagglutination (Rico *et al*., 2005). The presence of strong hemagglutinating activity has been detected in the aqueous sponge extracts of both heated and unheated fractions from sponge *Myrmekioderma styx* (7.2µg/mL). It suggests that the compound responsible for hemagglutinating activity may be lectin or a protein (Peng *et al*., 2002).

According to Plaza *et al.* (2009) sponges are known to be the source of novel bioactive peptides. Cyclic depsipeptides isolated from a number of marine sponges were active as HIV inhibitors and they showed hemagglutination property. Marine sponge *Siliquariaspongia mirabilis* derived compounds Celebesides A and Theopapuamide B blocked the entry of HIV-1 and neutralized HIV-1 respectively. These earlier results and the present experiments suggest that the strong hemagglutination nature of the sponge *S. pumila* against HSV-1 and HSV-2 could be due to the presence of peptides. The novel protein niphatevirin, from the sponge *Niphates erecta*, neither agglutinated nor lysed erythrocytes. It had the ability to bind to CD4 molecules and induced the cytopathic effects of HIV-1 (Corbeau *et al*., 1994). The hemagglutination effect induced by *S. pumila* and *H. atra* could be due to inhibiting action of the viral proteins on HSV. Previous studies on biologically active proteins from the sponges have reported the isolation of either toxic polypeptides or agglutinating lectins. Lytic proteins of a size similar to that of
niphatevirin have been isolated from the sponges *Tethya lycinurium*, *Zotrochota birotulata* and *Suberites dornuncula* (Mangel et al., 1992).

Lectins found in the body wall extracts of sea cucumbers like *Stichopus japonicas*, *Cucumaria echinata* and *Cucumaria japonica* were favourable for hemagglutination of erythrocytes (Bulgakov et al. 2000). This suggested the importance of *Holothuria atra* body wall extract in inducing hemagglutination. Callipeltin A, a novel antiviral cyclic depsidecapeptide from sponge of the genus *Callipelta* and Homophymine A from *Homophymia* sp exhibited the inhibition of cytopathic effects induced by HIV-1 in CEM4 lymphocytic cell lines (Zampella et al., 2008). Similarly a potent effect was seen in the *S. pumila* against the HSV 1 and 2 on the Vero cell lines.

Sponge derived polycyclic guanidine alkaloids exhibited diverse biological activities, including antiviral activities. Batzelladines A and B isolated from the ethanol extracts of a bright red Caribbean sponge of genus *Batzella* were active in the cell based assay that measured the binding of gp120 to CD4-positive T-cells showing antiviral activity (Patil, 1995). Illimaquinone isolated from red sea sponge *Smenospongia* sp. inhibited specifically RNase H and involved in the leukotriene metabolism in monocytes infected with human immunodeficiency virus type 1 (Schroder et al., 1991). In plaque reduction assay, it was noted the bioactive extracts of *S. pumila* have the antiviral effects against HSV-1 and HSV-2. The *S. pumila* showed growth inhibitory activity as 1.7 x10^9 pfu/ml for HSV-1 and 2.3 x10^9 pfu/ml for HSV-2.

Several marine sponges from Okinawan waters have bioactive compounds. The sponges include *Polyfibrospongia* sp. which have novel metabolites hennoxazoles, are moderately cytotoxic. The sponge *Dysidea herbacea* afforded cytotoxic dimethyldeoxoscalarin along with known diterpenes. Two cytotoxic dimers of a sesquiterpene have been isolated from *Halichondria sp* (Higa et al., 1994). The efficient antiviral activity using plaque reduction assay against HSV-1 by the sponge *Sigmadocia pumila* could thus be attributed to the presence of the bioactive compounds. The nucleosides are the basis for the synthesis of Ara-C, the first marine-derived
anticancer agent and the antiviral drug Ara-A. Ara-C is currently used in the routine treatment of patients with leukemia and lymphoma (Pomponi, 1999). Anti-HIV activity of crude extract of *Sidonops microspinosa* was also evaluated for anti-HIV activity in a cell based *in vitro* assay. Microspinosamide was found to be effective at a concentration of 0.2 μg/mL in CEM-SS target cells (Boyd *et al*., 1998). At 10μg of extracts, the *S. pumila* showed prominent activity in plaque reduction assay and thus inhibited the expression of Herpes simplex virus.

Aqueous and organic extracts from an Indonesian sponge *Sidonops microspinosa* exhibited anti-HIV activity. Microspinosamide, a cyclic depsipeptide in *Sidonops microspinosa* was an inhibitor of HIV and evaluated for its anti-HIV activity in a cell based *in vitro* assay. It was found to be effective at a concentration of 0.2 μg/ml (Valeria *et al*., 1996). The crude extract from the Brazilian sponge *Aaptos sp.* found to inhibit 76% of HSV-1 replication in Vero cells at a concentration of 2.4 μg/ml was first reported by Coutinho *et al.* (2002). Isolation of the alkaloid 4-methylaaptamine from the marine sponge *Aaptos sp.* confirmed that anti-HSV-1 activity of 4-methylaaptamine was even more potent than acyclovir. The compound inhibited HSV-1-infection in Vero cells even 4 h after infection, suggesting the inhibition of initial events during HSV-1 replication (Souza *et al*., 2007). The present results of antiviral activity against the HSV-1 and HSV-2 using plaque reduction assay on Vero cells could be compared to the earlier findings.

The compound bromoindole alkaloid, dragmacidin F from the sponge genus *Halicortex* showed *in vitro* antiviral activity against HSV-1 and HIV-1 with an EC50 of 96 μM and EC50 of 0.9 μM respectively. Total synthesis of (+)-dragmacidin F has been described by Garg *et al*., (2004). Samoylenko *et al*., (2009) stated that manzamine A from the sponge *Pachypellina sp.* acted as the anti HSV-II activity compound with a minimal effective concentration of 0.05 μg/ml.

In the Western Mediterranean, among the 59 sponge species studied, 90% showed cytotoxic, antibacterial, antiviral or antifungal (Uriz *et al*., 1992). It is assumed that the *Sigmadocia pumila* and *Holothuria atra* collected from the south east coast of India act as the
efficient source to be used as antitumor and antiviral agents. Saponins the secondary metabolites which are triterpene glycosides present in sea cucumbers like *H. forskali* are reported to have antiviral property by *in vitro* and *in vivo* methods (Kerr and Chen, 1995). It was observed that the *H. atra* extracts exhibited antiviral activity on plaque reduction assay in which maximum effect was seen against the HSV-1 to the tune of 74%. Potential drug lead from the marine sponge *Discodermia calyx* collected from the Sikine Jima island, Japan led to the isolation of three compounds viz., Calyceramides A-C. The sulfated ceramides acted as neuraminidase inhibitors with the IC\(_{50}\) values of 0.2-0.8 μg/ml (Nako *et al*., 2001). It was identified that the neuraminidase activity was inhibited using the crude methanolic extracts of *S. pumila* and *H. atra*. The *S. pumila* extract showed maximum effect at 92.22% against HSV-1 and *Holothuria atra* at 78.60%.

The differences in activity could be attributed due to the purification and also the higher dose of 100μg used. *S. pumila* organic extracts were effective against the growth of HSV-2 in Vero cells. Earlier, the antiviral and cytotoxic compounds named muqubilone along with the known sigmosceptrellin-B and muqubilin were reported from the Red Sea sponge *Diacarnus erythraenus*. They showed *in vitro* antiviral activity against herpes simplex type 1 (HSV-1) with ED(50) values of 7.5 and 30 μg/ml, respectively (Elsayed *et al*., 2001). The sponge *Negombata magnifica* and *N. corticata* produced potent cytotoxic macrocidal called latrunculins which had the antitumor activity in addition to antimicrobial and antiviral effects (Eman *et al*., 2011). Isolation of manzamine A from the sponge *Pachypellina sp* collected at Manado Bay, Sulawesi, Indonesia has shown anti HSV-II activity at minimal effective concentration of 0.05μg/ml. However it was difficult to chemically synthesize most of these compounds due to their highly complex structures (Ichiba *et al*., 1994).

The major triterpene glycoside of the sea cucumber *Psolus patagonicus* and its desulfated analog showed antiproliferative, hemolytic activities and reduced the growth of cancer cell lines through NF-κB activation (Valeria *et al*., 2009). Inhibitory action rather than a stimulatory one of cucumariosides on phagocytosis with the release of tumor necrosis factor-α (TNF-α) was noted
from Cucumariosides (Aminin et al., 2004). Thus Holothuria atra extracts have the ability to arrest the multiplication of virus and suppress its growth by influencing the growth factors. This could have resulted in appearance plaques in the plaque reduction assay.

Different derivatives of avarol and avarone have been isolated from Dysidea cinerea. They included neoavarol, neoavarone, 4’-methoxyavarone from sesquiterpenoid hydroquinone and quinones derivatives including 6’-hydroxyavarol, 6’-acetoxyavarol, 3’-hydroxyavarone, and 6’-acetoxyavarone. These compounds showed cytotoxic, antimicrobial and anti-HIV properties (Hirsch et al., 1991). (Gul et al., 2006) indicated sponge metabolites such as avarol, avarone, ilimaquinone and several phloroglucinols have the potential for developing new drugs for treating HIV- and AIDS-related conditions.

Strongyline A, a metabolite isolated from the marine sponge Strongylophora hartmani, showed cytotoxic activity against P-388 leukemia cells with the concentration at IC50 of 13 μg/ml and antiviral activity against Influenza virus (Wright et al., 1991). In Sigmadocia pumila, the bioactive compound such as Isobenzofuran (heterocyclic compound) consisting of fused benzene and furan rings with antitumour property was detected through NMR analysis (Ref Fig 5.6 a&b). The heterocyclic compound C6H10ON6 with ketone group was noted. The presence of sulphated polysaccharides which also could induce antiviral action was confirmed by 1HNMR and 13C NMR analysis.

The new sesquiterpenoid aminoquinone, cyclosmenospongine, containing a dihydropyran ring, was isolated from an Australian marine sponge Spongia sp., along with the known metabolites, smenospongiarine, ilimaquinone and smenospongine showed cytotoxic activity (Utkina et al., 2003). Callipeltin A, a macrocyclic 22-membered depsipeptide isolated from a shallow water sponge of the genus Callipelta expressed antitumour properties (Gul et al., 2006).

Detailed studies for the anti-HIV activity of papuamides A and B from the sponges Theonella mirabilis and Theonella swinhoei have been performed by Andjelic et al., (2008). Inhibition of viral entry into cells using papuamides A was shown to be independent of CD4,
gp120, chemokine co-receptors and gp41, key proteins which were involved in the process of viral entry and are thus considered as the targets for the most of the FDA approved inhibitors of virucidal activity (Xie et al., 2008). The present data suggest that the extracts of *S. pumila* and *H. atra* have the ability to interact with the viral glycoproteins and inhibited their action.

Natural product lead compounds from sponges have often been found to be promising pharmaceutical agents. Several of them have successfully been approved as antiviral agents and have been advanced to the late stages of clinical trials. Most of these drugs are used for the treatment of human immunodeficiency virus (HIV) and herpes simplex virus (HSV). It inhibits viral DNA polymerase and DNA synthesis of herpes, vaccinica and varicella zoster viruses (Sunil et al., 2010). Hence *Sigmadocia pumila* and *Holothuria atra* can act as the potential target for the drug development from marine sources.

Several features of H and C spectra obtained from the sponge *Kirkpatrickia varialosa* identified the polycyclic aromatic alkaloids called the vaiolins. The compounds were active against the P388 cell leukemia cells and showed antiviral activity (Nigel et al., 1994). Similarly in the present study the importance of H and C spectra were used to analyse the presence of effective bioactive compounds inducing the antitumor and antiviral activities.

The crude extract containing 2% mycalamide A from the genus *Mycale* was active against A59 corona virus. It also inhibited the Herpes simplex type I and Polio type I viruses at a concentration of 5 ng/disc. The property of protein synthesis inhibition was attributed to their biological activity as antiviral agents (Gurel et al., 2009). The activity of the methanolic extracts of *Sigmadocia pumila* and *Holothuria atra* were effective against the HSV-1 and HSV-2 due to the protein synthesis inhibitory action. Four analogues of mycalamide A-D have been reported to bind the nucleoprotein (NP) of influenza virus and inhibited its multiplication. The compound might bind to the N-terminal 13 amino acid region of NP which mediate the nuclear transport of NP and its binding to viral RNA and hence may inhibit viral replication (Hagiwara et al., 2010). Neuraminidase plays a key role in the release of newly made virus particle from the infected cells.
Additionally, the enzyme activity was responsible for preventing self aggregation of virus particles by cleavage of sialic acids still bound to the virus surface.

Sea cucumber derived fucosylated chondroitin sulfates (FCS) which inhibited the growth of human immunodeficiency virus and also acted as a cytotoxic agent was initially obtained from *Stichopus badionotus* (Kaswandi *et al*., 2004). It could be predicted that high molecular weight compounds present in *Holothuria atra* detected by NMR analysis (Ref Fig 5.8 a&b and 5.9) could form a potent antiviral sources.

Bioactive peptides and hemolytic lectins have been reported from sea cucumber as a source of antiviral activity. Among Holothuroidea genera, *Cucumaria echinata* and *C. frondosa* contained lectin and peptide, respectively. They have been found in the body wall mucus (Hisamatsu *et al*., 2008). Fucoidans, polysaccharides containing substantial percentages of L-fucose and sulfate ester groups are generally considered as constituents of sea cucumbers. Fucoidan can inhibit the development of cytopathic effect (CPE) and protect cultural cells from infection caused by viruses (Hemmingson *et al*., 2006). This can induce the antiviral effects against the Herpes simplex viruses.

Clive and Wang (2003) revealed that the Hamigeran B compound isolated from the marine sponge *Hamigera tarangaensis* (family Anchinoidae) from the Hen and Chicken Islands in New Zealand showed 100% *in vitro* virus inhibition against both the herpes and polio virus with slight cytotoxicity action. Trikendiol, an alkaloid which is a red pigment, isolated from the sponge *Trikentrion loeve* was found to be active in a CEM 4 HIV-1 infection assay. It inhibited the cytopathogenic effect of the virus (Loukaci and Guyot, 1994). Takeda *et al*., (2006) reported that marine sponges such as *Asteropus simplex* contain bioactive peptides of non ribosomal peptide origin, such as asteropine A. It acts as the lead for antibacterial and even antiviral drug development Gymnochrome B, Gymnochrome D and Isogymnochrome D isolated from lithistid sponge *Callipelta* sp inhibited the dengue virus (Laillea *et al*., 1998). Previous observations
indicated that the *Sigmadocia pumila* and *Holothuria atra* have larvicidal effects against *Aedes egyptii*.

Zalilawati *et al.*, (2009) revealed that the exopolysaccharides extracted from sponge, *Celtodoryx girardae* exhibited a unique molecular weight of 800 kDa. They showed potential antiviral activity against *Herpes simplex* virus type 1 (HSV-1). The EC$_{50}$ value was 5.9µg/ml without cytotoxicity on the Vero cell line. A new sesquiterpene quinone, 21-dehydroxybolinaquinone together with two known related analogues, Bolinaquinone and dysidine had been isolated from the Hainan sponge *Dysidea villosa*. A wide range of remarkable biological activities including anti-HIV-I activity were seen (Yan *et al*., 2009).

Fucoidan of sea cucumber *Laminaria japonica* has anti RNA and DNA virus functions. The antivirus effects of fucoidan on infection was against poliovirus III, adenovirus III, ECHO6 virus, coxsackie B3 virus and coxsackie A16. Fucoidan inhibited the development of cytopathic effect (CPE) and protected the cultural cells from infection caused by the viruses (Li *et al*., 1995). Sulfated polysaccharides from sea cucumbers such as the *Cucumaria japonica, Holothuria impatiens* are reported to exhibit antiviral activity. Based on this fact, Japanese scientists have patented their scientific findings regarding the potential use of sea cucumber chondroitin sulfate to inhibit human immunodeficiency virus (HIV) infection (Beutler *et al*., 1993).

Triterpene glycosides, namely holothurinosides A, B, C and D as well as desholothurin A from sea cucumber (*Holothuria forskali*), have considerable antitumour activity against P388 cell lines. The saponins isolated from the aqueous and methanolic extract of sea cucumber (*Holothuria forskali*) have showed considerable antiviral activities (Mulloy *et al*., 2000). Considering these as well as the results of present investigations, the methanolic extracts of *Holothuria atra* could form effective antitumour and antiviral agents. Toido *et al*., (2003) demonstrated that sulphated polysaccharides such as glycosaminoglycans an inhibitor of human immunodeficiency virus binds to T lymphocytes and showed antiviral activity. In low
concentrations, the extract showed potent inhibitory effect towards Herpes simplex virus and thus has got significant drug value.

The antiviral activity using the sea cucumber *Ludwigothurea grisea* and *Thelenota ananas* derived fucosylated chondroitin sulfates (FCS), was recognized as the sulfated polysaccharides. It inhibited human immunodeficiency virus (HIV) infection (McClure *et al.*, 1992). The present work suggested that *Holothuria atra* extracts showed virucidal action through reduction in number of plaques formed during plaque reduction assay against the HSV-1 and HSV-2. The strong growth inhibitory activity found in the extract of *Sigmadocia pumila* and *Holothuria atra* might be the source for the development of antitherpetic compound. Thus the sponge *Sigmadocia pumila* and *Holothuria atra* collected from the south east coast of India and their extracts act as the promising sources of new compounds with potential antitumor and antiviral activity, with broad application in pharmaceuticals.

Sea cucumbers contain triterpene glycosides called saponins. In the Mediterranean species *Holothuria forskali*, the body wall and the Cuvierian tubules contained complex saponin mixture. They possess a wide spectrum of pharmacological effects which include hemolytic, antitumoral, anti-inflammatory, antifungal, antibacterial, antiviral, ichthyotoxic, cytostatic and antineoplastic activities (Dyck *et al.*, 2009). Similarly the *H. atra* extract had various compounds such as the flavonoids, phenolic components, terpenoids, saponins, alkaloids etc. Extracts of *H. atra* are thus capable of antitumour and antiviral activities. Findings of this study could reveal that the antiviral effect of extracts of *Sigmadocia pumila* and *Holothuria atra* noted could be due to the distinct mechanisms such as:

(i) Direct interaction of compounds in the extracts among HSV strains  
(ii) alteration of the adsorption phenomenon  
(iii) inhibition of penetration into the host cell or  
(iv) inhibition of the multiplication of the viruses in the cells.