CHAPTER 7. INVITRO ANTIVIRAL ACTIVITY

7.1. Introduction

Herpes simplex virus (HSV) was recognised as an infectious agent from the last quarter of the 19th century, and it was the first human virus to be discovered. It is also one of the most commonly studied human viruses due to their ability to cause a range of infections, remain latent in their host, and reactivate to cause lesions at or near the initial site of infection (Binder, 1977). HSV type 1 and herpes simplex virus type 2 are members of a subfamily of the alpha herpes viruses, with common biological activities, but they are different in many aspects. They can infect and establish latency in the neurons of the sensory ganglia. HSV can infect the central nervous system, causing meningitis and encephalitis. Viral latency is a problem in the management of HSV treatment. Lethal infections have also been reported in immune compromised patients (Whitley, 1990; Morfin and Thouvenot, 2003).

The clinical symptoms of primary HSV-1 infections vary greatly, infection can be asymptomatic, combination of fever, sore throat, ulcerative and vesicular lesions, gingivostomatitis, edema, localized lymphadenopathy, anorexia, and malaise. The onset of recurrent orolabial lesions is distinguished by pain, burning, tingling, or itching, which lasts for usually 6 hrs before a vesicle begins to form. The total area of involvement is usually localised where there may be three to five vesicles. Within 72 to 96hrs, lesions progress to pustular or ulcerative, crusting stages. Viral infections of the eye are usually caused by HSV-1 (Ostler, 1977). Recurrent infections are common, and parallel that of herpes labialis infection. Infections can also occur in the skin. Once viral DNA enters the nucleus, viral transcription, DNA replication, encapsidation, and egress take place. By remodelling the host cell nucleus, the virus
then has a structurally sound base for efficient viral DNA replication and late DNA transcription. Herpes simplex virus 1 HSV-1 and HSV-2 are responsible for a wide range of diseases, affecting the skin or mucous membranes (cold sores, genital herpes, and gingivostomatitis), the eye (herpetic keratitis), or the central nervous system (necrotizing encephalitis and meningitis). Ocular HSV infections are the leading cause of infectious blindness in developed countries, and neonatal HSV-2 infection has a mortality rate of approximately 30% when antiviral are used (HSV-1 and HSV-2).

Herpes viruses are widely distributed in nature and most animal species are susceptible to at least one herpes virus. The following herpes viruses, that have humans as their primary host, have been identified as: HSV-1, HSV-2, human cytomegalovirus (HCMV), Varicella Zoster Virus (VZV), Epstein-Barr virus (EBV), and Human herpes viruses 6, 7 and 8 (HHV-6, HHV-7, and HHV-8) (Cocchi et al., 2000).

More recently, Real Time PCR as diagnostic tools to identify the virus (Wu and Spear, 1989). HSV can enter the host cell via attachment to the cell surface. This is a reversible process and involves the binding of virion gC and gB to glycosaminoglycans (GAGs) (Herold et al., 1991; Shieh et al., 1992). The next step in this pathway involves the interaction of gD with a specific receptor and fusion of the envelope with the plasma membrane. gD can interact with nectins, herpes virus mediator (HVEM), and a selected form of 3-O-sulfated heparin sulphate (3-OS HS) (Geraghty et al., 1998) and together with gB, gH, and gL, allow the fusion of the
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envelope with the plasma membrane (Campadelli-Fiume et al., 2000; Spear and Longnecker, 2003).

The oceans represent an important resource for the discovery of novel bioactive compounds. 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). Aim of the present study was to assay the in vitro antiviral properties of crude and fraction extracts from sea slug *K. ornata* evaluated against HSV-1 and HSV-2.

7.2. Materials and Method

7.2.1. Cells and Viruses

The cell line used was the African green monkey kidney cells (Vero cell line) grown in Eagle's Minimum Essential Medium (MEM, HiMedia) supplemented with 8% fetal calf serum (FCS, HiMedia) and 1% of antibiotics PCS (10 000 IU/ml penicillin, 25000 IU/ml colimycin, 10 mg/ml streptomycin; Sigma). The Vero cell lines were grown and maintained in a humidified incubator at 37 °C, in a 95% air, 5% CO₂ (v/v) atmosphere. Virus stock of *Herpes simplex* virus type 1 (HSV-1) was obtained from patients (Kings Research Institute, Chennai). The virus stock was prepared by incubating with Vero monolayers (75 cm² culture flasks seeded with 3.5×10⁵ cells/ml) at low multiplicity and incubating at 37 °C, in a 95% air, 5% CO₂ (v/v) atmosphere (Yasin et al., 2000).
7.2.2. Collection and extraction of samples:

Live specimen of mollusc *K. ornata* were collected from Parangipettai landing centre (11°29'N 79°46'E). The collected fresh molluscs were preserved in an ice box and transported to the laboratory.

7.2.3. Viral sample preparation

The samples are collected from carnival swabs and small volume of Lysozyme enzyme for the rupturing the cell wall were added and homogenised with mortar and pestal. The collected extracts was centrifuged for 8,000 rpm for 10 min the supernatant was transferred through 0.22 µm Millipore filter and used for the antiviral activity test. Minimum Essential Medium (MEM) AT 018 (HiMedia) for suspension culture with spinner salts (11.5 gL⁻¹) are given in the following table:

<table>
<thead>
<tr>
<th>Contents of Minimum Essential Medium (MEM)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Dehydrated medium</td>
<td>1.15g</td>
</tr>
<tr>
<td>Double distilled water</td>
<td>86.4ml</td>
</tr>
<tr>
<td>Filter sterilized</td>
<td>0.22µ membrane</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>12.4ml</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>1.0ml</td>
</tr>
</tbody>
</table>

7.2.4. Preparation of Test Sample

The stock solution of MeOH-KO was prepared by dissolving in 10% dimethyl sulfoxide (DMSO) of MEM medium containing 2% FCS (Fetal Calf Serum) to reach a final concentration of 10mg/ml. The stock solution of MeOH-KO was passed through 0.22 µm Millipore filter and used for antiviral study.
7.2.5. Cytotoxicity

The cytotoxicity test for the MeOH-KO was evaluated using MTT method (Song et al., 2013). Vero cells were seeded (3 × 10^4/well) in 96-well plates in 100 μl of growth medium (MEM) containing 10 % FCS mixture in each well; incubated at 37°C in a 5% CO2 incubator. After 24hrs of monolayer cell cultivation, the medium was removed and replaced by a 100 μL of varying concentrations (25- 800 μg/ml) of the MeOH-KO in MEM medium containing 2 % FCS in respective wells. Control cell was maintained in MEM medium containing 2 % FCS at 37°C in a 5% CO2. After 72h of incubation, 20 μl of MTT (5 mg/ml) in PBS solution was added and incubated at above condition for 4h and then was observed for the crystal formation. The medium was replaced by 100μl of DMSO solution and thoroughly mixed using a multichannel pipette. The optical density (OD) of each well was measured by using an ELISA reader at 620 nm.

7.2.6. Cytopathic end-point assay (TCID\textsubscript{50} - Tissue Culture Infection Dose 50)

The Vero cells were seeded (3 × 10^4/well) in 96-well plates in 100 μl of growth medium containing 10% FCS mixture in each well incubated at 37°C in a 5% CO2 incubator. After 24 hrs of monolayer cell cultivation, the medium was removed and 100μL of viral solution (HSV-1 and HSV-2) prepared by 10-fold dilution (10^1 to 10^8) were added to each well of 96- well plates with four wells for each concentration of sample 1 and sample 2 along with control. The plate was incubated at 37°C in a 5% CO2 incubator. After 4 days, the cytopathic effect was observed and recorded. The TCID\textsubscript{50} value was calculated and used for the antiviral activity (Chen et al., 2010).

7.2.7. Cytopathic effect reduction assay (HSV-1 and HSV-2 viruses)
Confluent Vero cells, grown in 96-well plates were infected with HSV-1 and
HSV-2 at multiplicity of infection (MOI) of $10^2$TCID$_{50}$/ml/well. The medium
containing virus was removed and replaced with medium containing different
concentrations of sample 1 and sample 2, which were tested in triplicate, and were
incubated under 100% humidity and 5% CO$_2$ at 37°C for 24hrs. Cells were then fixed
with 10% formaldehyde for 20min at room temperature and stained with 1% crystal
violet solution for 30min. Cells were then washed and dried and the intensity of
crystal violet staining for each well was read at 570nm (Hung et al., 2009).

7.3. Results

7.3.1. Cytopathic reduction assay (HSV-1 and HSV-2 viruses)

The antiviral activity of the MeOH-KO was evaluated initially in a standard
CPE reduction assay where the extracts were added prior to virus infection. This
initial analysis of the MeOH-KO exhibited antiviral activity against HSV-1 and
HSV-2 at the concentrations listed in Table. The viral inhibition rate by the
MeOH-KO against HSV-1 virus was found to be 50.47 and 67.62% at the
concentration of 100 µg/ml. Similarly the HSV-2 showed 49.67 and 68.55% at the
same concentration.
### Table 12. Cytopathic reduction of Sample 1 & 2 against HSV-I and HSV-II

<table>
<thead>
<tr>
<th>Name of Strains</th>
<th>Sample</th>
<th>Percentage of reduction/Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td><strong>HSV-I</strong></td>
<td>Crude</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td>Fraction</td>
<td>Nil</td>
</tr>
<tr>
<td><strong>HSV-II</strong></td>
<td>Crude</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td>Fraction</td>
<td>Nil</td>
</tr>
</tbody>
</table>

### Fig. 35. Comparison among the percentages of inhibition of the HSV viruses by *K. ornata* extract, obtained by MTT assay.
Table. 13. Antiviral activity of Crude and Fractions of *Kalinga ornata* against HSV-I and HSV-II viruses

<table>
<thead>
<tr>
<th>Name of strains</th>
<th>Samples</th>
<th>Cytotoxicity CC₅₀ (µg/ml)ᵃ</th>
<th>Cytopathic reduction IC₅₀ (µg/ml)ᵇ</th>
<th>Selective Index ratio CC₅₀/IC₅₀ (SI)ᶜ</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-I</td>
<td>Crude</td>
<td>&gt;250</td>
<td>21.7</td>
<td>11.52</td>
</tr>
<tr>
<td></td>
<td>Fraction</td>
<td>&gt;250</td>
<td>13.2</td>
<td>18.93</td>
</tr>
<tr>
<td>HSV-II</td>
<td>Crude</td>
<td>&gt;250</td>
<td>20.5</td>
<td>12.19</td>
</tr>
<tr>
<td></td>
<td>Fraction</td>
<td>&gt;250</td>
<td>11.3</td>
<td>22.12</td>
</tr>
</tbody>
</table>

ᵃ Cytotoxic concentration 50% (CC₅₀), concentration required to reduce Vero cell viability by 50%, was measured by MTT method.
ᵇ Inhibitory concentration 50% (IC₅₀): concentration required to reduce virus cytopathic reduction by 50%.
ᶜ SI: Selectivity Index is defined as the ratio of CC₅₀ to IC₅₀ (SI = CC₅₀/IC₅₀)
Fig. 36. Antiviral effects on HSV-1 treated by Crude and Fraction of Sea slug K. ornata
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Fig. 37. Antiviral effects on HSV-II treated by Crude and Fraction of Sea slug *K. ornata*
7.4. DISCUSSION

Many potential compounds are isolated from marine mollusc in different species exhibiting anti bacterial, anti fungal and anti oxidant properties. In continuation with this, the anti viral activity was performed. In parallel, antiviral activities were assayed in vitro against Herpes simplex virus type I and II, Vero cells by cell cytopathic effects.

Due to natural selection, a wide range of antiviral agents may develop in marine invertebrates to protect them from viral infection. The innate defenses available to marine invertebrates can include secondary metabolites, bioactive peptides and proteins, thus serving as models for development of new drugs for treating life threatening diseases. Indeed, several marine invertebrates that produce important antiviral compounds have been reported so far, including sponges, tunicates, echinoderms and molluscs. In the case of molluscs, it can protect themselves against previous infection via innate immunity. Their enormous success indicates compensation for the lack of adaptive immunity with effective innate defenses. Therefore, molluscs represent a great resource for discovery of antiviral compounds (Hooper et al., 2007).

*K. ornata* crude and fractions were assayed for their efficiency to cause lethal effects in Herpes Simplex Virus type I & II, a common virus used for screening of marine antiviral compounds on the model Vero cell lines. The HSV –I were treated with the crude and fractions obtained from the sea slug, *K. ornata*. After 24 hrs, the cell inhibition or cytopathic effects were noticed. In the same way, the HSV – II were treated with the same crude and fractions.
Carriel-Gomes et al. (2006) reported the antiviral activity and cytotoxicity against Herpes simplex virus type 1. Cytotoxicity of SPE-fractions to Vero cells was also evaluated. No antiviral activity was determined for the 10% SPE-fractions even though they were shown to be cytotoxic on Vero cell lines.

Maximum percent inhibition of viral activity was detected in the 100 μg/ml in the crude extract of *K. ornata* using HSV I. At this concentration, 50.47% of the cells were reduced and the cytopathic effect was observed. While, a minor cytotoxicity was observed in 50 μg/ml as 38.24% followed by 25 μg/ml as 29.42%, 10 μg/ml as 21.39% and 5 μg/ml as 8.44% respectively.

The antiviral activity of the *K. ornata* fractions on HSV I was detected by applying lower concentration to higher. 100 μg/ml is the maximum concentration applied. In this, 67.62% of the cells were reduced and the cytopathic effect was observed in 100 μg/ml. While, a minor cytotoxicity was observed in 50 μg/ml as 58.73% followed by 25 μg/ml as 40.27%, 10 μg/ml as 27.44% and 5 μg/ml as 15.22% respectively. Comparing the crude and fraction extracts, the fraction exhibited good results, as the concentration of the dose increases; it lowers the viral cell count. In percentage wise, the 50 and 100 μg/ml of fraction were found to be the promising dosage for causing half of the viral cell death.

The anti viral activity was also performed by using HSV-II cell line by the crude and fraction extracts obtained from sea slug of *K. ornata*. 100 μg/ml is the maximum concentration applied in this experiment throughout the study. In crude extracts, maximum cell death was recorded in 100 μg/ml as 49.67% followed by 50 μg/ml as 39.55%, 25 μg/ml as 32.52%, 10 μg/ml as 20.42%, 5 μg/ml as 14.79%. In fraction, the maximum viral cell death was observed in 100 μg/ml as 68.55%
followed by 50 μg/ml as 57.32%, 25 μg/ml as 43.71%, 10 μg/ml as 29.45% and 5 μg/ml as 18.57%.

By comparing the crude and fraction in HSV I and HSV II, the maximum cytopathic reduction was seen in HSV II at 100 μg/ml as 68.55% of the fraction sample taken from sea slug of *K. ornata*.

Many researches had been undertaken for evaluating the cytotoxicity test for the HPV virus in cell line. According to Serkedjieva, (2000), the aqueous extract of *Polysiphonia denudate* exhibited selective inhibition on the reproduction of HSV-1 and HSV-2 at their effective concentration 50% (EC\textsubscript{50}) range of 8.7–47.7 mg/ml. The inhibition affected adsorption as well as intracellular stages of viral replication. Likewise, anti-HSV activities of *Symphyocladia latiuscula* were evidenced by Park *et al.* (2005). A MeOH extract of *S. latiuscula* and its fraction was effective against acyclovir and phosphonoacetic acid-resistant HSV-1 (APr HSV-1), thymidine kinase deficient HSV-1 (TK HSV-1), and wild type HSV-1 in vitro without cytotoxicity. Specially, the major component of CH\textsubscript{2}Cl\textsubscript{2} - Soluble fraction, 2,3,6-tribromo-4,5-dihydroxybenzyl methyl ether (TDB), inhibited wild type HSV-1, as well as (APr HSV-1) and (TK HSV-1) with their inhibitory concentration 50% (IC\textsubscript{50}) values of 5.48, 4.81, and 23.3 mg/ml, respectively. Paolin, a substance isolated from oysters and clams tissues showed potent antiviral activity against herpes virus (Li *et al.*, 1965; Prescott *et al.*, 1966).

Carriel-Gomes *et al.* (2006) showed the outstanding inhibition of HSV-1 replication by cellular fraction obtained from oyster *Crassostrea rhizophorae*, particularly in post-infection treatment assay. According to him both hemolymph fractions from *C. rhizophorae* and *C. gigas* did not inhibit 50% of HSV-1 and
RV-SA11 replication. SI values were not calculated. After 120 h of treatment, the maximum percentage of AdV-5 inhibition was obtained with 0.20 mg mL\(^{-1}\) of the cellular fraction from \textit{C. rhizophorae}, which inhibited 63.93\% (simultaneous) and 63.84\% (pre-infection of AdV-5 replication).

In simultaneous treatment assay, SI values ranged from 1.89 (\textit{C. rhizophorae cellular fraction}) to 2.00 (\textit{C. rhizophorae} and \textit{C. gigas acellular fraction}), and in pre-infection treatment assay, SI value ranged from 1.73 (\textit{C. gigas} cellular fraction) to 2.40 (\textit{C. rhizophorae} acellular fraction). In the most recent study, antiviral activity of abalone \textit{Haliotis laevigata} against HSV-1 has been assessed by adding hemolymph or lipophilic extract at different times during the plaque assay (Dang et al., 2011). The concentration range at which abalone extract was used for antiviral testing caused minimal (10\%) mortality in Vero cells. Haemolymph (20\%, v/v) and lipophilic extract of the digestive gland (3 g/mL) both substantially decreased the number and size of plaques.

In the present study, the selective index value was calculated for both the crude and fraction. The SI values are calculated for HSV-I treated in crude and measures about 11.52 and the maximum was 18.93 in fraction. In HSV-II, the SI values ranged from 12.19 in crude and 22.12 in fraction respectively. By comparing the both viral cell lines, the HSV-II have the maximum cytopathic effects in fraction of the sea slug of \textit{K. ornata}.

The present study significantly cytotoxic activity against HSV 1 and HSV 2 (Herps Simplex Virus) with an IC\(_{50}\) of 100 \(\mu\)g/mL concentration of \textit{K. ornata} crude extract. The compound shown to have an IC\(_{50}\) activation on 50.47\% and 67.62\% in HSV 1 and 49.67 \% and 68.55\% in HSV 2 viruses samples. The cytotoxic
concentration (CC$_{50}$) Vero cell viability shows there 50 % value for HSV 1 as 21.7 and 13.2 for the HSV2 it shows the value as 20.5 and 11.3. The selective index ratio between CC$_{50}$ and IC$_{50}$ for HSV1 is 11.52 and 18.93 and for HSV2 was about 12.19 and 22.12. As expected, the extract displayed a high antiproliferative effect against HSV viruses.

Recently, there are few drugs licensed for the treatment of HSV infections. Meanwhile, drug resistance is an important clinical problem that may lead to ineffective therapy. Therefore, the development of new antiviral agents for HSV with diverse kinds of antiviral actions is always required. *K. ornata* fraction had shown its antiviral activity against HSV we declare that the *K. ornata* fraction can be used for the discovery of future antiviral compounds. It will pave the way to succeed and will resolve the current challenges that facing from the HSV infection in human and also it is clear that marine environment stores numerous undiscovered organisms with their unique metabolites, thus numerous drugs can be discovered in the upcoming days that viruses have not yet developed resistance to the same.