Keywords: VZV, HEp-2 cell line, Sphagneticola calendulacea, Methanolic, Ethanolic, MTT

1. Introduction

Varicella-zoster virus (VZV) is a member of the Herpesviridae family. Primary infection causes varicella (chickenpox). The virus is not cleared from the body but persists in a dormant state in the dorsal root and/or cranial nerve ganglia. Subsequent reactivation of the latent virus, typically occurring years later and causes zoster (shingles). The virus contains a double stranded DNA genome. Herpes viruses are characterized by the persistence of infection after primary infection, a property known as latency. Cell-mediated immunity is critical in clearing the infection and in preventing secondary reactivation.[1]

Shingles (zoster) is due to reactivation of the virus, which has previously been infected with VZV. It is self-limiting, occurring over one to three contiguous unilateral dermatomes. Pain is a frequent complication and may persist after the rash resolves (post-herpetic neuralgia).[3]

Chickenpox (varicella) is characterized by a generalized vesiculopustular rash. Symptoms usually begin with 1 or 2 days of fever, flu-like symptoms and generalized malaise, although this may be absent. Complications of chickenpox infection occur in approximately 1% of cases, with the most common being secondary bacterial infection of the skin lesions. The average number of skin vesicles is usually 250–500 but >500 lesions may occur in severe cases.[2]

Shingles (zoster) due to reactivation of the virus, which has previously been infected with VZV. It is self-limiting, occurring over one to three contiguous unilateral dermatomes. Pain is a frequent complication and may persist after the rash resolves (post-herpetic neuralgia).[3]

Sphagneticola calendulacea (L.) Pruski belongs to the family Asteraceae which is a very useful herbal medicinal plant. The leaves can be used in treatment of dermatological disorders, cough, headache, hair loss, strengthening the nervous system, lack of blood, digestive system disorders.[5] The plant has great importance in Ayurvedic, Siddha and Unani systems of traditional medicine. The leaves are used in dyeing grey hair and in promoting the growth of hair.[6]

However, the developments of resistance towards the anti VZV drugs are prevailing among immunocompromised patients. Also numerous side effects coupled with these drugs are reported namely the renal failure, neurotoxicity, thrombocytopenia, renal dysfunction, nephrotoxicity and hypokalemia.[7] Therefore, there is a desideratum that necessitates the development of new-fangled potential antiviral agents for VZV infections.
2. Experimental

2.1 Collection of plant material

The leaves of *Sphagneticola calendulacea* were collected during the month of December from the natural habitats of Thiruvalluvar district, Tamil nadu, India. The leaf was identified by Botanical Survey of India (BSI), Coimbatore, India with ref no.BSI/SRC/5/23/2015/Tech./2626. The leaves were washed with running tap water and finally washed with distilled water to remove the dirt and dried under shade for two weeks.

2.2 Preparation of powder and extract

The leaves were pulverized to powder in a mechanical grinder. The powder was successively extracted with Ethanol and Methanol in soxhlet apparatus and it was left for eight hours. The extracts were concentrated under reduced pressure in a rotary evaporator [8].

2.3 Cell line

Human Epithelial cell line (HEp-2) was procured from National Centre for Cell Science (NCCS) Pune, India and reconstituted in Minimum Essential Medium (MEM) with 5% v/v Fetal bovine serum (FBS), Penicillin, Streptomycin, Amphotericin-B, L-Glutamine, Sodium bicarbonate and HEPES (2-[4-(2-Hydroxyethyl)1piperzinyl ethane sulphonic acid) buffer. Appropriate volume of CO₂ was passed into the medium till the pH reached the optimum range (i.e.) 7.1 - 7.5.

2.4 Cytotoxicity Assay

A monolayer of the cell line was taken and the medium was discarded and the wells were given a gentle wash with Phosphate buffered Saline. Cells were trypsinised with Trypsin Phosphate versene Glucose. 10 ml of 10% minimum essential medium was added to the flask and the cells were removed. 100 μl of cells were plated into the 96 well microtitre plate and 100 μl of 10% minimum essential medium was added into each wells. The microtitre plate was then incubated at 37 ºC for 12 hours under 5% CO₂ atmosphere.

About 1 mg of methanol and ethanol leaf extracts of *S. calendulacea* was weighed separately and dissolved in 1 ml of methanol and ethanol based on the solubility and 200 μl of the extract was added into the wells of first column of microtitre plate and was diluted into two fold manners till the last well. 100 μl from the last well was discarded. Number of viable cells, 0.1 ml (1.4 x 10⁶ cells) was counted and 100 μl from the respective dilutions were added into the respective wells containing cells. 100 μl of 2% MEM was added into the wells and control wells with cells were maintained. The plate incubated at 37 ºC in 5% CO₂ atmosphere for 48 hours and was observed under inverted microscope for determination of cell toxic free concentrations to the lower ranges of leaf extracts concentration were selected for anti-VZV study. The following concentrations of toxic free leaf extracts are 25µg, 12.5µg, 6.25µg, 3.125 µg were diluted with 0.2ml of 2% FBS MEM and transferred to the virus inoculated monolayer cell lines. The entire setup was incubated at 37 ºC temperature for three days to allow multiplication of virus and subsequent development of Cytopathic Effect (CPE). Each well was observed under Inverted microscope every day for presence or absence of Cytopathic effect. The standard antiviral agent of Acyclovir (1mg/mL) was used as Positive control. Later, MTT assay was carried out.

3. Results

3.1 Cytotoxicity Assay

Based on the calorimetric and MTT assay the ethanol and methanol leaf extracts showed toxic to the HEp-2 cell line at the concentration of 100µg/ml and 50µg/ml. The maximal toxic free concentration of leaf extracts of Ethanol and Methanol was attained at 25µg/ml, 12.25µg/ml, 6.25 µg/ml, and 3.125µg/ml. Cell control and DMSO did not exhibit any cytotoxicity. The results were tabulated Table -1 and Fig (1a-1c) and (2a-2c).

![Table 1: Cytotoxicity Profile of *Sphagneticola calendulacea*](image)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Concentrations (µg/ml)</th>
<th>Ethanol Toxicity</th>
<th>Ethanol Cell control</th>
<th>Methanol Toxicity</th>
<th>Methanol Cell control</th>
<th>DMSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>100</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>50</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td>25</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4.</td>
<td>12.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5.</td>
<td>6.25</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6.</td>
<td>3.12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(+) → Presence of cytotoxicity
(-) → Absence of cytotoxicity

5 mg/ml of MTT was dissolved in PBS (Phosphate Buffered Saline). 20µl of MTT solution was added into the respective wells and the plate were incubated for 4 hours until purple precipitate is visible. Later 100µl of DMSO (Dimethyl sulfoxide) was added to each well and kept for some time. Plates were removed and measured at 540 nm using Microplate reader (Biotek, USA).

2.6 Antiviral Assay

Clinical *VZV* strain was isolated from a patient and transferred to Virus Transport Medium (VTM). The VTM vial was immediately kept in a refrigerator at 4°C. 0.1 ml of the VTM supernatant and maintenance medium was transferred on to the monolayer of HEp-2 cell line and incubated at 37 ºC for four days. Complete Cytopathic Effect (CPE) in HEp-2 cell line observed on the fourth day. Thus, virus stock was used for the estimation of TCID₃₀ by end point dilution assay [9].

2.7 *In vitro* antiviral assay

Monolayer of HEp-2 cells was grown in 96 well microtitre plates. 0.1 ml of 10⁶ TCID₃₀ viral suspensions, diluted in 2% FBS MEM, was added into experimental wells. 0.1 ml of 2% FBS maintenance medium alone was added into cell control and incubated at 37 ºC in 5% CO₂ atmosphere for 60 minutes to facilitate adsorption of virus to the cell line. The highest cell toxic free concentrations to the lower ranges of leaf extracts concentration were selected for anti-VZV study. The following concentrations of toxic free leaf extracts are 25µg, 12.5µg, 6.25µg, 3.125 µg were diluted with 0.2ml of 2% FBS MEM and transferred to the virus inoculated monolayer cell lines. The entire setup was incubated at 37 ºC temperature for three days to allow multiplication of virus and subsequent development of Cytopathic Effect (CPE). Each well was observed under Inverted microscope every day for presence or absence of Cytopathic effect. The standard antiviral agent of Acyclovir (1mg/mL) was used as Positive control. Later, MTT assay was carried out.

Table 1: Cytotoxicity Profile of *Sphagneticola calendulacea*
3.2 In vitro Antiviral Assay

Inhibition of virus was observed in the methanol and ethanol extracts of *S. calendulacea* at a maximal nontoxic concentration range from 25µg/ml, 12.25µg/ml, 6.25µg/ml, 3.12µg/ml and 1.56µg/ml. Infected cells treated with Acyclovir indicated complete inhibition and observed at a concentration of 25µg/ml. The results were tabulated Table - 2 and Fig 3 & 4 (A – D).

### Table 2: Antiviral activity of leaf extracts of *Sphagneticola calendulacea* (Methanol and Ethanol)

<table>
<thead>
<tr>
<th>S. No</th>
<th>Concentrations (µg/ml)</th>
<th>Cytopathic Effect</th>
<th>Virus Control</th>
<th>Cell Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>50</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>12.5</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td>6.25</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4.</td>
<td>3.12</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5.</td>
<td>1.56</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>6.</td>
<td>Acyclovir 25µg</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

4. Discussion

The development of plant based antiviral preparations promises a more potential alternative in combating viral diseases. Over the years, the World Health Organization (WHO) advocated that countries should interact with traditional medicine with the view to identify and exploit aspects that provide safe and effective remedies for ailments of antiviral. In addition, there are limited antiviral drugs being tested against viral disease in clinical trials. The wide prescription of herbal drugs is mainly due to their effectiveness, less side effects and relatively low cost\(^{10}\). As such present study is aimed to screen and determine the antiviral activity of Ethanol and Methanol leaf extracts of *Sphagneticola calendulacea* on VZV.

Although a reduction in the incidence of chickenpox was reported with the introduction of the varicella vaccine in 1995 vaccination against *VZV* is not a routine policy in many countries and seasonal outbreaks of the wild type infection of the virus continue to occur.

Previous authors claimed that the Nuts of *Semecarpus anacardium* methanolic extract was tested for its inhibitory effect in 96 micro plate against HEp 2 cell line and showed potent cytotoxic activity\(^{12}\) whereas, in the current study methanolic leaf extract of *Sphagneticola calendulacea* experimented for its inhibitory effect against HEp-2 cell line, the cytotoxicity was evaluated by MTT assay and outcome
proved in an effective manner. Lin et al., (1999) have reported that five groups of biflavonoids (amentoflavone, agathisflavone, robustaflavone, rhisflavanone and succedanea flavanones) were isolated from medicinal plants of Rhus succedanea and Garcinia multilora and exhibited various antiviral effects against a number of viruses including respiratory viruses and herpes viruses (HSV-1, HSV-2 and VZV). The inhibitory activity against VZV was demonstrated with succedanea flavanones [13]. Earlier, antiviral activity of Liquorice powder extract from the roots of Glycyrhiza glabra against Varicella Zoster Virus and studied using Vero cell line, which showed minimal activity when compared to standard [14] whereas, the present study was performed to find out the maximum toxic free concentration of leaf extracts of S. calendulacea against VZV on HEp-2 cell line and showed a positive results. Anand et al., (2004) have reported the efficacy of isolated principle from seed kernel of Pongamia pinnata against VZV [15]. Rajarajan et al., (2006) have studied in vitro antiviral potential from Coleus amboinicus on Human Herpes type-3(VZV) by using HEp2 cell line [16]. Jabareen et al., (2013) have examined the in vitro antiviral property using aequous and ethanolic leaf extracts of Passiflora edulis. They had conducted in African green monkey kidney (Vero) cells [17]. One of the plant extracts, glycyrrhizin (GL) was investigated for its antiviral action on varicella-zoster virus in vitro. When Human Embryonic Fibroblast (HEF) cells were treated with GL after inoculation of virus (post-treatment), the average 50%-inhibitory dose (ID50) for five VZV strains. GL was reported as effective against VZV replication when HEF cells were treated 24 hours before the inoculation whereas, in the current study leaf extracts of S. calendulacea was scrutinize against VZV using HEp-2 cell line. The ethanol and methanol leaf extracts showed a positive result on infected cell line with VZV.

5. Conclusion
The present study demonstrates that the inhibitory activity of methanolic and ethanolic leaf extracts against VZV at 25µg/ml concentration. Studies on S. calendulacea should be further conducted extensively by isolation, purification and characterization of the active compounds in order to discover the potential anti-viral compounds and provide more insight into the inhibition of virus adsorption and replication and to explore its potential use as a therapeutic product to combat dengue.

6. Acknowledgement
We are honoured to thank Secretary and Principal Ramakrishna Mission Vivekananda College (Autonomous), Chennai, India for providing all facilities. We are thankful to National Centre for Cell Science (NCCS), Pune, India for providing HEp-2 Cell line. We acknowledge the help of Dr. S. Rajarajan, Vice Chancellor, SRM University, Sonepat, Haryana, India and Dr. S. Gunasekran, Director King Institute of Preventive Medicine, Guindy, Chennai 600 032, for their help on cell line and antiviral studies and Dr. S. Sasikala, P.G & Research Department of Microbiology and Biotechnology, Presidency College (Autonomous), Chennai, India for photography of cell line.

7. References
15. Anand D, Rajarajan S. Studies on the estimation of in vitro safety & in vitro antiviral activities of few potential organic components, isolated from the seeds of Indian medicinal plant for Herpes Simplex Virus-1(HSV-1), Herpes Simplex Virus-2(HSV-2) & Varicella-Zoster Virus (VZV) and chemical characterization of the antiviral components.(2004); Ph.D thesis, University of Madras.
16. Rajarajan S. In vitro studies on the antiviral activities of Coleus amboinicus on Human Herpes type-3(VZV) by using Heq2 cell line. 2006; 27th annual conference of Indian Association of Biomedical Scientists souvenier.