**INTRODUCTION**

Distributed worldwide, Herpes simplex virus -1 is a typical human pathogen causing life-threatening diseases such as orofacial herpes, skin and mucosal infection, herpetic whitlow, herpes encephalitis, neonatal herpes acquired during delivery (Akanitapichat et al., 2006). The latent viral infection is spontaneously reactivated in recurrent infection in HSV infected chronic patients. (Bo et al., 2013 and Barton et al., 2005). These recurrent infections can be effectively treated with acyclovir (ACV), ganciclovir (GCV), Foscarnet nucleoside analogs. These drugs have severe side effects such as nausea, vomiting, and headache. (Burrel S et al., 2013). Further, it is reported as drug-resistant viruses. (Celum C et al., 2008). But there is no such research for effective new anti-herpetic drugs. This is very essential due to the progress of ACV resistant herpes viruses mutants especially in immunocompromised patients (Chakraborty et al., 2001 and Chattopadhyay et al., 2008). Moreover, ACV and other nucleoside analogs incorporate into the cellular DNA, yielding adverse drug reactions and thus unsuitable for pregnant women (Chavan et al., 2013) and neonates (Chowdhury et al., 1987 and Churqui et al., 2018). There are no potent drugs for reactivated HSV infections (De Clercq et al., 2004 and Erlich et al., 1989). Therefore, there is a strong need for novel effective anti-herpetic compounds with different mode of action from nucleoside derivatives. Medicinal plants are prospective alternatives for many viral infections in folklore medicine and also sound their promising therapeutic potential in ayurvedic and siddha medicines. (Fiddian et al., 1984).
Justicia adhatoda is a medicinal plant belongs to acanthaceae family and is widely used in many medicines such as Ayurveda, Siddha and Naturopathy. It is a shrub found in all parts of South East Asia (Nikomtat et al., 2006). The leaves of this plant are widely used for all types of respiratory problems. It also possesses very good bioactivities such as antiallergic (Shrivastava N et al., 2006), antidiabetic (Sydiskis et al., 1991), antioxidant (Tragoolpua et al., 2007), antimicrobial (Vanden Berghe et al., 1991), anti-ulcer (Verma et al., 2008) and antiviral properties (Wagner et al., 1989). Till date there is no scientific validation of J. adhatoda ethno medicine for antiviral activity. Therefore, the present study is investigated on the anti-HSV-1 activity of crude extracts of Justicia adhatoda leaves through in-vitro antiviral assays.

MATERIALS AND METHODS

Cell culture Media Chemicals
Cell culture reagents and FBS (fetal bovine serum) were procured from Gibco BRL (Gaithersburg, MD, USA). Culture medium, dimethyl sulfoxide (DMSO), Sodium pyruvate, penicillin G, streptomycin and fungizone antibiotics and antifungal drugs was bought from Sigma Aldrich India. 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) was purchased from Hi Media, India.

PCR kits and primers
HSV-1 specific primers for PCR were obtained from Biocorporals, Chennai. PCR kits and other reagents bought from Invitrogen, India. Vero Cell line (Kidney cells of african green monkey) were procured from NCCS, Pune. It was grown and maintained in MEM (Minimum Essential Medium), containing non-essential amino acids, L-glutamine, sodium pyruvate, sodium bicarbonate, 10% FCS (Heat inactivated fetal calf serum) and antibiotics such as streptomycin (100µL/mL), penicillin (100IU/mL), complement with 10% FBS and 1X non-essential amino-acids.

HSV-1 Virus Standard
HSV-1 strain was obtained from NIV (National Institute of Virology), Pune. The viruses were grown in Vero Cells and virus stocks were quantified by TCID50 (50% tissue culture infective doses) by endpoint dilution, with the infectious titer. (RJ W, DW K et al., 2005). The sub-cultured viral stocks were stored at -80°C for further use.

Preparation of extracts Justicacaeadathoda (L.)
J. adathoda leaves were gathered during 2015 from the forest reserve range of Chengalpattu November, TamilNadu, India, identified and authenticated at the Department of Plant biology and Plant Biotechnology, Presidency College, Chennai, India. The fresh, healthy leaves without any fungal contamination were segregated and preceded for washing distilled water, shadow dried at normal room temperature for ten days and powdered with the help of mechanical grinder. Hot extracts were prepared by using Soxhlet apparatus (Borosil, Mumbai) as per the standard procedure. Fifty grams each of the respective samples were soaked in 500ml of aqueous, chloroform and ethanol separately and further extracted with Soxhlet apparatus. The crude extracts was purified and filtered through 0.45micron syringe filter and further concentrated with Buchi rotary evaparator under reduced pressure. The powder is dissolved in 0.2% DMSO and preserved at -20°C.

Cytotoxicity Studies
The evaluation of cytotoxicity of J. adathoda extracts (CC50) was carried out using MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. Briefly, triplicate wells of confluent monolayers of Vero cells at population of 1.0 x 105 cells/ml cultured in 96 well TC plates. Different concentrations (mg/ml) of chloroform, aqueous and ethanol crude extract were added
to vero cells seeded in wells at a final volume of 100µl, in triplicate, adding DMSO as a negative control. All the plates were incubated at 37°C with 5% CO₂ for 16-18 hours. Then 100 microliter of (10%) MTT was added to each well and incubated for 5h at 37°C for development of blue color product formazan. DMSO was added to each well and the absorbance values were read at 620nm using 96-well microplate reader (Thermo Multikan EX, USA). The cytotoxicity of the *J. adathoda* extracts in vero cells was determined by (Test OD - cell free sample blank)/mean media control OD / 100%.

*J. adathoda* extracts concentration required for 50% (CC50 value) reduction of cell viability with noticeable morphological alteration in 50% of Vero cells with respect to vero cells alone was detected by Standard Error Mean (SEM). (Oliver NM et al., 1989)

**Plaque Inhibition Assay**

Vero cells cultured in 24 well plate was developed for the confluent monolayer in MEM supplemented media with 10% FBS in 5% CO₂ at 37°C. Different dilutions of *J. adathoda* leaf extracts were incubated with equal volume 100 plaque forming units (PFU) HSV-1 for 1h at 37°C. The *J. adathoda* leaf extracts were detected for extent of inhibition of HSV-1 plaque formation on infected cells as a sign of anti-viral activity in vitro. Hundred microliters of respective dilutions were then placed in each well of 24 wells plate. The plates were incubated for 2h at 37°C with intermediate shaking after every 10 min to facilitate virus to establish cell infection. The infected cells were further overlaid with 2% CMC (carboxymethyl cellulose) and 2X MEM and further incubated at 37°C with 5% CO₂ for 3 days. After incubation, the CMC with 2XMEM was removed and washed with MEM and stained with Amido black solution. The plaques appearing as clear dots, were counted using a inverted phase contrast microscope and plaque inhibition (%) was calculated. The EC50 (effective concentration) reducing plaques formation by 50% was calculated using the plaque inhibition assay at various concentrations (0.5, 0.25, 0.1, 0.01 and 0.001mg/ml) of *J. adathoda* leaf extracts.

**RESULTS**

*Justicia adathoda* leaf extracts were evaluated for cytotoxicity in vero cell line. The aqueous, chloroform and ethanol extracts at different concentration viz 10,25,50,75,100,250 and 500µg were determined for its cytotoxic effects in vero cells (Figure-1).

![Cytotoxicity of *J. adathoda* leaf extracts in Vero cell lines](image)

**Figure 1:** Cytotoxicity of *J. adathoda* leaf extracts in Vero cell lines

The Cytotoxic activity of aqueous extract found as 10µg 8.047±4.3, 25µg 12.01±23.09, 50µg 33.9±9.27, 75µg 65.52±14.11 and 100µg 93.01±8.09. The Cytotoxic activity of ethanol extract
10µg 11.01±9.01, 25µg 25.66±5.6, 50µg 53.55±13.5, 75µg 70.14±17.5 100µg 96.23±5.62. Similarly the chloroform extract showed 10µg 6.98± 2.31, 20µg 15.22±3.29, 50µg 30.14± 3.3, 75µg 52.12±9.62 and 100µg 64.34±9.77. Among the extracts screened for the cytotoxicity in vero cells, Table 1: Cytotoxic Activity of Justicaeadathoda extracts in Vero cell line

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Justicaeadathoda Extracts</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; in µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aqueous</td>
<td>58.76</td>
</tr>
<tr>
<td>2</td>
<td>Chloroform</td>
<td>74.26</td>
</tr>
<tr>
<td>3</td>
<td>Ethanol</td>
<td>47.05</td>
</tr>
</tbody>
</table>

Highest cytotoxicity was observed in the ethanolic extract. The effective cytotoxic concentration 50% (EC<sub>50</sub>) calculated in all the extracts is tabulated in Table-1. The EC<sub>50</sub> revealed that the minimum concentration with effective toxicity is found in ethanolic extract 47.05µg, aqueous extract, 58.76 and chloroform extract 74.26µg. Figure-2 shows the cytotoxic effect of Justicaeadathoda leaf extracts in the vero cell line. Figure 2a is the normal vero cell line and figure 2b vero cell line treated with aqueous extract and figure 2c with chloroform extract and figure 2d is with ethanolic extract. Amid all the extracts treated, ethanolic extract showed the highest cytotoxicity when compared to chloroform and aqueous extracts. All the extracts were treated with a constant concentration of 50µg /100μl.

**Figure 2.** Cytotoxic effect of J. adhathoda leaf extracts in different time interval on Vero cell lines

Further from the above results, the ethanolic extracts alone were chosen and investigate to the cytotoxic effect in different time interval on Vero cell lines (figure-3). In figure-3 the cytotoxic effect was observed from 24hrs to 72hours.

**Figure 3.** Cytotoxic effect of J.adhathoda leaf ethanolic extracts in different time interval on Vero cell lines.

The extract with stand its activity upto 72hrs. The cytotoxic effect was initially observed 30% in 24hrs, 45% in 36hrs and 56% in 48hrs and in 72hrs 70% toxicity was sustained. The cell morphology was completely shrunken, more gaps found in the cells revealed that cell population was decreased and cell cycle was also arrested and reached its termination. The ethanolic extract revealed that the extract has dose dependent effect and sustains its effect up to 72hrs. The ethanolic...
extract indicates a potent cytotoxic and hence it was chosen to screen the antiviral activity against HSV-1 virus through plaque reduction assay. The nontoxic dose concentration was optimized from EC_{50} and fixed dose was kept as 50 μg /100 μl of the J.a extracts. Figure-4 reveal the antiviral activity of J.a extracts based on the cytopathic effect (CPE). The cytopathic effect was observed in all the extracts, but more CPE was found only in ethanolic extract than in chloroform and aqueous extract. The TCID_{50} was observed in 10^{-5} and the mechanism of the antiviral activity through the plaque reduction assay was calculated as shown in table-2. The viral absorption, replication and protection were carried out in J.a extracts. The absorption of aqueous extract showed 100(19± 13) % and replication 85±16 and direct 50(15±6) and the chloroform extract revealed that replication showed 90±0(34± 18) and replication 91±16(>50) and direct 60(22±3) and the ethanolic extract indicated that 99 ± 5 (46 ± 15) of absorption, replication of 49 ± 21 (< 50) and direct found 100 (14 ± 4).

a-Cell control:Vero cell lines infected with HSV-1, b-Aqueous extract with HSV-1, c-Chloroform with HSV-1,d-Ethanol with HSV-1

**DISCUSSION**

Many drugs treated for Herpes simplex viruses are generating high side effects than the therapeutic efficacy against virus. This adverse effect is due to their in efficiency and viral resistance. (Yang CM et al., 2005). Therefore, at present the pharmaceutical and medical industry need new drugs in combating the viral infection and with no side effects. Traditionally many medicinal plants were treated for various viral diseases and the novel bioactive compounds were identified for the non toxic dose concentration develops new drugs. (Yoosook C et al., 2000 ).
Hence the present study was focused to evaluate the antiviral activity of *Justicaeadathoda* leaf extracts against herpes simplex virus 1 through in vitro studies. As reported earlier medicinal plants such as *Acacia, Terminalia, Curcuma, Terminalia mulleri* possess antiviral activity against Herpes simplex viruses (HSV). The results indicate that the ethanolic extract of *Justicaeadathoda* possess well pronounced cytotoxicity and antiviral activity against HSV-1 in vero cell lines. The nontoxic minimal dose with effective toxicity found in ethanolic extract was 47.05 µg than the aqueous extract and chloroform extract. Further the cytotoxic effect was initially observed 30% in 24 hrs and sustained 70% upto 72 hrs. The cell morphology was also completely changed by the appearance of shrunken and more gaps. The mechanism of action is that, the extracts of *Justicaeadathoda* act upon the envelope regions of the virus cells, decreases the cell population by arresting the cell cycle and initiate termination which leads to cell gaps and reduction of plaque forming Units. All the extracts in the study revealed that extract possess dose dependent effect. But when comparing all the extracts, the ethanolic extract indicates it is a potent cytotoxic and antiviral agent against HSV-1 virus through plaque reduction assay. The study done in the medicinal plant *Phyllanthus urinaria* Linn extracts inhibits HSV-2 infection at the early stage of virus infection and establishment in the host.(Yoosook C et al., 1999 ). Moreover, similar HSV-1 antiviral activity was reported in the aqueous extract of *Swertia chirata*. The plaque reduction assay of the present study indicated that 50% of plaques were effectively reduced in the replication of HSV-1 virus, when treated with ethanolic extract than the chloroform and aqueous extracts. Likewise, 95% of plaques were reduced and eradicated when treated with direct contact of *J.a* ethanolic extract in HSV-1 on vero cell lines.

**CONCLUSION**

Thus the present study concludes that ethanolic extract is proven as potential drug candidate for the molecular targets against the Herpes Simplex Virus-1 using Plaque Inhibition Assay. Hence the present study surmises that the ethanolic extract of *Justicaeadathoda* is a promising chemotherapeutics for the treatment HSV-1 infection.

**REFERENCE**