

## Anti-HSV and Cytotoxic activity of *J. adhatoda* leaf extracts against Human Herpes Simplex Virus type-1

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### ABSTRACT

The aim of the present study is to observe the cytotoxic activity of crude extracts of leaf of *Justicia adhatoda* a plant belonging to the family Acanthaceae. The leaf of *Justicia adhatoda* was extracted with organic solvent and the extracts (aqueous, chloroform and ethanol) were used for the observation of cytotoxic activity. Crude extracts of plant, were screened for cytotoxic activity using MTT assay. The Cytotoxic activity of aqueous extract found as 10 $\mu$ g 8.047 $\pm$ 4.3, 25 $\mu$ g 12.01 $\pm$ 23.09, 50 $\mu$ g 33.9 $\pm$ 9.27, 75 $\mu$ g 65.52 $\pm$ 14.11 and 100 $\mu$ g 93.01 $\pm$ 8.09. The ethanol extract 10 $\mu$ g 11.01 $\pm$ 9.01, 25 $\mu$ g 25.66 $\pm$ 5.6, 50 $\mu$ g 53.55 $\pm$ 13.5, 75 $\mu$ g 70.14 $\pm$ 57 100  $\mu$ g 96.23 $\pm$ 5.62. Similarly the chloroform extract showed 10 $\mu$ g 6.98 $\pm$  2.31, 20 $\mu$ g 15.22 $\pm$ 3.29, 50 $\mu$ g 30.14 $\pm$  3.3, 75 $\mu$ g 52.12 $\pm$ 9.62 and 100 $\mu$ g 64.34 $\pm$ 9.77. Hence proved the ethanolic extract showed highest cytotoxicity. The effective cytotoxic concentration 50% (EC<sub>50</sub>) is calculated.

**Keywords:** *Justicia adhatoda*, cytotoxic activity, MTT assay, Vero cells

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### INTRODUCTION

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 the delivery.<sup>[1]</sup> The latent viral infection is spontaneously reactivated in recurrent infection in HSV infected chronic patients.<sup>[2,3]</sup> 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.<sup>[4]</sup> Further it is reported as drug-resistant viruses.<sup>[5]</sup> 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 immune suppressed and deficient syndrome patients.<sup>[6, 7]</sup> Moreover ACV and other nucleoside analogs incorporate into the cellular DNA, yielding adverse drug reactions and thus, unsuitable for pregnant women<sup>[8]</sup> and neonates<sup>[9,10]</sup>. There are no potent drugs for reactivated HSV infections.<sup>[11, 12]</sup> Therefore, there is a strong need for novel effective anti-herpetic compounds with different mode of action from nucleoside derivatives. Medicinal plants are prospective alternates for many viral infections in folklore medicine and also sound its promising therapeutic potential in ayurvedhic and siddha medicines.<sup>[13]</sup>

The HSV vaccine developed from the animal experiments has not succeeded in human and the major immune determinants are also not well determined.<sup>[14,15]</sup> The vaccines administered for HSV is failed in some cases to influence the humoral immune responses to prevent the recurrent infection.<sup>[16]</sup> Hence the present study is focused on the evaluation of cost effective, readily available, less toxic, reduced side effects with good bioactive compounds to control and prevent HSV-1 infection and its transmission from the medicinal plants. Medicinal plants such as *Eugenia caryophyllus*<sup>[17-19]</sup> and *Scoparia dulcis*<sup>[20,21]</sup> were studied and reported for anti HSV activity using plaque and antiviral assay.

*Justicia adhatoda* is a medicinal plant belonging to acanthaceae family. It is widely used in many medicines such as ayurvedha, siddha and Naturaopahty. It is a shrub found in all parts of south east asia.<sup>[22]</sup> The leaves of this plant are widely used for all types of respiratory problems. Moreover it possess very good bioactivities such as antiallergic<sup>[23]</sup>, antidiabetic<sup>[24]</sup>, antioxidant<sup>[25]</sup>, antimicrobial<sup>[26]</sup>, anti-ulcer<sup>[27]</sup> and antiviral properties.<sup>[28]</sup> Till date there is no scientific validation of *J. adhatoda* ethnomedicine for antiviral activity. Therefore the present chapter is investigated on the anti-HSV-1 activity of crude extracts of *Justicia adhatoda* leaves through invitro 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 (100 $\mu$ L/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 TCID<sub>50</sub> (50% tissue culture infective doses) by endpoint dilution, with the infectious titer.<sup>[29]</sup> The sub-cultured viral stocks were stored at -80°C for further use.

### **Preparation of extracts *Justicae adathoda* (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 extracted 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 evapaporator 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 (CC<sub>50</sub>) was carried out using MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay.<sup>[33]</sup> Briefly, triplicate wells of

confluent monolayers of Vero cells at population of  $1.0 \times 10^5$  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 100 $\mu$ l, in triplicate, adding DMSO as a negative control. All the plates were incubated at 37°C with 5% CO<sub>2</sub> 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%.

The *J. adathoda* extracts concentration required for 50% (CC50value) 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).<sup>[20]</sup>

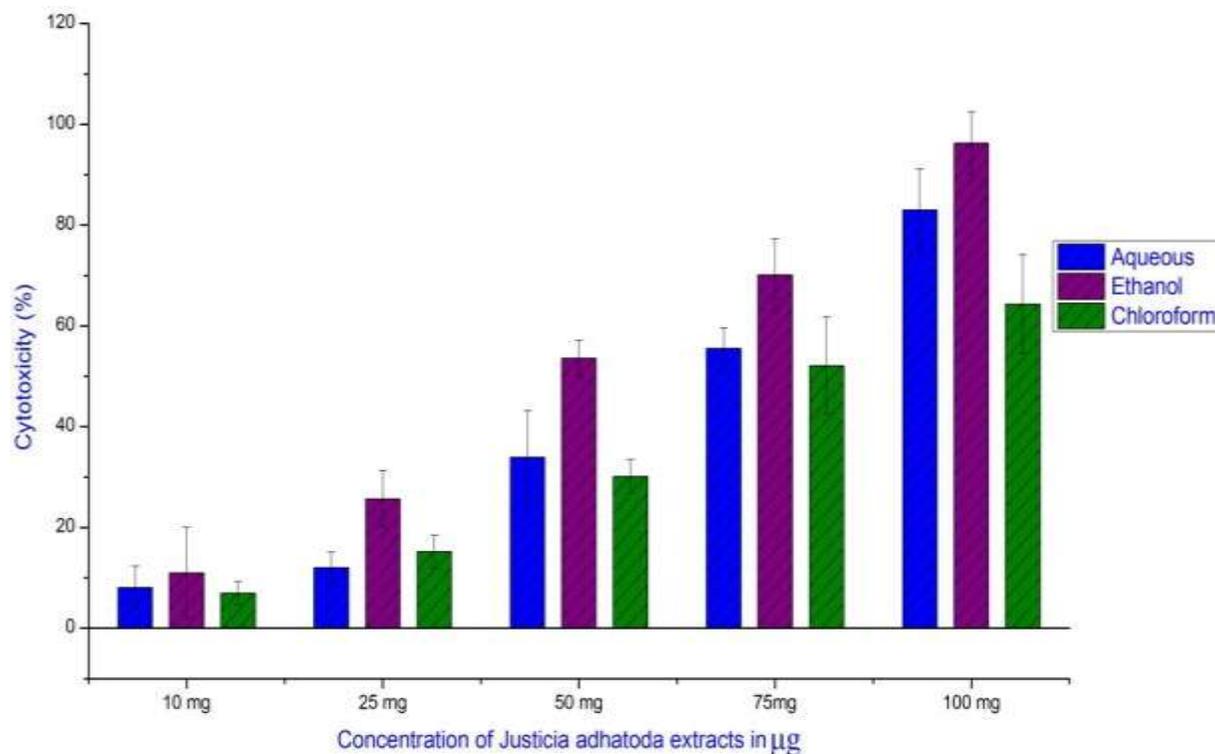
### **Plaque Inhibition Assay**

Vero cells cultured in 24 well plate were developed for the confluent monolayer in MEM supplemented media with 10% FBS in 5% CO<sub>2</sub> 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 was 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% CO<sub>2</sub> 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**

The cytotoxicity of *Justicae adathoda* leaf extracts were evaluated in the vero cell line. The aqueous, chloroform and ethanol extracts at different concentration viz 10,25,50,75,100,250 and

500 $\mu$ g were determined for its cytotoxic effects in vero cells (Figure-1). The Cytotoxic activity of aqueous extract found as 10 $\mu$ g 8.047 $\pm$ 4.3, 25 $\mu$ g 12.01 $\pm$ 23.09, 50 $\mu$ g 33.9 $\pm$ 9.27, 75 $\mu$ g 65.52 $\pm$ 14.11 and 100 $\mu$ g 93.01 $\pm$ 8.09. The Cytotoxic activity of ethanol extract 10 $\mu$ g 11.01 $\pm$ 9.01, 25 $\mu$ g 25.66 $\pm$ 5.6, 50 $\mu$ g 53.55 $\pm$ 13.5, 75 $\mu$ g 70.14 $\pm$ 57 100 $\mu$ g 96.23 $\pm$ 5.62. Similarly the chloroform extract showed 10 $\mu$ g 6.98 $\pm$  2.31, 20 $\mu$ g 15.22 $\pm$ 3.29, 50 $\mu$ g 30.14 $\pm$  3.3, 75 $\mu$ g 52.12 $\pm$ 9.62 and 100 $\mu$ g 64.34 $\pm$ 9.77. Among the extracts screened for the cytotoxicity in vero cells,

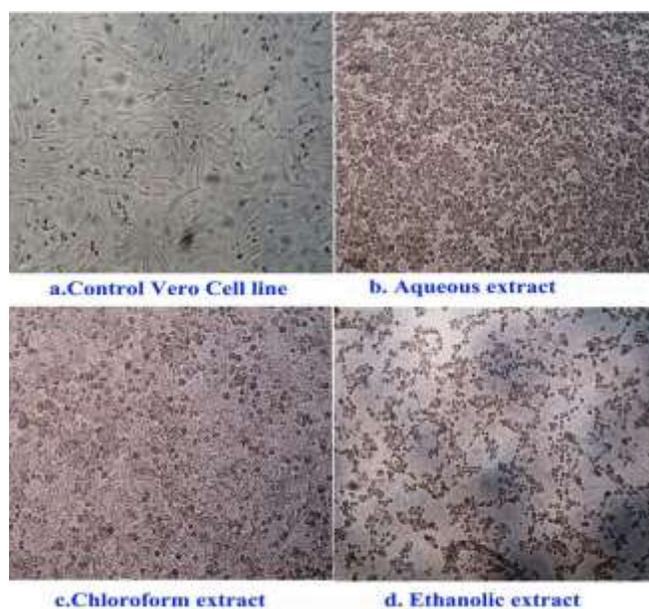


**Figure 1:** Cytotoxicity of *J. adhatoda* leaf extracts in Vero cell lines

**Table 1:** Cytotoxic Activity of *Justicae adathoda* extracts in Vero cell line

Sl.No	<i>Justicae adathoda</i> Extracts	EC <sub>50</sub> in $\mu$ g
1	Aqueous	58.76
2	Chloroform	74.26
3	Ethanol	47.05

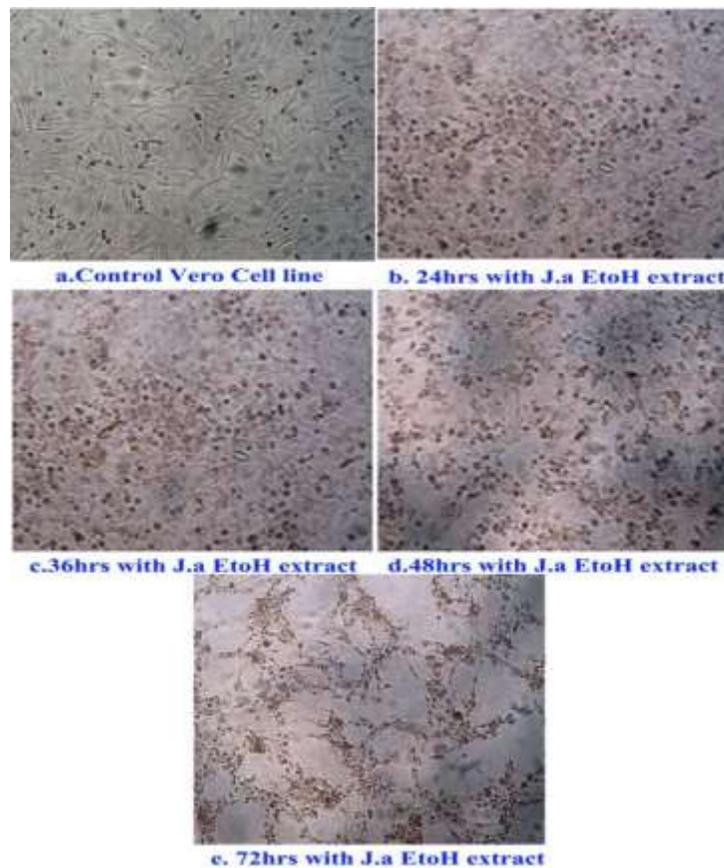
The ethanolic extract showed highest cytotoxicity. The effective cytotoxic concentration 50% ( $EC_{50}$ ) is calculated in all the extracts tabulated in the table-1. The  $EC_{50}$  revealed that the minimum concentration with effective toxicity is found in ethanolic extract 47.05 $\mu$ g, aqueous extract 58.76 and chloroform extract 74.26 $\mu$ g. The figure-2 showed the cytotoxic effect of *Justiciae adathoda* leaf extracts in the vero cell lines. The 2a is the normal vero cell line and figure 2b is vero cell line treated with aqueous extract and figure 2c is treated with chloroform extract and figure 2e is treated with ethanolic extract. Amid all the extracts treated the ethanolic extract showed the highest cytotoxicity when compared to the chloroform and aqueous extracts. All the extracts were treated with a constant concentration of 50 $\mu$ g /100 $\mu$ l.



**Figure 2.** Cytotoxic effect of *J. adathoda* 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. The extract with stand its activity upto 72hrs. The cytotoxic effect was initially observed 30% in the 24hrs, 45% in the 36hrs and 56% in the 48hrs and in the 72hrs it was 70% toxicity was sustained and the cell morphology was completely shrunken and more gaps was found in the cells revealed that cell population was decreased and cell cycle also arrested and it reaches termination. The ethanolic extract revealed that the extract

has dose dependent effect and sustains its effect upto 72hrs. The ethanolic extract indicated a potent cytotoxic and hence it was chosen to screen the antiviral activity against HSV-1 virus through plaque reduction assay.



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

The nontoxic dose concentration was optimized from the  $EC_{50}$  and it was kept fixed dose as  $50 \mu\text{g} / 100 \mu\text{l}$  of the *J.a* extracts. The figure-4 revealed the antiviral activity of the *J.a* extracts based on the cytopathic effect (CPE). The cytopathic effect was observed in all the extracts but more CPE is found only in the ethanolic extract than the chloroform and the aqueous extract. The  $TCID_{50}$  is observed in the  $10^{-5}$  and the mechanism of the antiviral activity through the plaque reduction assay was calculated in the table-2. The viral absorption, replication and protection were carried out in the *J.a* extracts. The absorption of aqueous extract showed  $100 (19 \pm 13) \%$  and replication  $85 \pm 16$  and direct  $50 (15 \pm 6)$  and the chloroform extract revealed that replication showed  $90 \pm 0 (34 \pm 18)$  and replication  $91 \pm 16 (> 50)$  and direct  $60 (22 \pm 3)$  and the ethanolic

extract indicated that  $99 \pm 5$  ( $46 \pm 15$ ) of absorption, replication of  $49 \pm 21$  ( $< 50$ ) and direct found 100 ( $14 \pm 4$ ).



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

**Figure 4:** Antiviral activity of *J.adhathoda* leaf extracts on Human herpes simplex virus-1 in Vero cell lines.

Sl.No	<i>J.adhathoda</i> Leaf extracts	Antiviral activity <sup>a</sup> of <i>J.a</i> extracts through Plaque Reduction assay (%)			
		Protection <sup>b</sup>	Absorption <sup>c</sup>	Replication <sup>d</sup>	Direct <sup>e</sup>
1	Aqueous	NA	95 (19± 13)	85 ±1 6 (> 50)	50 (15 ± 6)
2	Ethanol	NA	99 ± 5 (46 ± 15)	49 ± 21 (> 50)	95 (14 ± 4)
3	Chloroform	NA	90 ± 0 (34 ± 18)	91 ± 16 (> 50)	60 (22 ± 3)

NA Inhibition of lysis plaques  $< 50\%$  was considered not active, Tx Cytotoxic against Vero cells at  $100 \mu\text{g mL}^{-1}$

Table 2: Anti HSV activity of *J.adhathoda* Leaf extracts through Plaque Reduction assay <sup>a</sup>Inhibitory percentage of lysis plaque induced by HSV-1 at a *J.a* ethanolic extract

concentration of  $25\mu\text{g mL}^{-1}$  (top row), and effective concentration ( $\mu\text{g mL}^{-1}$ ) inhibiting 50% (EC50) of lysis plaque (bottom row; in parentheses), Vero cells were pretreated with compounds prior infection, <sup>c</sup>Vero cells and viruses were incubated together with compounds during the absorption period, <sup>d</sup>compounds were added after absorption and during the replication period, <sup>e</sup>Viruses were incubated directly with compounds prior infection of Vero cells.

## 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.<sup>[30]</sup> 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.<sup>[31]</sup> Hence in the chapter the present study was focused on the antiHSV viral activity of *Justicae adathoda* leaf extracts was studied for in the vero cell line by invitro study. The medicinal plants such as *Acacia*, *Terminalia*, *Curcuma*, *Terminalia mulleri* possess antiviral activity against Herpes simplex viruses (HSV). Similarly in the current study *Justicae adathoda* leaf extracts were evaluated against HSV-1. The results indicated that the ethanolic extract of *Justicae adathoda* possess well pronounced cytotoxicity and antiviral activity in the presence of HSV-1 in vero cell lines.

The Nontoxic minimal dose with effective toxicity is found in ethanolic extract  $47.05\mu\text{g}$  than the aqueous extract and chloroform extract. Further the cytotoxic effect was initially observed 30% and sustained upto 72 hrs. The cell morphology was also completely changed by the appearance of shrunken and more gaps. This is the mechanism of action the extracts of *Justicae adathoda* act upon the envelope regions of the virus cells and it decreases the cell population by cell cycle also arrested and initiate termination leads to cell gaps and reduction of plaque forming Units. All the extract in the study revealed that extract possess dose dependent effect. But when comparing all the extracts, the ethanolic extract indicated it is a potent cytotoxic and antiviral agent against HSV-1 virus through plaque reduction assay. Many medicinal plants was reported study was done in the medicinal plants *Phyllanthus urinaria* Linn extracts inhibits HSV-2 infection at the

early stage of virus infection and establishment in the host.<sup>[32]</sup> Moreover similar HSV-1 anti viral activity was reported in the aqueous extract of *Swertia chirata* inhibited with well pronounced.

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 surmise that the ethanolic extract of *Justicae adathoda* is a promising chemotherapeutics for the treatment HSV-1 infection.

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