INTRODUCTION

Herpes simplex virus type 2 (HSV-2) is a sexually transmitted pathogen that infects more than 500 million people worldwide and causes an estimated 23 million new infections each year. Genital herpes simplex virus infection is a recurrent, lifelong disease with no cure. The strongest predictor for infection is a person’s number of lifetime sex partners. The natural history includes first-episode mucocutaneous infection, establishment of latency in the dorsal root ganglion, and subsequent reactivation. In India, the prevalence of HSV-2 seropositivity was found to be associated significantly with low or moderate levels of sexual activity, urban middle class community and older age (Chawla, 2008). HSV-2 is the most common cause of genital ulcer disease, it is one of the most important biologic predictors of HIV acquisition with infected persons having a 2.1-fold increased risk of HIV. While most herpes infections do not cause serious complications, infections in infants and in people with weakened immune systems, or herpes infections that affect the eyes, can be life threatening. Transmission of HSV from mother to infant during birth is the most serious complication of genital herpes, and women who acquire HSV during pregnancy are at the highest risk of transmitting the infection (Looker et al., 2012). Effective oral antiviral medications are available for the treatment of genital herpes. These agents offer clinical benefit but do not cure the disease despite early animal models suggesting that antiviral medications may affect the long-term natural history of HSV infection. However, extensive use of this drug has led to the emergence of acyclovir resistant virus strains, particularly in immunocompromised patients (Piotr et al., 2014). Treatment of herpes infection is thus cause of major concern owing to the difficulty in eliminating it from the ganglion, high cost of treatment, increasing drug resistance, and association with HIV-1 (Priyanka et al., 2015). A substantial increase in antiviral usage in immunocompetent populations would have a substantial beneficial impact on herpes epidemics in terms of preventing new infections, and have a negligible detrimental impact in terms of generating drug resistance.

The use of herbal medicines has been steadily increasing over the past decade to cure some of the disorders in human. In India, medicinal plants are traditionally used in the treatment of infectious diseases, as they are inexpensive, efficacious and safe (Martins et al., 2013). The screening of plant extracts and plant products for antimicrobial activity has shown that plants represent a potential source of new anti-infective agents. (Parekh and Sumitra, 2007). The present study was aimed to

**ARTICLE INFO**

**Article History:**
Received 12th August, 2019
Received in revised form 23rd September, 2019
Accepted 7th September, 2019
Published online 28th November, 2019

**Key Words:**
Herpes simplex virus, *Andrographis paniculata*, CPE, TCID50, GC-MS.

**ABSTRACT**

Herpes simplex virus-2 (HSV-2) is a leading cause for genital ulcers worldwide and it is widespread even among people with low or moderate levels of sexual activity. In contrast to other sexually transmitted infections, HSV-2 is a prime biologic source of HIV transmission. Though effective oral antiviral medications are available that can be used only as episodic treatment or as long-term suppressive therapy. The current research was aimed to assess a novel antiviral compound from *Andrographis paniculata* against HSV-2. The antiviral activity of lyophilized methanolic extract of *Andrographis paniculata* was evaluated by in vitro cytotoxicity test by MTT assay, Anti HSV-2 activity by CPE reduction assay and HSV-2 was quantified by TCID50 assay. The in vitro cytotoxicity assay performed on vero cell lines, the highest concentration of methanolic extract of *Andrographis paniculata* exhibited 50% of cytotoxicity, CPE reduction assay showed 100% inhibition even at the lowest concentration of the extract. GC-MS analysis of methanolic extract of *Andrographis paniculata* exhibited five major active compounds responsible for the anti-viral activity.

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find out the antiviral activity of *Andrographis paniculata* against HSV - 2 as several researchers investigated significant antiviral activity against limited viruses, such as dengue virus serotype 1 (DENV-1), human papilloma virus type 16 (HPV16) herpes simplex virus type 1 (HSV-1), Influenza A virus and HIV (Reddy et al., 2005), their findings were very encouraging and noteworthy considering the life threatening role of these viruses in human community.

**MATERIALS AND METHODS**

**Collection and processing of Plant Material**

The Leaves of *Andrographis paniculata* were collected from Presidency College Medicinal garden and authenticated by Department of Botany. After the taxonomy identification, the leaves were washed and cleaned and shade dried for 10-15 days. Upon drying, 100 g of the plant materials were ground to powder and extracted consecutively with 500 mL of absolute methanol (Fisher Scientific, UK) for three to four days in dark condition. The resulting suspension was filtered and evaporated under reduced pressure at 50°C until dryness.

**Preparation of Methanolic extract and lyophilization**

20gms of *Andrographis paniculata* powdered leaf extract was weighed and mixed with 200ml of methanol and kept in a shaking incubator for 48h and the filtered methanolic extracts were transferred to lyophilization flask and were frozen at -80°C to -20°C prior to lyophilization. After an overnight (12 h) freezing, the flask was immediately lyophilized and the final lyophilized extract was stored at 4°C until use.

**Preliminary Phytochemical analysis**

The preliminary phytochemical screening of methanolic extract of *A. paniculata* was carried out to check for the presence of alkaloids, carbohydrates, glycoproteins, sterols, triterpenes and flavonoids (Sofowora, 1993; Evans and Trease, 1989).

**Phytochemical analysis of *A. paniculata* by GC-MS**

A phytochemical analysis were performed at Sophisticated Analytical Instrument Facility center IIT- Madras by JEOL GCMATE II GC-MS with Data system is a high resolution, double focusing instrument. Methanolic extract of *A. paniculata* was subjected for compound identification by injecting 1µl of extract into GC-MS instrument, after running for 40 minutes, major compounds were identified by comparing with standard reference library.

**Propagation of HSV- 2 in Vero cell lines**

Vero cells were obtained from Tamil Nadu Veterinary and Animal Science University (TANUVAS) and maintained in Dulbecco’s Modified Eagle’s Medium (MEM) supplemented with 10% fetal bovine serum (FBS) and penicillin (1000U/ml) streptomycin (10µg/ml). The HSV-2 strain was propagated in the 25-cm² tissue culture flask for 72 h at 37°C in CO₂ incubator at multiplicity of infection (MOI) of 0.01 PFU/cell. After three cycles of freezing/thawing, the supernatant was titrated on the basis of Plaque Forming Unit (PFU) as previously described (Blahe, 2005) and stored in aliquots at -80°C until use.

**Assessment of cytotoxicity by MTT assay**

Cytotoxicity activity of methanolic extract of *A.paniculata* was estimated using Vero cells (as described in Mosmann et al., 1983). Briefly cells at a density of 5×10⁴ cells/well were plated in 96 well tissue culture plates and incubated for 24 hours at 37°C with 5% CO₂. After 24 hours cells were treated with 2mg, 1mg, 500µg and 250 µg concentration in triplicate with vehicle [dimethyl sulfoxide (DMSO) and/or ethanol] control for 48 h. Negative control included cells with medium only. After incubation, 20 µl of MTT (5 mg/ml in 50 mM PBS) was added per well and incubated for 4 h at 37°C, followed by addition of MTT solvent (100 µl/well; 0.04 N HCl in absolute isopropanol). The absorbance (OD) was read at 540 nm with reference filter at 630 nm by using microplate spectrophotometer. The Percentage cell viability was calculated by dividing the OD obtained in treatment group by OD in the respective vehicle control multiplied by 100.

**Quantification of HSV-2 (TCID₅₀)**

HSV-2 quantifications were done by TCID50 assay by end point dilution assay method and were analyzed by spearman and karber method (Spearman, 1908; Karber, 1931). A total of 5×10⁴ cells/well seeded into two 96-well plates for HSV-2 and incubated at 37°C with 5% CO₂. After 24 hours, medium was discarded, cells were visualized for monolayer confluence, then total volume of 100µl of ten-fold serially diluted HSV was inoculated into each well with 10 replicates for each dilution. Plates were incubated later at 37°C for 3 days along, with controls. The presence of CPE in each well was marked as ‘+’, while its absence was marked as ‘-’. The proportion of wells with CPE in each serially diluted HSV was calculated and the TCID50 was estimated using the formula: Log TCID50 = L - d (s - 0.5), whereby L = lowest dilution factor; d = difference between dilution steps; s = sum of proportion in TCID50 units per milliliter (TCID50/ml). The value of TCID50 determined was applied in the antiviral assay.

**Anti-HSV-2 activity by CPE reduction assay**

Antiviral assay was performed 24 h prior to assay, cells were trypsinized, counted and seeded at a density of 5×10⁴ cells per well. After reaching 80% confluence the media was removed and 100µl of 10⁻³ TCID50/ml virus particles were added and incubated for 1 h for viral adsorption. Unadsorbed viral particles were removed by washing the cell sheet with SFM. After adsorption cells were treated with 2mg, 1mg, 500µg and 250 µg concentrations along with cell control and virus control afterwards incubated for 3 days at 37°C in 5% CO₂ atmosphere. Plates were examined microscopically for CPE daily. The antiviral effects of NPs were assessed using the grading system described by Kudi and Myint (1999). Degree of CPE inhibition upon treatment was marked at the following order: ‘++++’ represented a total inhibition, ‘+++’ for 75% inhibition, ‘++’ for 50% inhibition, ‘+’ for less than 50% inhibition, ‘-’ as no inhibition.

**RESULTS**

**Cytotoxicity Assay**

Cell viability was determined by exposing Vero cell lines to increasing concentrations of *A. paniculata* extracts. After 7 days of incubations, the percentage of viable cells was
determined using MTT assay. Results shown in Graph 1 indicates that the accepted safe concentrations on Vero cells were found to be less than 1mg. The results also showed that the rate of cell death increased with increasing concentrations of the *A. paniculata* extracts.

**Graph 1** Cytotoxic activity of *A. paniculata* extract

### Anti-viral assay of *A. paniculata*

The current study also investigated the cytotoxicity of *A. paniculata* against HSV-2. Various concentrations of the extract starting from 250 µg up to 2mg were analyzed. All the above-said concentrations showed complete CPE reduction (100 % inhibition) of HSV-2 growth in Vero cell lines (Table 1).

**Table 1** Anti-HSV-2 activity and CPE inhibition by various concentrations of *A. paniculata* extract

<table>
<thead>
<tr>
<th>Concentration of <em>A. paniculata</em></th>
<th>Anti-HSV-2 activity</th>
<th>% of CPE reduction</th>
</tr>
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<tbody>
<tr>
<td>2mg</td>
<td>++++</td>
<td>100 %</td>
</tr>
<tr>
<td>1mg</td>
<td>++++</td>
<td>100 %</td>
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<tr>
<td>500µg</td>
<td>++++</td>
<td>100 %</td>
</tr>
<tr>
<td>250µg</td>
<td>++++</td>
<td>100 %</td>
</tr>
<tr>
<td>Virus control</td>
<td>-</td>
<td>No inhibition</td>
</tr>
</tbody>
</table>

**Fig 1** Antiviral activity of *Andrographis paniculata* on vero cell lines

### DISCUSSION

Plant based treatments continue to play an essential role in primary health care. The improvement of medicinal plants value can be achieved by searching newer, more effective and less toxic therapeutic molecules, which will add great values to the resources that can be later, integrated into the therapeutic arsenal (Latifa et al., 2013). Medicinal plants present a potential source of drugs or molecular models for new drugs. The importance of natural compounds from plants materials is also raising interest among scientists to discover new antiviral molecules.

In the current study of *A. paniculata* was assessed for their antiviral activity. The results of the study were encouraging on using the chosen medicinal plants against Herpes simplex virus-2. Perhaps the efficacy of the herbal extracts chosen in the present study could be due to the active principles of their phytochemical constituents such as alkaloids and flavonoids individually or synergistically.

However, before the therapeutic use, the drug toxicity studies of these medicinal plants extracts are warranted. Therefore, the current study also investigated the cytotoxicity of *A. paniculata* against HSV-2. Various concentrations of the extract starting from 250 µg up to 2mg were analyzed. All the above-said concentrations showed complete CPE reduction (100 % inhibition) of HSV-2 growth in Vero cell lines.

In earlier research studies (Sheeja et al., 2007) the effect of *Andrographis paniculata* extract (APE) and its isolated compound andrographolide (ANDLE) on cell-mediated immune responses in normal and tumor-bearing control animals was reported. Treatment with APE and ANDLE significantly enhanced natural killer cell activity in normal and tumor-bearing animals. Antibody dependent cellular cytotoxicity was also increased in APE (45.17 % cell lysis on day 11) as well as ANDLE (39.92 % cell lysis on day 11)-treated normal and tumor-bearing animals (APE, 47.39 % cell lysis on day 11).

**Fig 2** GC-MS analysis of *Andrographis paniculata*
lysis; ANDLE, 41.48% cell lysis on day 11) compared to untreated tumor-bearing control animals (maximum of 11.76% cell lysis on day 17).

A few earlier workers have reported that the in vitro antiviral effect of A. paniculata crude extracts against influenza virus by Hemaggulatination (HA) reduction in two different layouts of simultaneous and post treatment assay. The aqueous and methanolic extracts were used for antiviral activity in the non-cytotoxic range. Methanolic extract showed 100% reduction in HA in the simultaneous and post treatment assays at the concentration of 10mg/ml. The aqueous extracts at concentrations of 10mg/ml and 5mg/ml reduced the HA to 33% and 16.67%, respectively, in the simultaneous assay. The methanolic extract showed high cytotoxicity against influenza virus that they had tested. But in the present study the extracts did not show any cytotoxicity at low concentrations (Rahul et al., 2014). Therefore, the plants’ extracts unlike that of the plants used in the previous study could be very useful in the treatment of infectious diseases at lower dosages.

GC-MS analysis was carried out on a GC-MS - 5975C AGILENT (GC-MS- QP 2010, SHIMADZU, JAPAN) system and Gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument. By GC-MS analysis it was found that methanol extract of Andrographis paniculata have large amount of 1. Benzo [1-3] thiazine-2-one, 8a-cyano-4-pentamethylene-perhydro, 2. 3-heptadecanone, 3. Pyrrolidine-1[7- o xo2,4,6-trimethylheptanoyl, 4. 1 hexene-1- butoxy2-ethyl, 5. 7H-indeno [2,1-a] antracen-7-one] probably these compounds may be responsible for observed antiviral activity. Several studies have proved that methanolic extracts of A. paniculata have antimicrobial and antiviral activity and found as a valuable medicinal plant in many popular traditional system of medicine (Pushpendra et al., 2013). The results of the present study also proved that the methanolic extract of the plant possess bioactive compounds exhibiting antiviral activity against Herpes Simplex Virus -2.

References


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