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Original Research Article

Anti herpes simplex virus-2 activity of the extracts of *Sericostoma pauciflorum* stocks ex wight and *Lepidagathis trinervis* Nees

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ABSTRACT

Aims: To determine the anti-HSV 2 properties and mechanism of action of two important medicinal plants *Sericostoma pauciflorum* Stocks ex Wight (Boraginaceae) and *Lepidagathis trinervis* Nees (Acanthaceae).
Methods and Results: To identify natural products with anti-HSV-2 activity, the aqueous and 50% ethanolic extracts were prepared from whole plants of *S. pauciflorum* and *L. trinervis*. Cytotoxicity assay using Vero cells as target revealed CC_{50} values of these extracts ranging from 301.8 to 436.8 $\mu g\ ml^{-1}$. Extracts from both the plants showed dose dependent inhibition of HSV-2 infection using Plaque Reduction Assay. Among the tested extracts, 50% ethanolic extract of *L. trinervis* showed lowest IC_{50} value of 6.86 $\mu g\ ml^{-1}$. To study the possible mode of antiviral action, all the extracts showed 50 to 97% reduction in the formation of plaques in the attachment assay. Extracts prepared from *L. trinervis* also inhibited the HSV-2 penetration in a time and dose dependent manner. In post-attachment assay, both the extracts from *S. pauciflorum* as well as 50% ethanolic extract from *L. trinervis* showed significant inhibition in HSV-2 infection.
Conclusions: These studies document anti-HSV-2 activity of the two commonly used medicinal plants that will have implications in the development of an alternate remedy for HSV-2 infection. Significant and impact of the study: This work provides information on the anti HSV-2 properties of Indian medicinal plants that showed their significant inhibition of HSV-2 virus in *in vitro* system.

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1. Introduction

Herpes simplex virus (HSV) is a double stranded DNA virus belonging to family *Herpesviridae.* It is classified into HSV-1 and HSV-2 subtypes. HSV-1 infection shows primary symptoms of fever, headache and in severe form leads to meningitis, encephalitis and keratoconjunctivitis. On the other hand, HSV-2 causes genital herpes, contributing to the risk of HIV infection through sexual route (Whitley 1990; Brugha et al. 1997; Severson and Tyring 1999).[1–3] HSV-2 is sexually transmitted virus and infect 500 million people worldwide and 23 million new infections are reported annually (Looker et al. 2008).[4] After the primary infection, HSV tends to persists in the neuron of the ganglia and get reactivated from its latent state during deficiency of immunity, causing serious systemic illnesses (Whitley 1990).[1] Since virus is an intracellular parasite in neuronal ganglia, it is difficult to completely eliminate it (Brugha et al. 1997; Severson and Tyring 1999; Looker et al. 2008).[2–4] Nucleoside derivatives such as acyclovir, valaciclovir, famciclovir and cidafovir have been widely used for the treatment of HSV infection (Cassady and Whitley 1997).[5] In addition to high cost, treatment with acyclovir and other related drugs is also associated with the emergence of drug resistant strains (Englund et al. 1990).[6] Long term treatment and mutations are major obstacles in

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the treatment of immunocompromised patients, who are also infected with other opportunistic infections (Seang et al., 2014).[7]

Natural products form medicinal plants have been an important source of new biologically effective compounds exhibiting different modes of action against viral infection (De Clercq 2000; Newman et al. 2000).[8,9] The present study was focused on to evaluate the anti-HSV-2 activity of two important traditionally used medicinal plants, *Lepidagathis trinervis* Nees (Acanthaceae) is a prostrate to sub-erect, up to 30 cm tall undershrub and its ashes were used to cure eczema (Singh et al., 2002).[10] *L. trinervis* shows anticancer activity against L1210 lymphoid leukemia and hypotensive effect (Iwu, 1993).[11] Antioxidant and antibacterial properties were also prove significant in this plant (Jain et al., 2010; 2012).[12,13] Immunosuppressive tryptophan-derived alkaloid was isolated from *L. cristata* (Ravikanth et al., 2001).[14]

Sericostoma pauciflorum Stocks ex Wight (Boraginaceae) is a short straggling undershrub growing widely throughout sea coast of Saurashtra and Maharashtra. Plant is generally used in dehydration and acidity. It is used in making an important drug in Ayurveda named "Krishnavalli". It is anticancer, antidiabetic, used in dehydration, acidity, and health promoting drug (as described in "Nighantu Ratnakar"). Phytochemically fernane, hopane and other type of triterpenoids were isolated from the plant (Afza et al., 1992; Ayatollahi et al., 1992).[15,16] Recent observations from our laboratory have documented anti-HIV activity, which is primarily mediated by inhibition of HIV-1 protease and Tat-long terminal repeat transactivation (unpublished observations).

Keeping in view that both plants possess effective anti-HIV properties, the 50% ethanolic and aqueous extracts prepared from whole plant of *S. pauciflorum* and leaves of *L. trinervis* were evaluated for anti-HSV-2 activity using *in vitro* plaque reduction assay. Subsequently, these extracts were also evaluated using different assay formats to determine at what stages of virus infection these may be acting.

2. Materials and Methods

2.1. Collection of plant materials and preparation of extracts

S. pauciflorum whole plant and *L. trinervis* leaves were collected from University campus, shed dried, grinded and strained through 30 mesh (0.5 mm). The botanical identity was confirmed by Herbarium, Department of Botany, University of Rajasthan, Jaipur. Voucher specimens of these plants, *S. pauciflorum* (110) and *L. trinervis* (64) has been deposited at the Herbarium and laboratory for further reference.

To prepare aqueous and 50% ethanolic extract finely grounded plant material (100 gm) was treated with 500 ml MilliQ water (60-75ºC for 6-8 h) and with ethanol: water (1:1 v/v; 500 ml at 25-30ºC overnight) respectively. The procedure was repeated three more times. The resultants were filtered through Whatman filter paper number 1, vacuum dried and lyophilized powder stored at -20ºC

2.2. Viruses and cell lines

Vero cells (a cell line from a normal adult African green monkey's kidney) were obtained from National Centre for Cell Science, Pune and grown on Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich Inc., St. Louis, MO, USA) supplemented with 10% Fetal bovine serum (FBS) and the antibiotic-antimycotic solution [penstrep - ampho sol; Penicillin (10000 units ml^{-1}), Streptomycin (10 $\mu g\ ml^{-1}$) and Amphotericin B (0.025 mg ml^{-1}) Biological Industries, Kibbutz Beit Haemek, Israel]. The cells were maintained at $37^{o}C$ in a humidified atmosphere of 5% CO_2. Herpes simplex virus type 2 (HSV-2) G strain, ATCC VR-734 (obtained from ATCC, Rockville, USA), was propagated in Vero cells. After three cycles of freeze/thaw, the supernatant was collected. The supernatant at increasing dilution was used to infect the Vero cells and plaque forming units (PFU) were determined as described previously (Burleson et al. 1992)[17] and virus stock stored in aliquots at -80°C until use.

Acyclovir (commercial name acycloguanosine) was purchased from Sigma-Aldrich Inc. It was dissolved in dimethyl sulphoxide (DMSO) and then diluted with sterile deionized distilled water before use. The final concentration of DMSO was <0.1%.

2.3. Cytotoxicity assay

Cytotoxicity of the plant extracts on Vero cells was assessed using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich Inc.] assay (Mosmann 1983).[18] Briefly, Vero cells (15×10^3/well 100 μl^{-1}) were seeded in a 96-well culture plate (Greiner Bio-One, GmbH, Frickenhausen, Germany) and grown overnight at 37°C in a humidified atmosphere of 5% CO_2. Next day, culture medium with increasing concentrations of various extracts was added in duplicate and further incubated for 72 h. Appropriate solvents, used to prepare various extracts were included as negative controls. After incubation, 10 μl of MTT reagent (5 mg ml^{-1}) was added per well, incubated at 37°C for 3 h followed by addition of MTT solvent (100 μl $well^{-1}$; 20% SDS and 50% dimethyl formamide in 50 mM PBS). The absorbance (OD) was read at 570 nm with reference filter at 690 nm. Percent cell viability was calculated by dividing the absorbance observed in experimental group by the absorbance in untreated group multiplied by 100. Values are expressed as

CC_{50} that represent the concentration of the extract showing 50% cell viability.

2.4. Plaque reduction assay

This assay was carried out using standard protocol as described previously (Shigeta et al. 1992).[19] Vero cells were grown in 24-well plate (FALCON 3046; 8×10^4cells well^{-1}) and incubated for 24 h at 37°C. After incubation, medium was removed, cells were inoculated with 300 μl of virus suspension (200 PFU per well) and the plates were further incubated for 1 h at 37°C under humidified atmosphere of 5% CO_2. After virus adsorption, excess viral suspension was removed by washing twice the cells with fresh medium and cells were overlaid with an agar overlay medium containing varying concentrations of the test compounds (6.25, 12.5, 25 and 50 μg ml^{-1}). Agar overlay with solvent used as negative control whereas the well containing Acyclovir (10 μM) was used as positive control. Each plant extract/compound was tested in duplicate in two separate experiments. After 72 h incubation, 24 well test plates were fixed with 10% formalin (in 50 mM PBS), followed by staining with 0.2% crystal violet. The number of plaques was counted. The average plaque count from duplicate wells at each concentration of test compound was plotted against the average plaque count of three untreated virus infected wells. The concentration required to reduce the plaque number by 50% (IC_{50}) was calculated from the mean dose response curves of at least two independent plaque reduction assays.

2.5. Attachment assay

Vero cells (80,000 cells well^{-1}) growing in 24 well plates were pre-chilled at 4°C for 15 min before experiment. Cells were infected with HSV-2 (200 PFU) in serum-free DMEM for definite time periods of 0.5, 1, 1.5 and 2.0 h at 4°C in the presence or absence of serial dilutions of plant extracts (6.25, 12.5, 25 and 50 μg ml^{-1}). Unadsorbed virus was then removed by washing with sterile 50 mM PBS pH 7.4, cells overlaid with medium and nutrient agar. After 72 h cells were fixed, stained and processed for counting the plaques as described above (Piret et al. 2002).[20]

2.6. Penetration assay

In penetration assays, HSV-2 (200 PFU) was adsorbed on Vero cells growing on 24 well plates for 2 h at 4°C. The medium was replaced with pre-warmed fresh medium containing plant extracts (6.25, 12.5, 25 and 50 μg ml^{-1}) and the temperature was increased to 37°C to maximize virus penetration. Cells were incubation for 0.5, 1, 1.5 and 2 h with plant extract. After incubation, cells were treated for 1 min with 50 mM PBS (pH 3.0) to inactivate the unpenetrated virus. After washing three times with serum-free medium, cells were overlaid with DMEM-0.5% agarose to quantitate surviving virus versus time of plant extracts treatment by counting plaque formation as described above (Piret et al. 2002) [20].

2.7. Post-attachment virus neutralization assay

Briefly, prechilled cells (2 h at 4°C) were incubated with HSV-2 (200 PFU) at 4°C for 2 h to allow stable attachment of virus without fusion with cell membranes. Medium was removed and plant extracts in DMEM were added in different concentration (6.25, 12.5, 25 and 50 μg ml^{-1}) and incubated further for 2 h at 4°C. Cells were then washed and overlaid with DMEM plus 0.5% agarose and incubated at 37°C for 24, 48 and 72 h in humidified atmosphere of 5% CO_2. For cell control, HSV-2 virus was incubated with serial dilutions of extracts for 2 h at 4°C prior to adsorption to cells (pre-attachment neutralization). Cells were treated as described earlier for plaque number reduction assay (Piret et al. 2002).[20]

2.8. Statistical analysis

The values are expressed as mean ± standard error of two independent experiments performed in duplicate. For determination of the IC_{50}value, nonlinear regression of concentration-response curves was prepared using GraphPad Prism 4 (GraphPad Software Inc., CA, USA).

3. Results and Discussion

3.1. Cellular toxicity

The inhibitory activity of the plant extracts against HSV-2 infection may be as a result of their toxic effect and therefore might result in an erroneous conclusion. Hence to exclude the non-specific antiviral effect, the toxicity of plant extracts on HSV-2 target Vero cells was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann 1983).[18] The CC_{50} values of 50% ethanolic and aqueous extracts prepared from *S. pauciflorum* and *L. trinervis* ranged from 301.8 to 436.8 μg ml^{-1} (Table 1).

3.2. Antiviral activity

The activity of aqueous and 50% ethanolic extracts prepared from the leaves of *L. trinervis* and whole plant of *S. pauciflorum* have been evaluated for their anti HSV-2 activity using plaque reduction assay (Shigeta et al. 1992).[19] These extracts have been tested at 4 concentrations ranging from 6.25 to 50 μg ml^{-1}, whereas their toxicity analysis was done from 62.5 to 500 μg ml^{-1}. The results are summarized in Table 1. Among the tested extracts, *L. trinervis* 50% ethanolic extract demonstrated lower IC_{50} value (6.9 μg ml^{-1}), whereas both 50% ethanolic and aqueous extracts of *S. pauciflorum* showed activity with IC_{50} value of 11.4 μg ml^{-1} and 13.8 μg ml^{-1}, respectively (Table 1). Acyclovir

was used as standard reference drug and at 10 μM was completely inhibiting plaque formation by HSV-2 virus. The therapeutic index (TI) of a drug is the ratio between the toxic and the therapeutic dose and is used as a measure of its relative safety. The 50% ethanolic leaves extract of *L. trinervis* was more potent against HSV-2 as TI of the extract was 44.0 (Table 1).

3.3. Attachment assay

According to the results of time course studies, virus attachment was significantly inhibited both the extracts of *S. pauciflorum* (IC_{50} values of 26.8 to 7.6 μg ml^{-1} by 50% ethanolic and 69.2 to 20.7 μg ml^{-1} by aqueous extracts, respectively; Figure 1). Further, both 50% ethanolic and aqueous extracts from *L. trinervis* also inhibited the attachment of HSV-2 to Vero cells in a dose dependent manner. After exposure of Vero cells with *L. trinervis* extracts (50 μg ml^{-1}) for either 1.5 or 2.0 h at 4°C, ≥ 90% inhibition in the attachment of HSV-2 was observed. In contrast, acyclovir which inhibits HSV replication, failed to show any significant inhibition in the virus attachment to Vero cells when tested up to 10 μM (data not shown).

Anti-HSV activity of natural products from other plants has been shown previously (Nawawi et al. 1999, 1999, 2001).[21–23] Antimicrobial, antioxidant activities were evaluated and α Amyrin, β Amyrin, β-Sitosterol, friedelin and caffeic acid were isolated form *S. pauciflorum* (Pancholi, 2022).[24] The interaction between legend and receptor is a complex step involved in virus infectivity, and our results showed that both 50% ethanolic and aqueous extracts effectively inhibit attachment of the virus particle.

3.4. Penetration assay

In penetration assay, plant extracts were added to the Vero cells after the initial binding of HSV-2 at 4°C. Both 50% ethanolic and aqueous extracts of *S. pauciflorum* failed to inhibit penetration in *in vitro* assay system (data not shown). Previously *Cordia americana* of family Boraginaceae were prove effective against HSV-2 viral penetration (Costa et al. 2012)[25] but due to antagonistic effects of some other complex phytochemicals, *S. pauciflorum* plant extracts (50% ethanol as well as aqueous) does not show inhibitory activity on virus penetration. According to previous studies, caffeic acid derivative reduce penetration of HSV via disturbing viral glycoproteins (Ikeda et al. 2011).[26]

L. trinervis extracts showed activity in dose and time dependent manner (Figure 3). After virus adsorption, Vero cells treated with 50% ethanolic extract for 2 h at 37°C showed an IC_{50} value of 12.6 μg ml^{-1}, which was more effective then aqueous extract (IC_{50} value of 33.7 μg ml^{-1}; Figure 3). *Clinacanthus nutans* and *C. siamensis from family Acanthaceae show potent inhibition of HSV* . Monoglycosyl diglycerides and digalactosyl diglyceride were the active ingredients that possess the anti-viral potentials (Janwitayanuchit et al., 2003; Kunsorn et al., 2016).[27,28] This may be one of the possible reasons for the efficacy of both the extracts of *L. trinervis* in inhibition of HSV virus penetration.

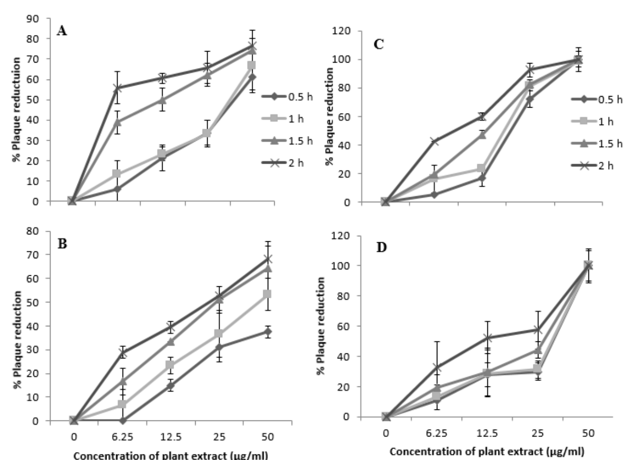

Fig. 1: Effectsof *S.pauciflorum* and *L. trinervis* 50% ethanolic (**A** and **C**) and aqueous extracts (**B** and **D** respectively) on attachment assay of HSV-2: Prechilled Vero cells were infected with 200 PFU of HSV-2 in presence or absence of serial dilution of the extracts at different time period at 4°C. *S. pauciflorum* 50% ethanolic extract showed 76.32% inhibition at 50 μg ml^{-1} concentration. On the other hand aqueous extract demonstrated 68.42% inhibition at 50 μg ml^{1} concentration with respect to cell control. *L. trinervis* both extract showed ≥ 90% inhibition at 50 μg ml^{-1} concentration. The results showed that HSV attachment was significantly inhibited at concentration and time dependent (IC_{50} values 7.56 and 11.74μgml^{-1} in 50%ethanolic and aqueous extract respectively at 50 μg ml^{-1} concentration).

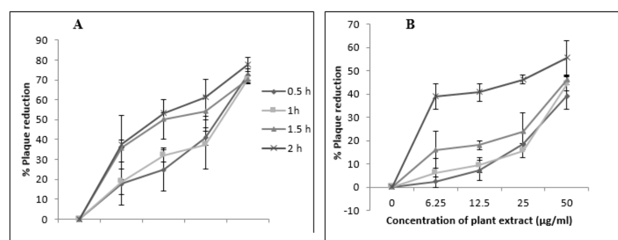

Fig. 2: Effects of *L. trinervis* 50% ethanolic (**A**) and aqueous extracts (**B**) on penetration assay of HSV-2: Prechilled Vero cells were infected with 200 PFU of HSV-2 for 2 h at 4°C. After removal of unbound virus cells were shifted to 37°C in presence and absence of serial dilution of extract. Test results shown dose dependent inhibition of plaque formation (77.78 and 55.56% inhibition in 50% ethanol and aqueou sextract respectively). Reduction in IC_{50} value was time dependent 12.61 and 33.74 μg ml^{-1} for 50% ethanol and aqueous extract respectively.

Table 1: In vitro cytotoxicity and anti-HSV-2 activity of the extracts derived from stem bark of *S. pauciflorum* and leaves of *L. trinervis*

Plants tested	Extracts used	CC_{50} (μg/ml)*	IC_{50} (μg/ml)*	TI
S. pauciflorum	50% ethanol	319.9	11.4	28.0
	Aqueous	334.2	13.8	24.3
L. trinervis	50% ethanol	301.8	6.9	44.0
	Aqueous	436.8	21.0	20.8

*CC_{50}: The cytotoxic concentration of the extracts that caused the reduction of viable cells by 50%. All data presented are averages reading of two independent experiment performed in duplicate.

*IC_{50}: The concentration of the extracts that resulted in 50% inhibition in HSV-2 infection. All data presented are averages of duplicate readings and of two independent experiments.

TI: Therapeutic index is CC_{50}/IC_{50}

3.5. Post-attachment virus neutralization assay

In post attachment assay, a dose dependent inhibition of HSV-2 infection was recorded in both the extracts of *S. pauciflorum* (Figure 3). The ethanolic extract was more effective as compared to aqueous extract (71.5 and 31.5% inhibition at 50 μg ml^{-1}concentrationrespectively) at 72 h incubation period (Figure 3 A, 3B). However, 85.5% inhibition by 50% ethanolic extract from *L. trinervis* was observed after 72 h (Figure 3C), whereas aqueous extract did not show positive results (data not shown). Due to higher amounts of polyphenols and flavonoids in 50% ethanolic extracts may be one of the reasons of its higher activity on compared to aqueous extract (rich in saponins and triterpenoids; Pancholi, 2022).[23]

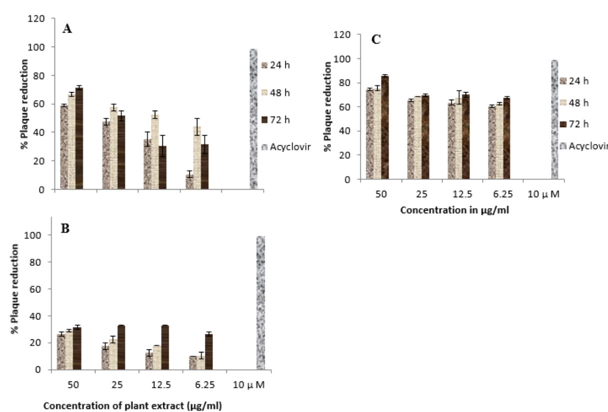

Fig. 3: Effects of *S. pauciflorum* 50% ethanolic (**A**) and aqueous extracts (**B**) and *L. trinervis* 50% ethanolic extracts (**C**) on post-attachment assay of HSV-2: Prechilled Verocells were infected with 200 PFU of HSV-2 for 2 h at 4°C and after the removal of unbound virus cells were incubated with serial dilution of extract in different time period at 4°C. *S. pauciflorum* 50% ethanolic extract showed 71.5% inhibition at 50μg ml-^{1} concentration on 72 h incubation. On the other hand aqueous extract demonstrated 31.5% inhibition at 50 μg ml^{-1}concentration with respect to Acyclovir (98.78% at 10μM concentration). *L. trinervis* 50% ethanolic extract showed 85.55% inhibition at 50 μg ml^{-1} concentration. However, aqueous extract does not show dose dependent inhibition upto 50 μg ml^{-1} concentration with respect to cell control (data not shown).

4. Conclusions

The present study has demonstrated that 50% ethanolic and aqueous extracts prepared from *S. pauciflorum* and leaves of *L. trinervis* possess potent *in vitro* activity against HSV-2. The TI values suggest that the antiviral activity of these plant extracts was not due to the cellular cytotoxicity. The 50% ethanolic extract from *L. trinervis* inhibit attachment of virus to host cells, prevent virus penetration and reduces HSV-2 infection even after virus attachment. These studies are encouraging and may help in devising alternate strategies for the treatment of HSV-2 infection. Further isolation of key compounds responsible for the Anti-HSV property is in active progress.

5. Source of Funding

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6. Conflict of Interest

None.

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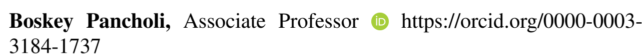
Author biography



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