

ANTIVIRAL ACTIVITY OF THE INDIAN MEDICINAL PLANT EXTRACT, *SWERTIA CHIRATA* AGAINST HERPES SIMPLEX VIRUSES: A STUDY BY *IN-VITRO* AND MOLECULAR APPROACH

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Abstract

Purpose: The antiviral activity of Indian Medicinal plant extract *Swertia chirata* was tested against Herpes simplex virus (HSV) type-1, using multiple approaches both at cellular and molecular level. **Methods:** Cytotoxicity, plaque reduction, virus infectivity, antigen expression and polymerase chain reaction (PCR) assays were conducted to test the antiviral activity of the plant extract. **Results:** *Swertia* plant crude extract (1gm/mL) at 1:64 dilution inhibited HSV-1, plaque formation at more than 70% level. HSV antigen expression and time kinetics experiments conducted by indirect immunofluorescence (IFA) test, revealed a characteristic pattern of small foci of single fluorescent cells in *Swertia* extract treated HSV-1 infected cells at 4 hours post infection dose, suggested drug inhibited viral dissemination. Infected cell cultures treated with *Swertia* extract at various time intervals, tested by PCR, failed to show amplification at 12, 24-72 hours. HSV-1 infected cells treated with Acyclovir (antiviral drug) did not show any amplification by PCR. **Conclusions:** In this preliminary study, the Indian medicinal plant extract, *Swertia chirata* showed antiviral properties against Herpes simplex virus type-1.

Keywords: Antiviral activity, cytotoxicity assay, herpes simplex virus, immunofluorescence, polymerase chain reaction

Medicinal plant products have been used as folk remedies for different kinds of ailments including viral diseases.^[1] There is a need to search for new compounds for treatment of viral infections since there is an increasing resistance to antiviral drugs.^[2] The problems of viral resistance and viral latency leading to recurrent infections in immunocompromised patients has been documented earlier.^[3-5] Recently, a number of medicinal plant products have been shown to have antiviral activity.^[6-7] Traditional plant extracts having anti-infective properties, have been screened for their antiviral activity.^[8]

Acute and recurrent herpes simplex virus (HSV) infections are distributed worldwide and cause wide range of diseases from mild to severe and in some cases they may become life threatening in immunocompromised patients.^[4,9,10] Also, several antiviral compounds have been tried as therapeutic use in earlier decades.^[11,12] Nucleoside derivative drugs such as acyclovir (AVC), gancyclovir (GCV) and pencyclovir have been widely approved drugs for the treatment of HSV infections.^[7] However, widespread use of these drugs has shown resistance especially in immunocompromised and bone marrow transplant recipients.^[3,10] In order to circumvent the problem of viral resistance, development of new antiviral products with different mechanism of action are very much required.

The present study describes the antiviral activity of the Indian medicinal plant extract, *Swertia chirata* against herpes simplex virus type-1 (HSV-1) using multiple approaches.

Materials & Methods

Cells and virus stocks

Vero E6 cells were obtained from cell repository of tissue culture department, National Institute of Virology, Pune, India. The cells were grown in Eagles minimum essential medium (EMEM) containing Earles salts, L-glutamine, non-essential amino acids (Hi-Media Co., Mumbai India), Sodium bicarbonate, Sodium pyruvate (Sigma Chem. Co., St. Louis, USA), 10% heat inactivated foetal calf serum (Gibco BRL Co., Germany) and antibiotics, (penicillin (100 IU/mL) streptomycin (100 µL/mL). HSV-1 strain was obtained from the virus repository of National Institute of Virology (NIV), Pune, India. Virus stocks were propagated in Vero E6 cells and used at a concentration of $10^{6.5}$ TCID₅₀ in all *in vitro* experiments.

Standard antivirals

Acyclovir (Sigma Chem. Co., St. Louis, USA) dissolved in distilled water and used as a standard antiviral drug (1 mg/mL) with respect to test compounds at a concentration of 500 µg/mL (1:2) to 3.9 µg/mL (1:256).

Preparation of plant extracts

Water extracts were prepared from dried powder of

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Table 1: Plaque inhibition of *Swertia*

Virus	Drug	Effective dilution of drug used	Titre (PFU/0.1mL)		% Reduction of plant extract in log titre
			Untreated	Treated	
HSV-1	<i>Swertia</i>	(1:64)	15×10^4	2×10^4	70
	Acyclovir	0.1mg/mL (1:10)	12×10^4	0	100

leaves and stem of *Swertia chirata* of family *Renunculaceae*. Ten grams of powder was mixed in 100 mL of distilled water and kept at 37°C overnight and filtered with Whatman filter paper #1, 1 μ , 0.45 μ , 0.22 μ filters. After filtration, *Swertia* extract was concentrated ten folds by using vacuum concentrator (Stock of 1 gm/mL). *Swertia* plant extract at a concentration of 500 mg/mL, 1:2 dilution and further double dilutions were made upto 1:1024 for conducting different assays.

Cytotoxicity assay

To evaluate the cytotoxicity of test compounds the following experiments were carried out. In the first experiment quadruplicate wells of confluent monolayers of vero-E6 cells were grown in 96 well tissue culture plates. Cells were incubated with various concentrations of the test compounds and cell viability was examined by ability of the cells to cleave the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide], Sigma Chem., Co. St. Louis, USA], by the mitochondrial enzyme succinate dehydrogenase which develops a formazan blue colour product and the procedure was followed as described earlier.^[13]

The 50% cytotoxicity concentration (CC₅₀) was defined as the test compound concentration required for reduction of cell viability by 50% and CC₅₀ values were calculated by regression analysis. In the second experiment plating efficiency was checked with the subtoxic dose of test compound.

Plaque reduction assay

Vero E6 monolayer cells grown in 24 well tissue culture plates were infected with HSV-1 with 10 PFU/0.1 mL. Virus adsorption was carried out for 1 hour at 37°C in the presence or absence of test compound. Virus dilutions were prepared in MEM(E) consisted of 1:1 (2x MEM). Cells were overlaid with carboxy methyl cellulose (CMC), 2x MEM containing plant extracts and infected cell cultures were incubated at 37°C, CO₂ incubator for 24-48 hours. The infected cells were stained and observed for plaque reduction as described earlier.^[14]

Virus infectivity and antigen expression assay

Vero E6 cells cultured in 24 well plates were

inoculated with effective dose of test compound and acyclovir along with various dilutions of HSV-1. The infected cell cultures were incubated further at 37°C and observed up to 72 hours. Acyclovir treated HSV-1 infected culture was used as a control. Vero E6 cells grown and infected with HSV-1 (10⁻³ dilution) with an effective dose of test compound were incubated at 37°C for 3 days. Culture supernatant was removed by scraping the cells after 0, 4, 12, 24, 48, and 72 hours intervals. Smear was prepared on cavity slides after washing with phosphate buffered saline (PBS, pH 7.2). Cells were fixed with chilled acetone for 10 minutes at 2-8°C and incubated with FITC labeled anti-HSV-1 mouse monoclonal antibodies at 1:1000 dilutions (Light diagnostics Chemicon international, UK) and observed under fluorescent microscope.^[15]

Polymerase chain reaction (PCR)

DNA extracted from the culture supernatant (drug treated/untreated) was subjected to PCR using HSV-1 type specific primers (RL2 region) as described earlier.^[16]

Results

Cytotoxic effect of *Swertia*

Cytotoxic concentration (CC₅₀) of the test compound was determined by conducting MTT assay. The CC₅₀ value of *Swertia* was calculated at 1:54 dilution. The CC₅₀ value calculated by regression analysis and the biphasic curve showing percent cell viability by MTT assay was represented in fig. 1. In the plating efficiency assay toxicity of cells was checked by serial passaging of the cells for three times and no effect on cell growth was noticed in the treated cells.

Plaque inhibition of *Swertia*

The inhibitory effects on the plaque formation of HSV-1 with *Swertia* plant extract was carried out by plaque reduction assay. For this purpose, 10^{6.5}TCID₅₀ dilution of virus stock was seeded with various dilutions of plant extract (1:32 to 1:256 and also subtoxic dilution, 1:54) and observed for plaque inhibition. *Swertia* plant extract at 1:64 dilution inhibited HSV-1 plaque formation at more than 70% inhibitory level (Table 1).

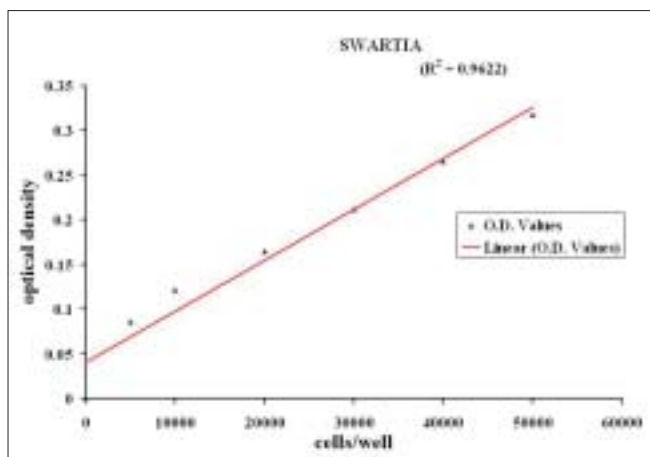


Figure 1: Correlation of percent cell viability and *Swertia* concentration by MTT assay

Virus infectivity and antigen expression

To determine the time kinetics of *Swertia* extract on HSV-1 antigen expression, indirect immunofluorescence assay was carried out. Infected Vero E6 cells were treated with *Swertia* plant extract (1:64 dilution) at 4, 8 and 24 hours of post infection dose (PID) and results were represented in Fig. 2a, 2b, 2c, 2d. Maximum reduction in number of fluorescent cells was observed at 4 hours PID, a characteristic pattern of small foci of positive cells and even single fluorescent cells observed suggested the drug inhibited viral dissemination (Fig.2c). *Swertia* plant extract subsequently added at 8 and 24 hour PID showed a significant reduction of positive fluorescent cells (Fig.2d). Also, HSV-1 infected cells treated with acyclovir indicated a gradual reduction of infected cells and a complete inhibition was observed at a concentration of 0.1 mg/mL compared with controls (Fig.3a, 3b, 3c).

Amplification of *Swertia* treated HSV-1 infected cells by PCR

HSV-1 infected cell cultures treated with *Swertia* extract at various time intervals (0, 12, 24, 48, 72 hours) were harvested. Nucleic acid (DNA) extracted from the tissue culture fluid (TCF) was subjected to PCR using HSV-1 type specific primers. Drug treated infected cultures at 12 and 24-72 hours duration failed to show any PCR amplification (Fig. 4a, lanes 11-15). However HSV-1 infected cultures showed a 147 bp product in 2% agarose gels at 24, 48, 72 hour of time period (Fig. 4a, lanes 6-10). Acyclovir treated virus cultures did not show any amplification (Fig. 4a, lanes 16-20) and HSV-1 virus control showed amplification (Fig. 4a, lane-5). Also, antiviral activity of various concentrations of acyclovir against HSV-1 (10^{-3}) was carried out. Acyclovir treated HSV-1 infected cells at low concentration showed PCR amplification (Fig. 4b, lanes 10-12). In infected cells, amplification of β -globin gene (internal control) indicated

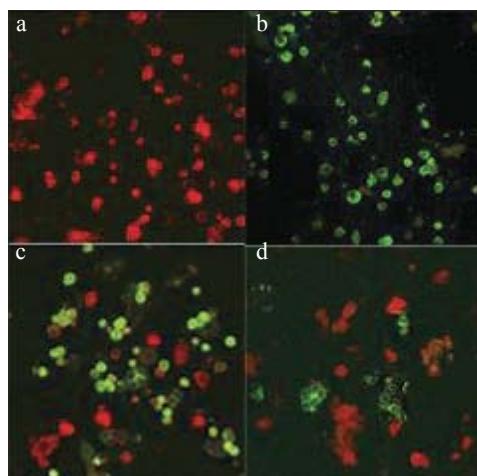


Figure 2: Effect of *Swertia* extract on HSV-1 antigen expression (Immunofluorescence assay) (a) Cell Control, (b) Virus Control, (c) Infected cells at 4hrs PID, (d) Infected cells at 24 hours PID

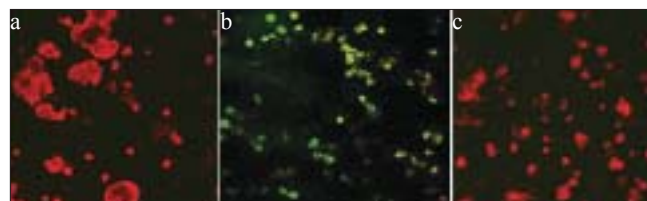


Figure 3: Effect of *Swertia* extract on HSV-1 antigen expression (Immunofluorescence assay) (a) Cell Control, (b) Virus Control, (c) Infected cells treated with acyclovir (0.1 mg/mL)

the integrity of the gene. A PCR product of 246 bp was identified in 2% agarose gel indicative of β -globin gene (Fig.4c, lanes 3-6).

Discussion

The present study was carried out to test the antiviral activity of medicinal plant extracts *Swertia chirata* of family *Renunculaceae* against herpes simplex virus type-1 (HSV-1) using cytotoxicity assay, plaque reduction assay, virus infectivity and antigen expression assay and PCR based approach. Antiviral activity has been reported earlier on plant products against DNA viruses, including herpes viruses.^[5,6,17-19] However, antiviral activity of plant products is so far been tested by using cytotoxic/plaque reduction assays.^[6,15] In this study, Indian medicinal plant i.e., *Swertia chirata* showed antiviral properties against HSV-1. Basic experiments conducted such as, plaque reduction assay and time kinetics of HSV-1 antigen expression showed that *Swertia* plant product has a potential to have antiviral activity as compared to acyclovir drug treated virus control. Similarly, non-amplification of *Swertia* drug treated HSV-1 infected cells by PCR further complemented and strengthened the antiviral activity of *Swertia chirata*. Detection and amplification of β -globin gene (control gene) in drug treated and virus infected cells indicated that there was no cytotoxicity observed after treatment of cells with plant products.



Figure 4a: Detection of HSV-1 in drug treated and untreated (*Swertia*) by PCR, Lane 1: 50 bp Marker, Lane 2: PCR control, Lane 3: cell control, Lane 4: cell+drug control, Lane 5: Positive control (HSV-1), Lanes 6-10: cell+virus (10^3) 0 hr., 12 hrs., 24 hrs., 48 hrs., 72 hrs., Lanes 11-15: cells + drug + virus (10^3), 0 hr., 12 hrs., 24 hrs., 48 hrs., 72 hrs., Lanes 16-20: cells +virus+drug (Acyclovir), 0 hr., 12 hrs. 24 hrs., 48 hrs., 72 hrs.



Figure 4b: Antiviral activity of acyclovir against HSV-1 (10^3) by PCR, Lane 1: 50 bp marker, Lane 2:PCR control, Lane 3: Cell Control, Lane 4: 500 µg \ml, Lane 5: 250 µg \ml, Lane 6: 125 µg \ml, Lane 7: 62.5 µg \ml, Lane 8: 31.25µg\mL, Lane 9: 15.6 µg\mL, Lane 10: 1.25µg\mL, Lane 11: 7.8 µg\mL, Lane 12: 3.9µg\mL

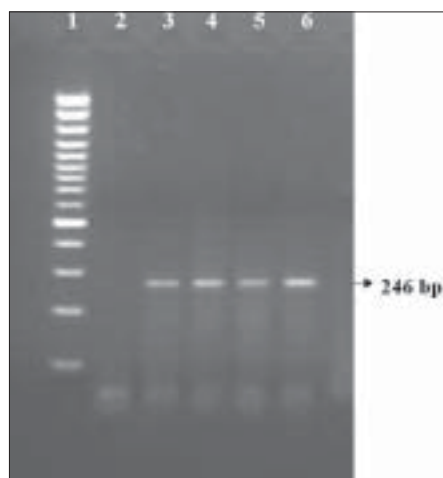


Figure 4c: Amplification of β -Globin gene in *Swertia* treated cells. Lane 1: 100 bp Marker, Lane 2: PCR control, Lane 3: cell+drug 12 hrs., Lane 4: cell+drug, 24 hrs, Lane 5: cell+drug, 48 hrs, Lane 6: cell+drug., 72 hrs.

In conclusion, this is a preliminary report on antiviral activity of *Swertia chirata*, an Indian medicinal plant against HSV-1. Further studies are required to know the mechanism of action using suitable animal models.

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