In-Vitro Antiviral Screening of Sphaeranthus Indicus Linn Leaf Extract against Herpes Simplex 1 & 2 Viruses

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ABSTRACT

Background: Many human skin diseases have been attributed to viral etiology. Few of them are well recorded from ancient times and remedies have been described in traditional medicines. Due to mutation rate of viruses which allowed them to rapidly and continuously develop, as well as create new strains that are resistant to the existing commercially available antivirals. There is an increasing demand for new antivirals due to drug resistance and mutations, development of new targeted antiviral drugs is in need to control these viral infections.

Objectives: The aim of the present work was studying the antiviral action of the herb sphaeranthus in their original form of therapeutic formulation administered traditionally.

Methods and materials: Aqueous and ethanolic extracts of *Sphaeranthus indicus* were evaluated for antiviral activity against Herpes simplex 1& 2 virus by micro tissue culture assay (MTCA).

Results: The results showed that a minimum concentration of 125 and 75 µg of both aqueous and ethanolic sphaeranthus extracts exhibited anti HSV-1 & 2 activities.

Conclusion: The aqueous and ethanolic extracts of sphaeranthus showed recordable antiviral activity against HSV 1 and 2 viruses.

Keywords: Anti HSV-1, Anti HSV - 2, Aqueous extracts, Ethanolic extracts, Herbal extracts

INTRODUCTION

Various human skin ailments have been attributed to viral etiology[1]. Few of them are well recorded from ancient times and remedies have been described in traditional medicines through the civilizations[2]. The development of targeted antiviral drugs has not been able to keep a check in controlling these virus infections while there is an increasing demand for new antivirals due to drug resistance and mutations observed. This is probably because of the innate morphological and physiological attributes of the virus[3,4]. The exact mechanisms of the herbal products including secondary metabolites exhibiting virucidal actions have not been determined due to multiple metabolite involvement from the crude formulations used in the therapeutical applications[5]. Most of the drugs currently showing therapeutic promise against viral diseases are synthetic compounds[6]. The traditional medical practitioners of Unani, Siddha, Ayurveda, Folklore and tribal medicine have successfully used plants and herbs for treatment of many diseases without known their viral etiology[7]. Sphaeranthus indicus is a well-known Asian herb widely used for treatment of various diseases like epilepsy, leprosy, skin diseases, hemicrania, jaundice, hepatopathy, diabetes, mental illness, fever, pectoralgia, cough, gastropathy, hernia, hemorrhoids, helminthiasis and dyspepsia[8]. Further, recent studies have identified the antioxidant/anti-inflammatory[9] properties of sphaeranthus extracts with additional hepatoprotective[10] and immunomodulatory activities[11]. Sphaeranthus plants are commonly found in the hilly semi-arid regions of south western Saudi Arabia and also from India to Australia[12].

Herpes Simplex, a DNA virus is known to present with varying symptoms of skin/oral lesions to encephalitis[9]. The morbidity of HSV 1 and 2 is a high concern in young adults and has been more implicated with the HIV infection[13]. Though antiviral drugs are available for HSV 1 and 2 like acyclovir, valacyclovir and famciclovir, their residual toxicity and resistance are widely reported[14]. Therefore, in the current study we investigated the antiviral activity of aqueous and ethanolic extracts of sphaeranthus leaves in-vitro on Vero cell line by micro tissue culture assay (MTCA). The effective concentration possessing the maximal anti-viral activity was determined by the standard cell viability assay.

MATERIALS AND METHODS

Cell Line: The Vero cell line was retrieved from department of Microbiology cold storage and propagated and maintained in DMEM supplemented with 10% Fetal Calf Serum (FCS) supplemented with 100 U/ml penicillin, and 100 µg/ml streptomycin, 2 mM L-glutamine, 2.5 µg/ml amphotericin B in 25 cm^2 Tissue culture flask.

HSV 1 and 2 Viruses: Clinical strains of HSV 1 and 2 viruses were retrieved from department of Microbiology Virology section. The virus strain was grown in Vero cells in 25 cm^2 Tissue culture bottles. $TCID_{50}$ was determined by standard procedure[15]. $TCID_{50}$ of $10^{5.7}$ for HSV-1 and $10^{5.5}$ for HSV-2 were stocked in -80°C till further use. The working stock virus was stored at –20°C for antiviral assay.

Preparation of 24 Well Microtiter Plates for Toxicity and Antiviral Assay: Briefly TC bottles with complete monolayer of Vero cells were washed, trypsinized, harvested and seeded in 24 well TC plates at a concentration of 10^5 cells /ml. The plates were incubated for 24 hours

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at 37°C in 5% CO_2. The well with complete monolayer were selected for toxicity and antiviral screening assay[16].

Herbs and Extracts: The herbs *Sphaeranthus indicus*, was collected and identified by Taxonomist. Fresh leaves of the herbs were cleaned, shade dried and coarsely pounded. The powder was stored in dark airtight containers for further extraction. Extraction was done by standard procedure with modification to the local laboratory standards[17]. Briefly 1 gm of the prepared powdered leaves was soaked in 100 ml of distilled water and 100 ml of absolute ethanol separately. The mixture was kept in the rotary shaker for 48 hours. The contents were filtered through muslin cloth and the filtrate was dried at 55°C. The sediments were re-extracted as mentioned above. The dried extract was scraped and stored at 4°C in airtight vials. Working concentrations of extracts ranging from 10 micrograms to 1 mg were prepared fresh and filter sterilized through 0.45 microns filter before each assay.

Cytotoxicity Assay: *Briefly the growth medium from 24 well plate prepared with Vero is decanted and washed once with PBS. Increasing concentration of herbal extracts (from 5µg to 1 mg concentrations/ml of DMEM W/O FCS) was added in tetrads. Cell control containing extract free medium, solvent control and neat extract controls were included. The plates were incubated at 36°C in 5% CO_2 environment, were examined for cytotoxicity at 3rd, 5th and 7th day of incubation. The highest concentration of drug showing no cytotoxic effect was recorded by microscopic examination for the morphological changes induced by extracts[18] and MTT assay as previously described elsewhere[19].*

Direct Pre- Infection Incubation (DPI) Assay or Virus Inactivation Assay: *This assay was performed with minor modifications from method described else were. The filtered extracts (100mg/10ml) were first diluted to nontoxic concentration of 5-400 µg/0.5ml and 5-300 µg/0.5ml for aqueous and ethanolic extracts respectively. To three aliquots of each concentration of each type of extract, 500µl of 1, 10, 100 $TCID_{50}$ of HSV 1 and 2 viruses were added, vortexed and incubated for 30 minutes at 37°C. 100µl was overlaid on four wells of 48-hour monolayer of Vero culture after the incubation period. Cell control, solvent control, extract control and virus control were included in the assay. The plates were incubated for seven days at 37°C in 5% CO_2 environment. The plates were observed on day 3,5 and 7[20,21]. The effect of virus inactivation with reference to the $TCID_{50}$ of the virus used for the assay was determined by macroscopic observation for CPE followed by MTT assay.*

Statistical Analysis: Mean and standard deviation were calculated for MTT assay from the GM values the results of toxicity and antiviral assay were interpreted. One-way ANOVA, Tukey was used to determine the significance ($p < 0.05$).

RESULTS

The solvents used for the extraction and dilution (Distilled water 70% ethanol and 0.25% DMSO) of the extracts did not show any observable residual toxicity to Vero cells. The nontoxic concentration of aqueous and ethanolic extracts of sphaeranthus determined by the cytotoxicity assay were used for in-vitro antiviral activity against 1,10 and 100 $TCID_{50}$ of HSV 1 and 2 viruses (Table 1). Concentrations of ≤400µg of aqueous and ≤ 300µg ethanolic extracts of sphaeranthus were observed to be nontoxic to Vero cells. The cytotoxicity was confirmed by MTT assay (Figure 1).

Out of the various concentrations of the extracts tested, a minimum concentration of 125µg of aqueous sphaeranthus extract showed complete inhibition of 10 $TCID_{50}$ of HSV 1 virus while partial

Table 1: Results of cytotoxicity assay for Sphaeranthus extracts on Vero Cells

S.N	Name of the Herbal Extracts	5 µg to 100µg	200µg	300µg	400µg	500µg	600µg	800µg	900µg	1 mg	Neat
1	Sphaeranthus indicus aqueous extract	NT	NT	NT	+/-	T	T	T	T	T	T
2	Sphaeranthus indicus ethanolic extract	NT	NT	+/-	+/-	T	T	T	T	T	T

(NT - Nontoxic, T - Toxic, +/- means Equivocal)

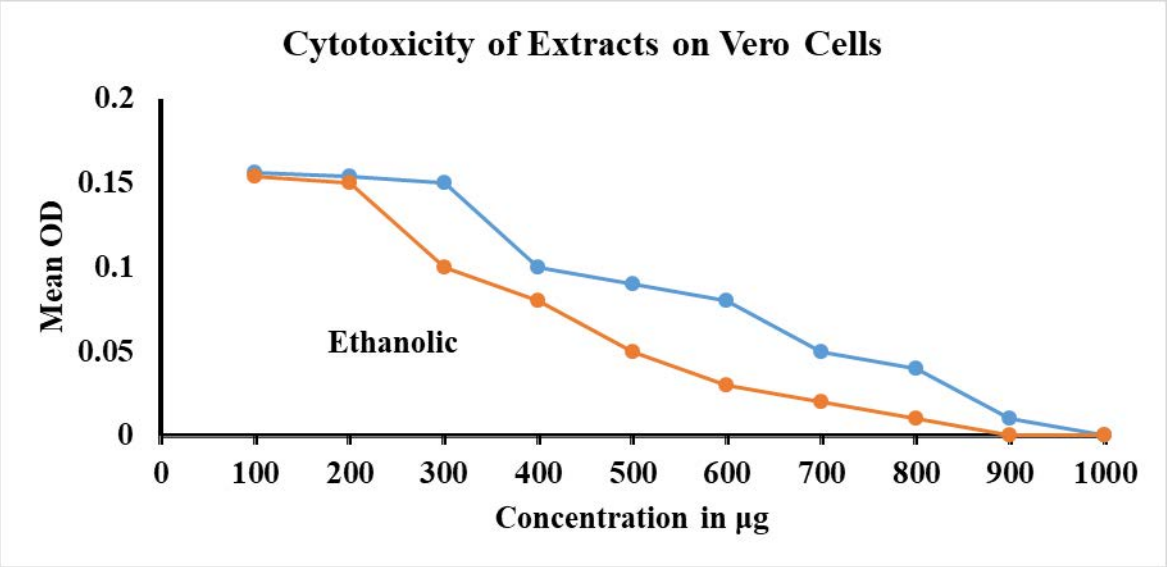

Figure 1: Cytotoxicity pattern of varying concentration of the aqueous and ethanolic extracts of Sphaeranthus on Vero cell line

inhibition of 100 $TCID_{50}$ HSV1 virus. HSV 2 virus was completely inactivated at 125µg. On the other hand, 75µg ethanolic extract of sphaeranthus showed complete inhibition of 100 $TCID_{50}$ HSV 1 and 2 viruses (Table 2).

Residual viral titer was observed with minimum concentration of 125µg of aqueous sphaeranthus extract as determined by MTT assay (Figure 2) which was able to inhibit only 10 $TCID_{50}$ of the virus but not completely 100 $TCID_{50}$. A steep fall of HSV 1 titer from $10^{1.5\pm0.02}$ was observed against the initial titer of $10^{6.7}$ (Table 3).

DISCUSSION

The HSV 1 and 2 virus inhibition on Vero cells by aqueous and ethanolic extracts of sphaeranthus was concentration dependent. This can be evidenced by the partial inhibition of virus i.e., Low titer and reduced titer observed in few concentrations of the extract incubated with the virus. The complete inhibition of HSV 1 and 2 viruses by virus inactivation assay clearly indicates that the drug has virucidal activity. This is in concordance with few of the other studies done with plant extracts[21,22]. However, the dose of the extracts which showed partial inhibition indicates that the extracts are not only virucidal but they are also acting as inhibitory to viral replication or virus protein translation or post viral translational assembling which needs to be investigated further[23]. Since the concentration more than 300µg of ethanolic sphaeranthus extract was toxic to Vero cells, higher concentrations of this extracts for anti HSV 1 & 2 activity have to be studied in vivo. In a nutshell the virus inactivation assay showed lower concentration of the sphaeranthus extracts were potent virus inhibitors and can be further evaluated for prophylactic effect on the HSV 1 and 2 infections

Table 2: Results of antiviral screening of aqueous and ethanolic extracts of Sphaeranthus by Virus inactivation assay

Extracts	Conc (µg)	HSV- 1			HSV-2		
		1TCID $_{50}$	10TCID $_{50}$	100TCID $_{50}$	1TCID $_{50}$	10TCID $_{50}$	100TCID $_{50}$
Aqueous	5 to 100		NA			NA	
	125	A	A	P/A	A	A	A
	150 to 400	ND	ND	A	ND	ND	A
Ethanolic	5 to 50		NA			NA	
	75	A	A	A	A	A	A
	100 to 300	ND	ND	A	A	A	A

Results based on the microscopic observation for cytopathic effect (CPE) compared to control cells (NA – No Activity, A – Active, P/A – Partially Active, ND – Not Done).

Table 3: Determination of the Viral titer from the supernatant after 7th day post virus inactivation assay

Extract Concentrations (µg)	HSV1 ($10^{6.7}$) 100 $TCID_{50}$		HSV 2 ($10^{5.8}$) 100 $TCID_{50}$	
	Aqueous	Ethanolic	Aqueous	Ethanolic
50	$10^{6.0\pm0.02}$	$10^{2.7\pm0.03}$	$10^{5.7\pm0.01}$	$10^{3.7\pm0.02}$
75	$10^{6.5\pm0.02}$	$10^{1.5\pm0.02}$	$10^{5.4\pm0.05}$	0
100	$10^{5.6\pm0.04}$	0	$10^{3.9\pm0.02}$	0
125	0	0	0	0
150	0	0	0	0

0 – Nil Titer

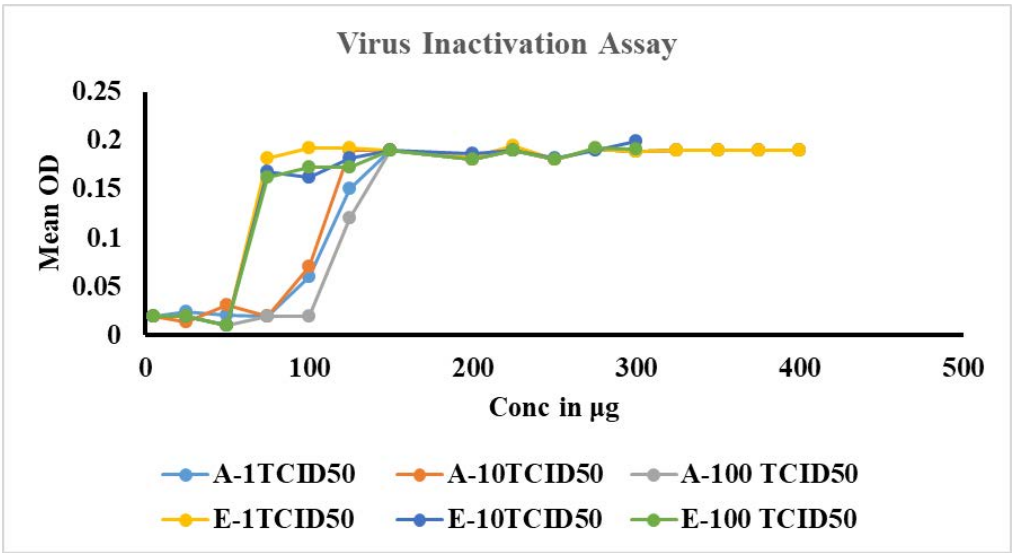

Figure 2: Virus inactivation assessment of nontoxic concentration of aqueous and ethanolic extracts on varying concentration of HSV 1 and 2 viruses (A - Aqueous Extract E - Ethanolic Extract)

when the cells have been pretreated with varying concentrations of the extracts.

Though literature evidence is not available to hold that sphaeranthus is active against only DNA viruses, a similar study done on antiviral activity of Garlic showed activity against HSV-1 but not against RNA viruses[24].

The antiviral activity seen in both extracts of sphaeranthus against HSV-1 and 2 may be due to the macromolecules, proteins, carbohydrates and other substance along elevated levels of sterols, alkaloids, glycosides, saponins and tannins particularly in the ethanolic extract[25]. The higher bioactivity observed in the ethanolic extracts can be attributed to the presence of secondary metabolites which are extracted with non-polar solvents[26]. Further it is seen from the literature that the antiviral activity is a synergistic effect of two or more metabolites along with glycoproteins rather than one active principle[27,28]. This was the soul idea of using the crude extracts as routinely used in traditional medical practices.

CONCLUSION

The aqueous and ethanolic extracts of sphaeranthus showed recordable antiviral activity against HSV 1 and 2 virus. Ethanolic extract exhibited higher activity at lower concentrations compared to the aqueous extracts. The microscopic observations and MTT assay were comparable. The partial activity of aqueous extracts may be dose/concentration dependent of the active ingredients present in the extracts.

The antiviral activity found to occur at concentrations non-toxic to cell line may be used to screen large groups of herbs and natural products, yet the therapeutic dose for commercial exploitation must be standardized by in vivo studies in animals. Nevertheless, these studies lend credence to the scientific authentication of the herbal plants as novel antiviral agents.

LIMITATION OF THE STUDY

This study describes possible antiviral properties of sphaeranthus against HSV 1 and 2. The current study is a preliminary screening using In-vitro cell culture. There are other advanced techniques like viral adsorption assay, viral pretreatment assay and molecular tools to deduce where the components of the extracts are preventing the viral propagation cycle. Further to add in-vivo animal models can be added advantage to move the testing of plant extracts to know exact biological action and side effects if any were not possible for us this time.

FUTURE DIRECTION

Further work is required to do enumeration of the secondary metabolites present in the both aqueous and ethanolic sphaeranthus extracts. The phytochemical profile will used to identify the active ingredient which is virucidal or anti-viral. On other hand, screening for viral adsorption or entry, prophylactic effects and molecular mechanism involved in viral inhibition can be worked on. However, escalation of the studies involving animals are necessary for these extracts to be up to required pharmacological benchmarks like therapeutic window.

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Competing Interest: None

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