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In vitro evaluation of the *Sphagneticola calendulacea* against VZV using Hep-2 Cell Line

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Abstract
Due to the high preponderance of viral infections having no peculiar medication and the perpetual appearance of resistant viral strains, the development of novel antiviral agents is imperative. The aim of this study was to evaluate the antiviral potential of methanol and ethanolic crude extract of an ethnomedicine *Sphagneticola calendulacea* against *Varicella Zoster Virus* (VZV) using HEp-2 cell line. To assess the reduction of viability of infected or uninfected cell cultures, the MTT [3-(4,5-dimethylthiazol–2-yl)2,5-diphenyltetrazolium bromide] colorimetric assay were used, and the capacity of the extracts to inhibit the lytic activity of VZV. The therapeutic index of the positive extracts for the antiviral activity was determined by calculating the ratio CC_{50} (50% cytotoxic concentration) over IC_{50} (50% inhibitory concentration of the viral effect). The results endorse that ethanol and methanol extract of *S. calendulacea* showed a maximum relative activity at 25µg/ml compared to the standard drug Acyclovir.

Keywords: VZV, HEp-2 cell line, Sphagneticola calendulacea, Methanolic, Ethanolic, MTT

1. Introduction
Varicella-zoster virus (VZV) is a member of the Herpesviridae family. Primary infection causes varicella (chickenpox). The virus is not cleared from the body but persists in a dormant state in the dorsal root and/or cranial nerve ganglia. Subsequent reactivation of the latent virus, typically occurring years later and causes zoster (shingles). The virus contains a double stranded DNA genome. Herpes viruses are characterized by the persistence of infection after primary infection, a property known as latency. Cell-mediated immunity is critical in clearing the infection and in preventing secondary reactivation [1].

Chickenpox (varicella) is characterized by a generalized vesiculopustular rash. Symptoms usually begin with 1 or 2 days of fever, flu-like symptoms and generalized malaise, although this may be absent. Complications of chickenpox infection occur in approximately 1% of cases, with the most common being secondary bacterial infection of the skin lesions. The average number of skin vesicles is usually 250–500 but >500 lesions may occur in severe cases [2].

Shingles (zoster) is due to reactivation of the virus, which has previously been infected with VZV. It is self-limiting, occurring over one to three contiguous unilateral dermatomes. Pain is a frequent complication and may persist after the rash resolves (post-herpetic neuralgia) [3]. Shingles is found worldwide and has no seasonal variation. Around 20 percent of the general population will experience shingles during their lifetime and an estimated 500,000 episodes of shingles occur annually in the U.S. Approximately four percent of individuals will experience a second episode of shingles [4].

Sphagneticola calendulacea (L.) Pruski belongs to the family Asteraceae which is a very useful herbal medicinal plant. The leaves can be used in treatment of dermatological disorders, cough, headache, hair loss, strengthening the nervous system, lack of blood, digestive system disorders [5]. The plant has great importance in Ayurvedic, Siddha and Unani systems of traditional medicine. The leaves are used in dyeing grey hair and in promoting the growth of hair [6].

However, the developments of resistance towards the anti VZV drugs are prevailing among immunocompromised patients. Also numerous side effects coupled with these drugs are reported namely the renal failure, neurotoxicity, thrombocytopenia, renal dysfunction, nephrotoxicity and hypokalemia [7]. Therefore, there is a desideratum that necessitates the development of new-fangled potential antiviral agents for VZV infections.

2. Experimental

2.1 Collection of plant material

The leaves of *Sphagneticola calendulacea* were collected during the month of December from the natural habitats of Thiruvalluvar district, Tamil nadu, India. The leaf was identified by Botanical Survey of India (BSI), Coimbatore, India with ref no.BSI/SRC/5/23/2015/Tech./2626.The leaves were washed with running tap water and finally washed with distilled water to remove the dirt and dried under shade for two weeks.

2.2 Preparation of powder and extract

The leaves were pulverized to powder in a mechanical grinder. The powder was successively extracted with Ethanol and Methanol in soxhlet apparatus and it was left for eight hours. The extracts were concentrated under reduced pressure in a rotary evaporator [8].

2.3 Cell line

Human Epithelial cell line (HEp-2) was procured from National Centre for Cell Science (NCCS) Pune, India and reconstituted in Minimum Essential Medium (MEM) with 5% v/v Fetal bovine serum (FBS), Penicillin, Streptomycin, Amphotericin-B, L-Glutamine, Sodium bicarbonate and HEPES (2-[4-(2-Hydroxyethyl)1piperzinyl ethane sulphonic acid) buffer. Appropriate volume of CO_2 was passed into the medium till the pH reached the optimum range (i.e.) 7.1 - 7.5.

2.4 Cytotoxicity Assay

A monolayer of the cell line was taken and the medium was discarded and the wells were given a gentle wash with Phosphate buffered Saline. Cells were trypsinised with Trypsin Phosphate versene Glucose. 10 ml of 10% minimum essential medium was added to the flask and the cells were removed. 100 µl of cells were plated into the 96 well microtitre plate and 100 µl of 10% minimum essential medium was added into each wells. The microtitre plate was then incubated at 37 °C for 12 hours under 5% CO_2 atmosphere.

About 1 mg of methanol and ethanol leaf extracts of *S. calendulacea* was weighed separately and dissolved in 1 ml of methanol and ethanol based on the solubility and 200 µl of the extract was added into the wells of first column of microtitre plate and was diluted into two fold manners till the last well. 100 µl from the last well was discarded. Number of viable cells, 0.1 ml (1.4 x 10^5 cells) was counted and 100 µl from the respective dilutions were added into the respective wells containing cells. 100 µl of 2% MEM was added into the wells and control wells with cells were maintained. The plate incubated at 37 °C in 5% CO_2 atmosphere for 48 hours and was observed under inverted microscope for determination of toxic free concentration [9].

2.5 MTT Assay

5 mg/ml of MTT was dissolved in PBS (Phosphate Buffered Saline). 20µl of MTT solution was added into the respective wells and the plate were incubated for 4 hours until purple precipitate is visible. Later 100µl of DMSO (Dimethyl sulfoxide) was added to each well and kept for some time. Plates were removed and measured at 540 nm using Microplate reader (Biotek, USA).

2.6 Antiviral Assay

Clinical *VZV* strain was isolated from a patient and transferred in to Virus Transport Medium (VTM). The VTM vial was immediately kept in a refrigerator at 4˚C. 0.1 ml of the VTM supernatant and maintenance medium was transferred on to the monolayer of HEp-2 cell line and incubated at 37 ˚C for four days. Complete Cytopathic Effect (CPE) in HEp-2 cell line observed on the fourth day. Thus, virus stock was used for the estimation of $TCID_{50}$ by end point dilution assay [10].

2.7 *In vitro* antiviral assay

Monolayer of HEp-2 cells was grown in 96 well microtitre plates. 0.1 ml of 10^6 $TCID_{50}$ viral suspensions, diluted in 2% FBS MEM, was added into experimental wells. 0.1 ml of 2% FBS maintenance medium alone was added into cell control and incubated at 37 ºC in 5% CO_2 atmosphere for 60 minutes to facilitate adsorption of virus to the cell line. The highest cell toxic free concentrations to the lower ranges of leaf extracts concentration were selected for anti-VZV study. The following concentrations of toxic free leaf extracts are 25µg, 12.5µg, 6.25µg, 3.125 µg were diluted with 0.2ml of 2% FBS MEM and transferred to the virus inoculated monolayer cell lines. The entire setup was incubated at 37 ºC temperature for three days to allow multiplication of virus and subsequent development of Cytopathic Effect (CPE). Each well was observed under Inverted microscope every day for presence or absence of Cytopathic effect. The standard antiviral agent of Acyclovir (1mg/mL) was used as Positive control. Later, MTT assay was carried out.

3. Results

3.1 Cytotoxicity Assay

Based on the calorimetric and MTT assay the ethanol and methanol leaf extracts showed toxic to the HEp-2 cell line at the concentration of 100µg/ml and 50µg/ml. The maximal toxic free concentration of leaf extracts of Ethanol and Methanol was attained at 25µg/ml, 12.25µg/ml, 6.25 µg/ml, and 3.125µg/ml. Cell control and DMSO did not exhibit any cytotoxicity. The results were tabulated Table - 1 and Fig (1a-1c) and (2a-2c).

Table 1: Cytotoxicity Profile of *Sphagneticola calendulacea*

S. No.	Concentrations (µg/ml)	Ethanol Toxicity	Ethanol Cell control	Methanol Toxicity	Methanol Cell control	DMSO
1.	100	+	-	+	-	-
2.	50	+	-	+	-	-
3.	25	-	-	-	-	-
4.	12.5	-	-	-	-	-
5.	6.25	-	-	-	-	-
6.	3.12	-	-	-	-	-

(+) → Presence of cytotoxicity
(–) → Absence of cytotoxicity

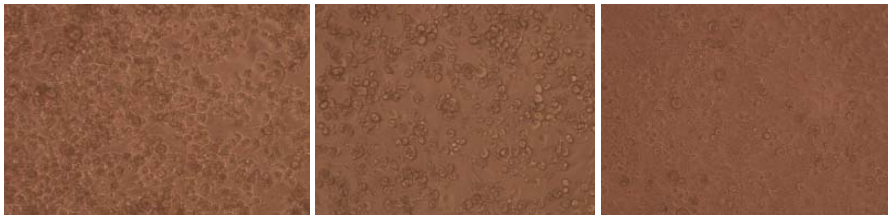

Fig 1a: 100µg/ml **Fig 1b:** 50 µg/ml **Fig 1c:** 25µg/ml

Fig 1: Cytotoxic activity of methanolic leaf extracts of *S. calendulacea* against HEp-2 cell line

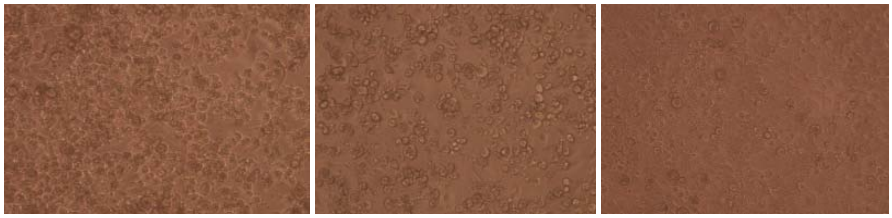

Fig 2a: 100µg/ml **Fig 2b:** 50 µg/ml **Fig 2c:** 25µg/ml

Fig 2: Cytotoxic activity of ethanolic leaf extracts of *S. calendulacea* against HEp-2 cell line

3.2 *In vitro* Antiviral Assay

Inhibition of virus was observed in the methanol and ethanol extracts of *S. calendulacea* at a maximal nontoxic concentration range from 25µg/ml, 12.25µg/ml, 6.25µg/ml, 3.12µg/ml and 1.56µg/ml. Infected cells treated with Acyclovir indicated complete inhibition and observed at a concentration of 25µg/ml. The results were tabulated Table - 2 and Fig 3 & 4 (A – D)

Table 2: Antiviral activity of leaf extracts of *Sphagneticola calendulacea* (Methanol and Ethanol)

S. No	Concentrations (µg/ml)	Cytopathic Effect	Virus Control	Cell Control
1.	50	+	+	-
2.	12.5	+	+	-
3.	6.25	+	+	-
4.	3.12	+	+	-
5.	1.56	+	+	-
6.	Acyclovir 25µg	-	+	-

Fig 3a: Control **Fig 3b**: 100µg/ml **Fig 3c**: 25µg/ml **Fig 3d**: 12.5µg/ml

Fig 3: Maximal Toxic free concentration of methanolic leaf extracts of *S. calendulacea* on HEp-2 cell line

Fig 4a: Control **Fig 4b:** 100µg/ml **Fig 4c:** 25µg/ml **Fig 4d:** 12.5µg/ml

Fig 4: Maximal Toxic free concentration of ethanolic leaf extracts of *S. calendulacea* on HEp-2 cell line

4. Discussion

The development of plant based antiviral preparations promises a more potential alternative in combating viral diseases. Over the years, the World Health Organization (WHO) advocated that countries should interact with traditional medicine with the view to identify and exploit aspects that provide safe and effective remedies for ailments of antiviral. In addition, there are limited antiviral drugs being tested against viral disease in clinical trials. The wide prescription of herbal drugs is mainly due to their effectiveness, less side effects and relatively low cost[11]. As such present study is aimed to screen and determine the anti-viral activity of Ethanol and Methanol leaf extracts of *Sphagneticola calendulacea* on VZV.

Although a reduction in the incidence of chickenpox was reported with the introduction of the varicella vaccine in 1995 vaccination against *VZV* is not a routine policy in many countries and seasonal outbreaks of the wild type infection of the virus continue to occur.

Previous authors claimed that the Nuts of *Semecarpus anacardium* methanolic extract was tested for its inhibitory effect in 96 micro plate against HEp 2 cell line and showed potent cytotoxic activity [12] whereas, in the current study methanolic leaf extract of *Sphagneticola calendulacea* experimented for its inhibitory effect against HEp-2 cell line, the cytotoxicity was evaluated by MTT assay and outcome

proved in an effective manner.

Lin *et al.,* (1999) have reported that five groups of biflavonoids (amentoflavone, agathisflavone, robustaflavone, rhusflavanone and succedanea flavanones) were isolated from medicinal plants of *Rhus succedanea* and *Garcinia multiflora* and exhibited various antiviral effects against a number of viruses including respiratory viruses and herpes viruses (HSV-1, HSV-2 and VZV). The inhibitory activity against VZV was demonstrated with succedanea flavanones [13].

Earlier, antiviral activity of Liquorice powder extract from the roots of *Glycyrrhiza glabra* against *Varicella Zoster Virus* and studied using Vero cell line, which showed minimal activity when compared to standard [14] whereas, the present study was performed to find out the maximum toxic free concentration of leaf extracts of *S. calendulacea* against VZV on HEp-2 cell line and showed a positive results.

Anand *et al*., (2004) have reported the efficacy of isolated principle from seed kernel of *Pongamia pinnata* against VZV [15]. Rajarajan *et al*., (2006) have studied *in vitro* antiviral potential from *Coleus amboinicus* on Human Herpes type-3(VZV) by using HEp2 cell line [16]. Jabareen *et al*., (2013) have examined the *in vitro* antiviral property using aqueous and ethanolic leaf extracts of *Passiflora edulis*. They had conducted in African green monkey kidney (Vero) cells [17].

One of the plant extracts, glycyrrhizin (GL) was investigated for its antiviral action on *varicella-zoster virus in vitro*. When Human Embryonic Fibroblast (HEF) cells were treated with GL after inoculation of virus (post-treatment), the average 50%-inhibitory dose (ID50) for five VZV strains. GL was reported as effective against VZV replication when HEF cells were treated 24 hours before the inoculation [18] whereas, in the current study leaf extracts of *S. calendulacea* was scrutinize against VZV using HEp-2 cell line. The ethanol and methanol leaf extracts showed a positive result on infected cell line with VZV.

5. Conclusion

The present study demonstrates that the inhibitory activity of methanolic and ethanolic leaf extracts against VZV at 25µg/ml concentration. Studies on *S. calendulacea* should be further conducted extensively by isolation, purification and characterization of the active compounds in order to discover the potential anti-viral compounds and provide more insight into the inhibition of virus adsorption and replication and to explore its potential use as a therapeutic product to combat dengue.

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