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A STUDY ON THE INVESTIGATION OF *IN VITRO* CYTOTOXIC AND ANTIVIRAL ACTIVITIES OF AQUEOUS, AQUEOUS ETHANOL AND ETHANOL EXTRACTS OF *HEMIDESMUS INDICUS*.

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Objective

The aim of this study is to find out the cytotoxicity and antiviral activities of aqueous, aqueous ethanol, ethanol extracts of *Hemidesmus indicus* against Varicella zoster virus (VZV) isolated from chicken pox and zoster patients

Methods

Fifty VZV vesicular swab specimens from patients clinically diagnosed were collected and viral strains were isolated using HEp2 cell line.VZV isolates were identified using Elisa blotting technique. Cytotoxicity of the plant extracts was determined for evaluating their safety on the cell line. The antiviral activity of lyophilized plant extracts and the standard drug acyclovir (ACV) were evaluated against VZV using antiviral assay following the Julia Serkedjieva et al (1999) method.

Results

The toxic concentrations of lyophilized Aqueous, aqueous ethanol extracts root extracts of the plant *Hemidesmus indicus* and Acyclovir on the Hep-2 cell line ranges from 500µg/ml to 250µg/ml but, ethanol extract of the plant toxicity ranges from the concentration 250µg/ml, to 125µg/ml respectively in Hep2 cells. The Maximum Non Toxic Concentration (MNTC) of all the extracts ranged from 500 to 125µg/ml. The antiviral assay was performed for aqueous, aqueous ethanol and ethanol extracts against chicken pox and zoster at 120 hours. The antiviral assay results of aqueous, aqueous ethanol against chicken pox were (125µg/ml-62.5µg/ml), 125µg/ml, and against zoster were (125µg/ml-62.5µg/ml) and (125µg/ml-62.5µg/ml).But ethanol extracts did not exhibit any activity against both the viruses at any concentrations.

Conclusion

From the findings we suggested that the plant *Hemidesmus*

indicus can be used as a better herbal remedy for the treatment and prevention of chicken pox and zoster infections or associated diseases.

INTRODUCTION

Varicella-zoster virus (VZV) is a ubiquitous human alphaherpesvirus, which causes varicella (chicken pox) and herpes zoster (zoster). Chickenpox is a relatively mild disease in healthy children but may be life threatening in immunosuppressed patients, neonates, and normal adults especially smokers for whom the risk of varicella pneumonia is high [1]. In the industrialized countries of the West, varicella is largely a childhood disease, whereas reports from tropical countries indicate a significant incidence of varicella among adults. Primary VZV infection begins with inoculation of mucous membrane sites, after which the virus is presumed to spread to regional lymph nodes and elicits primary and secondary viremic phases in the early and late incubation periods [2].

Adults are more likely to die than children from chickenpox and have serious complications resulting from varicella infection. Immunocompromised people are more likely to have serious illness with complications as a result of chickenpox. If a pregnant woman gets varicella during the first 20 weeks of pregnancy, her baby has a 1 in a 100 risk of having serious birth defects such as shortening and scarring of limbs, cataracts, small head size, abnormal development of the brain and mental retardation.

Varicella can also reactivate without rash or neurological symptoms or signs, as evidenced by a 5-fold increase in VZV-specific antibodies [3]. Reactivation of the virus causes zoster. Initial infection with varicella-zoster cause chickenpox but the virus can then remain silent in the body for decades. Zoster causes a painful, blistering rash that usually appears on just one side of the body, most often on the torso or face. Pain and numbness may occur in the location of the rash two to four days before the rash appears. The chances of getting zoster increase with age.

Acyclovir is a synthetic nucleoside analogue that interferes with the replication of herpes viruses. It is used to treat chickenpox and zoster. Acyclovir is a safe and effective drug with rare side effects when used as short term treatment [4], [5],[6].

A majority of the world's population in developing countries still relies on herbal medicines to meet its health needs. Herbal medicines are the only available health service often used to provide first-line and basic health service, to people living in remote and undeveloped areas at an affordable cost. The interest on herbal medicines and their utilization have been increasing rapidly in recent years. *Hemidesmus indicus*, also known in ancient Ayurvedic medicine as Sugandi or Sariva, has been revered for its medicinal properties for nearly a thousand years. Traditional Ayurvedic medicine practitioners have used Sariva for hundreds and hundreds of years[7]; It was used as a healing herb as well as a magical-spiritual dream herb. It is used to treat stomach problems, cure rashes, skin related infections.etc.,[8].

Although some research has been done in the plant, the antiviral activity against varicella zoster virus has not been reported so far. Hence this study highlights the primary research on the antiviral efficacy of the plant in comparison to the standard drug acyclovir by adopting antiviral assay against vzv isolated from the clinical samples of chicken pox and zoster patients and confirming it by using Elisa technique .

MATERIALS AND METHODS

Plant materials and Cell line preparation

The plant part (roots) of *Hemidesmus indicus* were collected from suburbs of Chennai, India. The accuracy of the plant parts and family were ascertained with the Department of Plant Biology and Biotechnology, Presidency College, Chennai. The roots were shade dried and powdered

coarsely. Aqueous, aqueous ethanol (1:1) and ethanol extracts were prepared by cold maceration method and then filtered through a 0.22µm pore sized Millipore filter and lyophilised at -70^{o}C at reduced pressure.

Estimation of maximal cytotoxic free concentration by *in vitro* cytotoxic assay

Procurement of cell line

Human Epidermoid larynx carcinoma cell line (HEp2) were initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained in P.G. & Research Department of Microbiology and Biotechnology, Presidency College, Chennai, India.

Preparation of Cell culture medium

The Preparation of Cell culture medium and subculturing of Hep-2 cells were carried out by following the method as described by Gerald Collee *et al*.,1996 [9]. For 10% culture media 10ml of FBS(fetal bovine serum) was added in 90ml plain MEM and for 5% culture medium 5ml of FBS was added in 95ml media (Sigma Aldrich M4655) The prepared medium were transferred into sterile disposable storage medium bottle. Then the content was supplemented with Penicillin (100 IU/ml), Streptomycin (100µg/ml), Kanamycin (100µg/ml) and Amphotericin-B (100µg/ml) (Himedia A0011).

HEp2 cell lines

HEp2 cell lines were grown in MEM (Sigma Aldrich M4655) with 5ml of foetal bovine serum.

Subculturing of cells

Cultures were observed under inverted phase contrast tissue culture microscope to assess the confluence and the absence of bacterial and fungal contaminants of the cell monolayer. The confluent monolayer was twice washed gently with 2 ml of PBS and the wash solution was removed. 4ml of TPVG solution sufficient enough to cover the cell layer was delivered over the monolayer. The flask was incubated at 37^{o}C for 3-5 minutes until the cell sheet dissociate. At this stage cells had undergone rounding without detachment, which was observed under Inverted Phase Contrast Culture Microscope (Nikon). Then 5ml of 10% FBS growth medium was added into the flask and the content was aspirated several times with a micropipette. 100-200µl was removed to perform a cell count. The required number of cells was transferred to a new labelled flask containing pre-warmed HEp-2 medium and incubated at 37^{o}C.

Collection of clinical samples

Fifty clinical samples from vesicles of patients suffering from chicken pox and herpes zoster were collected from the Skin Outpatient Department, Kilpauk Medical College, Chennai, India in BD^{TM} Universal viral transport medium. The oozing fluid from the vesicles was collected using a sterile screw cap vial containing 4ml of virus transport medium(VTM),transported in ice pack and store at -80^{o}C until further use.

Detection of IgG and IgM antibodies

Serum sample collected from patients sufferings from chickenpox and herpes zoster were screened for the presence of IgG and IgM antibodies against chicken pox and zoster viruses by Enzyme linked immunosorbent assay (ELISA) using commercial kits (Calbiotech Catalog No.VZ081G and Calbiotech Catalog No. VZ082M).

Isolation of virus collected from chicken pox and zoster patients

The viral transport medium(VTM) with swab(containing the viruses collected from the patients' vesicles) was centrifuged at 2000rpm for 10min and 0.1ml of the supernatant was directly applied over the monolayer cell line after careful removal of the growth medium. It was kept undisturbed for 60 min at room temperature. 10ml of 5% FBS maintenance medium was added on to the HEp2 cell line. A control flask was also maintained. The culture flask was incubated at 33^{o}C for

7 days. The flask was observed daily for cytopathic effect (CPE) using an inverted phase contrast microscope

Estimation of tissue culture infective dose 50

Tissue Culture Infective Dose 50 ($TCID_{50}$) was estimated by Reed and Muench Method, (1938) [10]. 100µl of the diluted virus was suspended in 900µl of 2% MEM and it was serially diluted from 1 to 12^{th} concentration. 100µl of the diluted virus was added to the respective columns and incubated at 37°C for 1 hour and about 100µl of 2% minimum essential medium was added. After 48 hours, the cytopathic effect in the wells of each row was counted and $TCID_{50}$ were estimated as below

$$TCID_{50} = \log_{10} \text{dilution factor} \frac{\{\% \text{ infection at a next dilution above } 50\% - 50\}}{\{\% \text{ infection at next dilution } 50\% - \% \text{ infection at Next dilution below } 50\%\}}$$

Estimation of maximal cytotoxic free concentration by *in vitro* cytotoxic assay

The cytotoxicity of the plant materials were tested by using the Human epidermoid larynx carcinoma cell lines (HEp2) which were allowed to grow in tissue culture 96 well microtitre plates in the presence of the lyophilized plant test materials following the methods adopted and described by Julia Serkedjieva et al (1999) [11]. 0.1ml cellular suspension was transferred into each of the wells containing growth medium. The plate was incubated at 37°C in 5% CO_2 atmosphere in a CO_2 incubator for 12 hours. The growth medium was removed after confluence was obtained by micropipette. 1mg of the extracts was dissolved (standard/test) in 1ml 2% FBS MEM. The extract mixed medium was serially diluted in two fold manner in 2% FBS MEM, from an initial concentration of 500µg/ml to a final concentration of 3.9µg/ml. 0.1ml of the serially diluted extract was added into the wells. For standard, same procedure was adopted with acyclovir which was added to another row of cells. The plate was incubated at 37°C in 5% CO_2 atmosphere for 120 hours and was observed under inverted phase contrast microscope for determination of toxic free concentration. Confluent monolayer of HEp-2 cells was grown in 96 well microtitre plates. 0.1ml viral suspension, obtained by seven consecutive ten-fold dilutions in 2% FBS MEM, was added into five wells of microtitre plates (Four for antiviral, one for virus control). 0.1ml of 2% FBS maintenance medium alone was added into cell control (sixth row) and incubated at 37ºC in 5% CO2 atmosphere for 60 minutes to facilitate adsorption of virus to the cell line.

Estimation of maximal nontoxic concentration of the lyophilized extracts by *in vitro* cytotoxicity assay

A monolayer of the Hep-2 cell line was taken and the medium was discarded and the wells were given a gentle wash with PBS. Cells were trypsinized with 0.25% Trypsin Phosphate Versene Glucose (TPVG-Himedia). 10ml of the growth medium (10% MEM) supplemented with 10% FBS (Himedia),L-Glutamine and antibiotics (Penicillin & Streptomycin (100µl) of dislodged cells were plated into the 96 well tissue culture micro titre plate and 100 µl of 10% MEM was added to each wells. The microtitre plate was then incubated at 37°C for 12 hours under 5% CO_2 atmosphere.

One gram of the extract was weighed and dissolved either in ethanol/water based on the solubility and made up to 1ml in MEM (Sigma Aldrich, India) to obtain the final stock solution as 1mg/1ml by adjusting the pH to 7.0 with Hcl/NaOH. The dissolved extract was filtered using 0.22µm syringe filter. The stock solution was diluted from the concentration of 500µl/ml to 3.9of 500µg/ml (log_2 dilution).

About 100µl of the diluted aqueous, aqueous ethanol, ethanol extract of varying concentration (500µg/ml to 3.9µg/ml in log_2 dilution) was added to the confluent HEp2 cell line in

96 well tissue culture microtitre plates. 100µl of 2% MEM was added into the wells and control wells were maintained. It was incubated for 4 days at 37°C and the highest concentration of the compound that showed no morphological variations or alterations as observed under inverted phase contrast microscope (Nikon) were considered as Maximum Nontoxic Concentration of the drug.

Estimation of minimum inhibitory concentration of the lyophilised extracts to varicella zoster virus by inhibition of virus induced cytopathic assay

The antiviral assay of the lyophilised extracts was carried out from the maximum nontoxic concentration of the extracts and cells were infected with the virus at the multiplicity of infection of 1(MOI) of $TCID_{50}$ and incubated for 1 hour at 37°C. After adsorption of virus, maximum nontoxic concentration (MNTC) of the drug was added to the adsorbed virus and incubated at 37°C. The virus control with untreated drug and cell control were maintained. Each experiment was done in triplicates. Acyclovir (Sigma Aldrich, India) was used as a standard drug. The minimum concentration of the extracts that could inhibit the formation of CPE by Chicken pox and Zoster virus was estimated in comparison to the virus control.

RESULTS

Totally fifty samples for varicella zoster (12 chickenpox samples) and (38 zoster samples) were collected from the patients suffering from both the diseases. Among 12 chickenpox and 38 zoster samples collected from patients, 6 positive chickenpox samples and 4 positive zoster samples were isolated.

HEp-2 cell line showing cytopathic effect (CPE) of chicken pox virus & zoster virus isolated from patients suffering from varicella zoster. (Fig:1)

The viral isolates from the patients suffering from chickenpox and zoster were cultivated using the HEp-2 cell line (immortalized, human epithelial cells).The virulence and the pathogenicity of the chicken pox and zoster viruses were observed as CPE. The comparison of CPE of both the virus chickenpox and zoster was done by using *in vitro* experimentation.

Estimation of Maximal Nontoxic free Concentration of lyophilized extracts of selected plant on Hep-2 cell line at 120 hours.

The aqueous extract and aqueous ethanol extract of the *Hemidesmus indicus* showed toxicity at the first two highest concentrations 500-250µg/ml at 120 hours. .

The ethanol extracts of *Hemidesmus indicus* were toxic to the cells at first three concentrations.

The cytotoxic results of the standard drug Acyclovir were also tabulated and compared with each lyophilized extracts the plants.

***In vitro* antiviral activity of the lyophilized extracts of the plant *Hemidesmus indicus* against chicken pox at 120 hours based on the max nontoxic concentration assay.**

Aqueous extract

The lyophilized aqueous extract of the plant *Hemidesmus indicus* exhibited antiviral activity against chicken pox at 120 hours at 125µg/ml and 62.5µg/ml only but, at other remaining concentrations 31.25µg/ml to 3.98 µg/ml it showed no activity which was observed by the presence of cytopathic effect in the Hep-2 cells.

Aqueous ethanol

In vitro antiviral activity of the lyophilized aqueous ethanol extract of the plant *Hemidesmus indicus* on chicken pox at 120 hours was 125µg/ml and no other concentrations showed any change.

Ethanol extracts

The lyophilized ethanol extract of the plant *Hemidesmus indicus* did not show any activity against chicken pox virus 120 hours.

In vitro antiviral activity of the lyophilized extracts of the plant *Hemidesmus indicus* against zoster at 120 hours

Aqueous extract

In vitro antiviral activity of the aqueous extract of the plant *Hemidesmus indicus* on zoster at 120 hours was just the same as that it was against chickenpox i.e., 125-62.5µg/ml.

Aqueous ethanol extract

Antiviral activity of the aqueous ethanol extract of the plant *Hemidesmus indicus* on zoster at 120 hours was slightly different to that of the results against the chickenpox. Here it exhibited the antiviral activity at two concentrations 125µg/ml and 62.5µg/ml.

Ethanol extracts

The lyophilized ethanol extract of the plant *Hemidesmus indicus* here again did not show any activity against zoster virus 120 hours.

DISCUSSION

Even though the introduction of the varicella vaccine in 1995,[12] has reduced the incidence of chickenpox still there is a continuation of the outbreak of vzv infection in many countries where vaccination for vzv is not a routine policy and seasonal outbreaks of the wild type infection of the virus continue to occur. In addition there is an increasing number in the atypical cases of varicella zoster which has some serious effects in a group of people like immunosuppressed patients, neonatal, smokers with risk of varicella pneumonia. The incidence of zoster is considerably increased in HIV-positive adults[13] and children, and in AIDS patients, zoster can also produce multiple CNS and ocular disorders which are estimated to occur in up to 11% of HIV-positive subjects[14].Also when VZV reactivates all the neurological and ocular diseases occur in the absence of zoster rash [15]

Confirming the VZV infection and studying the characteristics of virus is very vital in finding an alternative to the present treatment for controlling the severity of the disease which is done by isolation and identification of the vzv virus.

In the present study, fifty vzv clinical samples were collected from the vesicular lesions and serum samples of patients with chicken pox and zoster Among them 6 positive chicken pox and 4 zoster were isolated.VZV isolated was propagated on the Human Epidermoid larynx carcinoma cell line (HEp2) [16].

The low percentage of recovered isolates might be attributed to virus loss because of filtration of specimens prior to inoculation onto Hep-2 cells. Virus loss may be due to the protein binding capacity of the membrane filters, as well as timing of the sample collection, which may be due the time between onset of infection and sample patient admission to hospital. Filtration of specimens using a (0.22µm) Sartorius syringe filter decreases the chance of success of VZV isolation [17] [18] Also, in this study, the rate of isolation was high in the first 3 days after the onset of the disease but, it declined gradually with time. The delayed detection of cytological changes may be attributed to the low viral load in collected specimens, Alternating passaging in cell cultures could be a supporting factor to maximize the viral load, Other researchers have noted that the infective varicella zoster virus can be isolated using cell cultures and a typical cytopathic effect (CPE) can be observed within 3 days to 3 weeks according to the viral load. These results showed that Hep2 cells could be used successfully for isolation and propagation of VZV. The antibodies to VZV could be detected using the same assay according to Leonardi *et al*. who reported that dot-ELISA could be used reliably for the detection of antibodies to varicella viruses in human sera [19]. The cytotoxicity of the lyophilized Aqueous, aqueous ethanol and ethanol root extracts of the plant *Hemidesmus indicus* and acyclovir were monitored by daily observing the changes in the cells such as morphology, eruption of monolayer, aggregation of cells under microscopy for detectable alterations and it was evaluated in order to determine the nontoxic concentrations that maintained 100% cellular viability. The toxic

concentration of Aqueous, aqueous ethanol extracts of *Hemidesmus indicus* and Acyclovir on Hep-2 were 500-250µg/ml but, the ethanol extract showed toxic even in lower concentration at 125µg/ml.

The antiviral activities of Aqueous, aqueous ethanol and ethanol extracts of *Hemidesmus indicus* and acyclovir against one positive isolate in chicken pox and one positive isolate in zoster were evaluated. The discrepancy in this study might be simply explained by the use of a three kinds of extracts of Hemidesmus indicus rather than the crude plant powder. The nontoxic concentration of acyclovir (125 µg/ml) showed good antiviral activity against the isolated virus.

In that context, Wutzler [20] reported that the IC50 for acyclovir against most laboratory strains and clinical isolates of VZV ranges from 0.12 to 10.8 µg/ml.

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CONCLUSION

VZV isolates were successfully isolated and propagated using Hep-2 cells. Isolates were identified using ELISA techniques. The cytotoxicity of Aqueous, aqueous ethanol and ethanol extracts of *Hemidesmus indicus* extracts and acyclovir on Hep-2 cells was determined using Maximum Non Toxic free Concentration followed by Julia Serkedjieva . Aqueous, aqueous ethanol and ethanol extracts of *Hemidesmus indicus* in the crude form has a low antiviral activity against VZV compared with acyclovir. Molecular biology techniques are recommended for genotyping of clinical isolates isolates. Screening of the antiviral activity of a wide range of herbal antiviral agents is also suggested in order to establish a safer, more economical and more efficient treatment, particularly for acyclovir resistant isolates.

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Results

Table: 1 Detection of IgG and IgM

Wells	Samples	IgG(Ab index)	Color Interpretation	IgM(Ab index)	Color Interpretation
1A	Blank	B	No Color	B	No Color
1B	Positive Control	2.5	Dark Yellow	2.3	Dark Yellow
1C	Positive Control	2.6	Dark Yellow	2.0	Dark Yellow
1D	Calibrator	1.3	Pale Yellow	1.0	Pale Yellow
1G	Negative Control	0.2	No Color	0.4	No Color
1H	Negative Control	0.5	No Color	0.1	No Color
2A	VZ23	1.7	Pale Yellow	1.3	Pale Yellow
2B	VZ18	1.2	Yellow	1.7	Pale Yellow
2C	VZ9	1.3	Pale Yellow	1.5	Pale Yellow
2D	VZ15	1.6	Pale Yellow	1.6	Pale Yellow
2G	VZ 12	1.8	Yellow	1.7	Yellow
2H	VZ 36	1.5	Yellow	1.8	Yellow
3A	HZ41	1.7	Pale Yellow	1.2	Pale Yellow
3B	HZ42	1.3	Pale Yellow	1.3	Pale Yellow

3C	HZ3	1.6	Pale Yellow	1.5	Pale Yellow
3G	HZ8	2.3	Dark Yellow	2.5	Dark Yellow
3H	HZ7	2.2	Dark Yellow	2.4	Dark Yellow

Table 2: Results for the Maximum Nontoxic free Concentration & Antiviral assays of the lyophilized aqueous ,aqueous ethanol, ethanol extracts of the *Hemidesmus indicus* against chickenpox and zoster virus @ 120 hours and Physiochemical properties of the lyophilised plant extracts subjected for *in vitro* analysis.

Conc (µg/ml)		Aqueous		Aqueous/ethanol		Ethanol		ACYCLOVIR	
		MNTC	ANTIVIRAL	MNTC	ANTIVIRAL	MNTC	ANTIVIRAL	MNTC	ANTIVIRAL
500	C.P	+	*	+	*	+	*	+	*
	ZOSTER	+	*	+	*	+	*	+	*
250	C.P	+	*	+	*	+	*	+	*
	ZOSTER	+	*	+	*	+	*	+	*
125	C.P	_	+	_	+	+	*	_	+
	ZOSTER	_	+	_	+	+	*	_	+
62.5	C.P	_	+	_	_	_	+	_	+
	ZOSTER	_	+	_	+	_	+	_	+
31.25	C.P	_	_	_	_	_	_	_	+
	ZOSTER	_	_	_	_	_	_	_	+
15.62	C.P	_	_	_	_	_	_	_	+
	ZOSTER	_	_	_	_	_	_	_	+
7.86	C.P	_	_	_	_	_	_	_	+
	ZOSTER	_	_	_	_	_	_	_	+
3.98	C.P	_	_	_	_	_	_	_	-
	ZOSTER	_	_	_	_	_	_	_	-
PH VALUES		7.0		7.0		7.5			
PHYSICAL PROPERTIES		Brownish color sticky texture		Brownish Black colour crystals		Black color sticky texture			

C.P= chicken pox virus, For MNTC (+) = TOXIC to Hep-2 cells , (-) = NONTOXIC to Hep-2 cells

For antiviral activity (+) = Presence of antiviral activity, (-) = Absence of antiviral activity

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Fig1: Cytopathic effect of virus isolated from chickenpox patients on HEp-2 cell line.

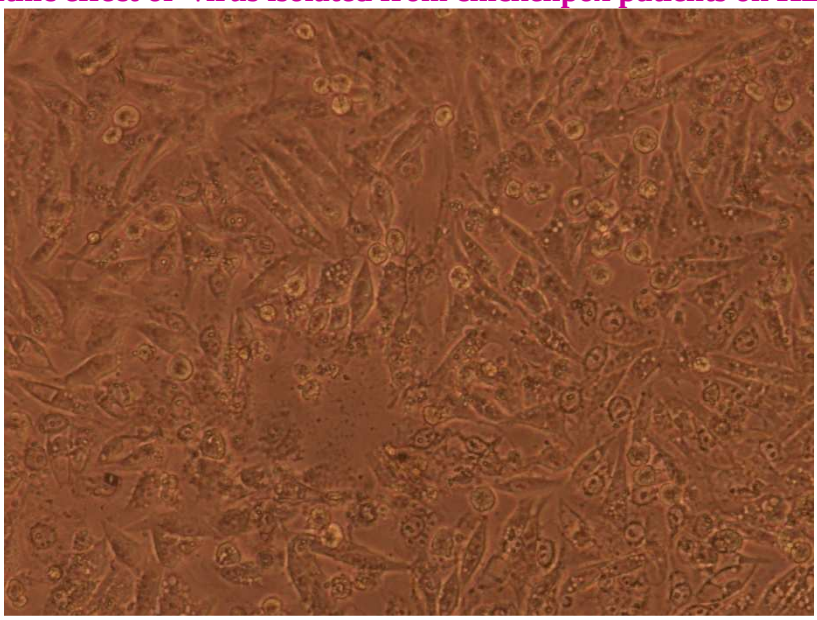

Fig2: Cytopathic effect of virus isolated from zoster patients on HEp-2 cell line.

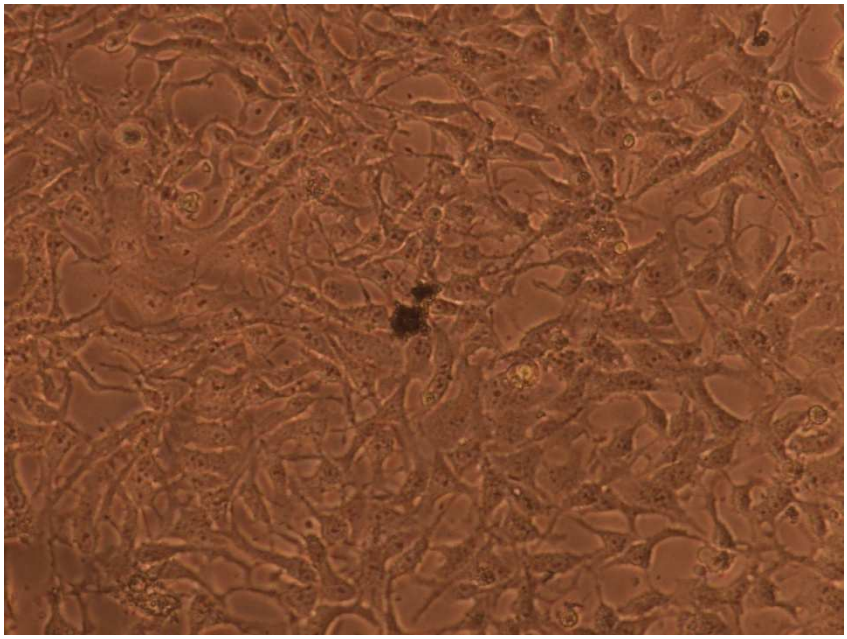

Fig 3: Normal cell control

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