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Evaluation of *in vitro* Antiviral Activity of *Hypericum mysorense* F. Heyne against HSV-2

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ABSTRACT
The medicinal plants have played a vital role in drug discovery. Many plants are used traditionally for the treatment of different types of viral disorders and other infectious diseases. *Herpes simplex virus* (HSV) is a ubiquitous organism that causes infections in human populations throughout the world. It causes a variety of diseases ranging in severity from mild to life-threatening. The present study was to evaluate antiviral activity of *Hypericum mysorense* F. Heyne ethanolic leaf extract against herpes simplex virus type 2 (HSV-2) using HEp-2 cell line. The toxic free concentrations of ethanolic extract were determined using MTT assay, followed by anti HSV-2 activity and a positive control was maintained. 50µg/ml of ethanol extract was observed to be maximum non toxic free concentration against HEp-2 cell line and also exhibited significant inhibitory activity against HSV-2.

Keywords: *Hypericum mysorense,* HEp-2 cell line, MTT assay, Antiviral.

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INTRODUCTION

Plants have been used as folk remedies and ethno botanical literature has described the usage of plant extracts, infusions and powders for century against diseases of viral origin. [1] Medicinal plants have been known for their disease-curing qualities for centuries. Nature has provided us with a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources. Tremendous advances have occurred in recent decades in development of safe and effective medications for the treatment of viral diseases. [2] The herbs used in traditional medicine are the goldmine for current therapeutics. According to the statistics of World Health Organization (WHO) that more than three forth of the people in the developing world depend on herbal medicines. *Hypericum mysorense* is a shrub and small tree native to the Nilgiri Hills in India. *H. mysorense* is reported in Ayurveda for having antiviral and nerve calming properties. [3] *H. mysorense* exhibited significant antiviral activity against herpes simplex virus type-1. [4]

Herpes simplex virus type 2 (HSV-2) is the most common cause of genital ulcer disease in worldwide. [5] WHO estimated that 417 million people aged 15-49 years have affected with HSV-2 infection. Taken together, the estimates reveal that over half a billion people between the ages of 15-49 years have genital infection caused by

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either HSV-1 or HSV-2. Acyclovir (AVC) and Pencyclovir have been widely approved drugs for the treatment of HSV infection. [6] However widespread use of these drugs has shown resistance especially in immunocompromised and bone marrow transplant recipient. [5] Hence there is a need to search for new antiviral drug with different mode of action. The current study was aimed to isolate the leaf from *Hypericum mysorense* to carry out its antiviral activity against HSV-2.

MATERIALS AND METHODS

Plant materials

The leaves of *Hypericum mysorense* F. Heyne were collected from in around Ootacamund, Tamil Nadu, India. The plant was identified and authenticated by Botanical Survey of India, (BSI) Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, with the voucher specimen (No.BSI/SRC/5/23/15/Tech-1145).

Preparation of leaf extracts

The leaf sample was shade dried at room temperature. Then pulverized to get a coarse powder and 30 gm of powdered leaves were extracted with 300ml of ethanol using soxhlet apparatus, the extract was then concentrated in vacuum under reduced pressure using rotary evaporator. The crude extract stored below 4°C until further studies.

Cell Culture

Human epithelial type 2 (HEp - 2) cell was obtained from National Centre of Cell Sciences (NCCS, Pune, India). The HEp -2 cell line was cultured with Eagle's Minimum Essential Medium (EMEM) supplemented with 10% FBS (Fetal Bovine Serum), with Earle's salts, L-glutamine, Sodium bicarbonate, HEPES buffer, Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin-B (2.5µg/ml). Cultured cell lines were incubated at 37°C in a 5% CO_2 atmosphere (pH 7.1-7.4). [7]

In vitro cytotoxicity assay

The cytotoxicity activity of leaf extract on HEp-2 cell line was carried out using MTT assay (3-(4, 5-dimethylthazol-2-yl)-2, 5-diphenyl tetrazolium bromide). [8] 0.1 ml of cell suspension at density of 1.4x 10^5cell/ml were seeded in 96 well microtitre plate and incubated at 37°C in a 5% CO_2 atmosphere for 24 hours. Then the medium was removed and 0.1µl of various concentrations (100µg, 50µg, 25µg, 12.5µg, 6.25µg, 3.125µg, 1.56µg/0.1 ml) of leaf extract prepared by diluting with 2% EMEM was transferred into the respective wells. Further 100µl of 2% EMEM was added to all the experimental wells. 0.1% DMSO and positive control (Acyclovir) were maintained and the entire setup was incubated at 37°C in a 5% CO_2 atmosphere for 96 hours. Then the cells were treated with 20µl MTT (5 mg/ml) and incubated at 37°C for 4 hours. Further 150µl of DMSO was added in to all the wells incubated at 37°C for 10 min. The plate was read on a microtitre plate reader (Biotek USA) at 540nm. Percentage viability of cell line was calculated using the following formula

$$\% \text{ Cell viability} = [(\text{O.D of control} - \text{O.D of test compound}) / (\text{O.D. of control})] \times 100$$

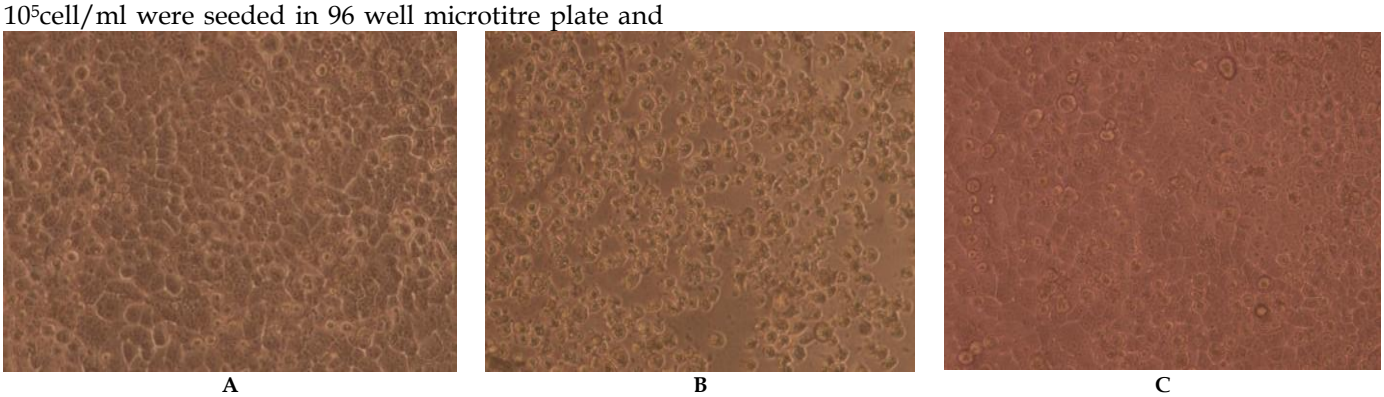
Antiviral study

Virus stock

HSV-2 (Strain No 753167) of National Institute of Virology (NIV), Pune, India, was propagated in HEp-2 cell line and incubated at 37°C for 96 hrs. Complete cytopathic effect virus stock was used for the estimation of $TCID_{50}$ by end point dilution assay and $10^{-6.5}TCID_{50}$/ml virus stock concentration used for the antiviral study. [9]

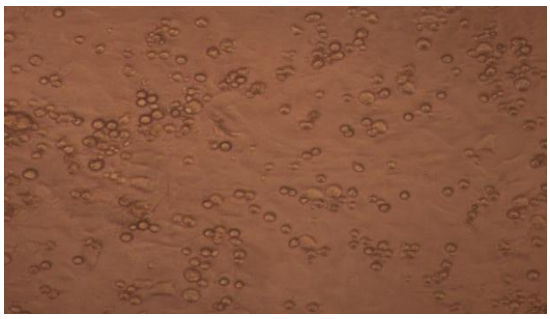
In vitro antiviral assay

The *in vitro* antiviral activity of leaf extract was performed by MTT assay. 100µl of $10^{-6.5}$ $TCID_{50}$ dose of virus suspension in 2% EMEM were added in the confluent monolayer of HEp-2 cells, 200µl of 2% EMEM medium alone was maintained as the cell control. Then the plates were kept for virus adsorption in a 5% CO_2 atmosphere at 37°C for 90 min. 100µl of non toxic concentrations of the leaf extract diluted with 2% EMEM was added with the virus inoculated monolayer cell line. Acyclovir was used as positive control and incubated at 37°C in a 5% CO_2 atmosphere for 96 hours. After incubation cells were treated with 20µl MTT (5 mg/ml) and kept at 37°C for 4 hours. Further 150µl of DMSO were added in to all the wells and placed at 37°C for 10 min. The plate was read on a microtitre plate reader at 540nm.

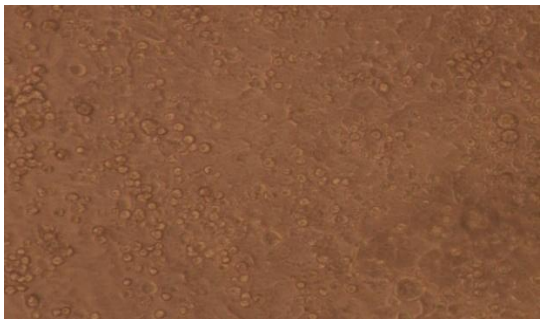


A B C

Fig. 1: Cytotoxic effect of *Hypericum mysorense* leaf extract on Hep-2 cells
A. Control , B. Presence of cytotoxic effect at 100µg , C. Absence of cytotoxic effect at 50µg



a. HSV-2 inoculated on Hep-2 cell line



b. Inhibitory concentration of ethanol extract on HSV-2 at 50µg

Fig. 2: Inhibitory activity of *Hypericum mysorense* leaf extract on HSV-2.

Table 1: Cytotoxicity and antiviral activity of *Hypericum mysorense*

S. No	Concentration of leaf extract µg/ml	Toxicity	Cell control	Antiviral CPE Inhibition assay			
				Cytopathic effect(CPE)	Virus Control	Cell control	Acyclovir
1	100	++	--	NP	+	-	-
2	50.00	--	--	+/-	+	-	-
3	25.00	--	--	+	+	-	-
4	12.50	--	--	+	+	-	+
5	6.25	--	--	+	+	-	+
6	3.12	--	--	+	+	-	+
7	1.56	--	--	+	+	-	+

(++) → Presence of cytotoxic effect, (--) → Absence of cytotoxic effect, (+) → Presence of cytopathic effect, (-) → Absence of cytopathic effect, (+/-) → 50 % of cytopathic effect, NP→Not performed

Each well was observed under inverted phase contrast microscope for every 24 hours. The percentage of cytopathic effect (CPE) was compared with virus control.

RESULTS

In vitro cytotoxic assay

The seven concentrations were performed for *in vitro* cytotoxicity assay, 100µg/ml showed toxic to HEp-2 cell line whereas from 50µg -1.56µg/0.1 ml exhibits non toxic to cell line. The CC_{50} value of ethanol extracts were exhibited at 50µg/0.1 ml (Fig.1a, 1b) (Table 1).

In vitro antiviral activity assay

The ethanol extract inhibited HSV-2 at 50µg/0.1 ml with an inhibitory concentration for IC_{50} value of 25µg/0.1 ml. The standard drug acyclovir showed antiviral activity at 25µg/0.2 ml (Fig.2a, 2b) (Table 1).

DISCUSSION

Many aromatic plants used in phytotherapy are considered to be important sources for the production of raw materials or preparations containing phytochemicals that have significant activity against Microorganisms. Plant extracts have been widely used in traditional medicine for treatment of many diseases.

Medicinal plants were bio assayed against various viruses and which proved to have antiviral activity against HSV-1 and 2. [10] *Hypericum mysorense* is traditionally used in the treatment for antiviral, anxiety and inflammation. Hypericin and Pseudohypericin, were obtained from *H. mysorense* had been reported to be effective as virucidal agents. [11] The two compounds have been shown to be active against a broad range of viruses such as HSV-1 and 2, vesicular stomatitis and influenza viruses, cytomegalovirus and human immunodeficiency virus 1 (HIV-1). [12] The plants extracts obtained from Turkish medicinal plants including *Hypericum capitatum* and *Hypericum scabrum* and their extracts obtained from cell cultures were investigated for the cytotoxic properties and antiviral activities against herpes simplex viruses (HSV-1 and 2) and HIV-1. [13] Vijayan *et al.,* (2003) had reported the methanol extracts of the aerial parts of *Hypericum mysorense* and *Hypericum hookerianum*, exhibited detectable antiviral effect towards HSV-1 with an inhibitory concentration for fifty percent (IC_{50}) of 100µg/ml and 50µg/ml. [3] Here in the present study, ethanol leaf extract of *H. mysorense* revealed antiviral activity (HSV-2) at the concentration of 50µg/0.1 ml by MTT. Though HSV infection is usually managed with effective anti herpes synthetic drugs like acyclovir (ACV), continuous exploitation of these drugs lead to the development of resistant strain. Thus the result of the current study leads to the alternative source of treating the viral infection. Further isolation and purification of molecules responsible for the activity, present in the ethanol extract of *H. mysorense* will be effective in future perception.

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