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Antiviral prospective of *Tinospora cordifolia* on HSV-1

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A B S T R A C T

HSV-1 also known as Oral herpes causes sores or lesions in the gums or near the mouth. Dry stem of *Tinospora cordifolia* is powdered and extraction is carried out in soxhlet apparatus using the solvent Methanol and Ethyl Acetate in the ratio 80:20. The crude extract was subjected to prelimnary phytochemical analysis which indicated the presence of Saponins, alkaloids, phytosterols and triterpenoids. The preparation of virus pool was carried out using Vero cell lines. MTT assay was conducted and the cytotoxicity level of *T.corfiolia* on the cells was obtained as 315.68±7.8. Viral titration was carried out followed by Virucidal assay and it was concluded that *T.cordifolia* inhibits the growth of HSV by 61.43% at 10TCID$_{50}$.

Introduction

Herpes is a Viral infection caused by *Herpes simplex Virus* (HSV). Herpes can be classified into two types as HSV-1 and HSV-2 based on the area of infection. At present no cure or vaccine is availiable for this particular disease. Paracetamol, lidocaine, acyclovir and valacyclovir are the commonly available antiviral medication which may help lessen the severity of the infection (1). The research work carried out aims at identification of a competent drug capable of inhibiting the growth of virus which inturn will lead to reduction of the symptoms faced during the outbreak of Herpes. Though controversies prevail research has proved that the use of food rich in Lysine, argenine and citric acid may lower the symptoms or severity of the outbreak (2). Many herbal extracts including ginger, garlic, onion, banana, honey, goldenseal, grape seed extract are known to reduce the blisters caused by *herpes simplex virus* yet not known to completely cure the disease (3). *T.cordifolia* is one among the most ancient herb used by the medical practitioners in cure of a wide variety of diseases. The plant has been used since time immemorial in curing of skin problems, allergies, inflammation and is used in

preparation of many ayurvedic medicines(4). Researchers have proved that the plant contains a wide range of secondary metabolites like the tannins, alkaloids, cardiac glycosides, tannins, saponins, triterpenoids, phytoserols and polyphenols which have proved to be of great medicinal value and act as immunomodulators, antidiabetic medicine, anti allergic, anti leprotic and anti inflammatory agents(5). Recent research was conducted and a remarkable activity has been proved on the activity of *T.cordifolia* extract on HIV. Also few studies have been conducted on the antiviral properties of *Tinospora* and have been reported to be of great value. Therefore, the objectives of this work were (i) to test the efficacy of crude extract of *T.cordifolia* on HSV-1 (ii) to calculate the percentage inhibition of HSV by *T.cordifolia* extract.

Materials and Methods

Collection of raw materials

Fresh plant material of *T.cordifolia* was procured from Tellicherry a town in Kannur District of Kerala state in south India. The plant material was shade dried until all the water molecules evaporated and plants became well dried for grinding(6). Fig 1.

Preparation of plant Extract

Dry stem of *T.cordifolia* was powdered and is used for extraction using soxhlet apparatus(7). The solvent used was methanol and ethyl acetate in the ratio 80:20. Crude extract is obtained and carried for phytochemical analysis.

Phytochemical analysis

Phytochemical analysis is conducted to analyze the crude extract obtained from *T.cordifolia*.

Preparation of culture media

Stock cells and Herpes Simplex Virus (HSV-1) was cultured in MEM along with 10% inactivated FBS, 100IU/ml of penicillin, 100µg/ml of streptomycin and 5µg/ml amphotericin B in a CO_2 incubator with 5% CO_2 at 37°C until confluency. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS) (8).

Preparation of virus pool

A monolayer cell culture was prepared and inoculated with 100µl of virus suspension and was incubated for 1 hour at 37^0C for virus adsorption. After 1 hour of incubation 5ml of MEM along with 2% serum was added onto the monolayer and incubated at 37^0C and observation of cytopathic effect (CPE) was practical 24 hour onwards. The cells were frozen at -70^oC and thawed at RT thrice repeatedly, on observation of 100% CPE and the supernatant i.e. cell free extract was collected by centrifuging the cell suspension (8).

MTT Assay

The test drug was weighed and dissolved in distilled DMSO and volume was made up using MEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration (9). Trypsinization was carried out and the cell count was adjusted to 1 x 10^5 cells/ ml by diluting it with MEM containing 10% FBS. 0.1ml (approx. 10,000 cells) of cell suspension was added to each well of the 96 well titre plate and left for 24 hours incubation (10). After the incubation the supernatant was removed and the partial monolayer was washed with fresh media. Subsequently 100 µl of different concentrations of the test drug were added to the microtitre plates with partial monolayer. The microtitre plates were

incubated in CO_2 incubator at 37^0C and 5% CO_2 for 3 days. Microscopic examination of the plates was done on regular interval of 24h and the observations were recorded. After 72 h, the drug solutions were discarded from the wells and 50 µl of MTT in PBS was added to each well (10). The plates were lightly shaken and incubated for 3hr at 37^0C in 5% CO_2 atmosphere. The supernatant was removed and the plate was treated with 100µl of propanol to solubilise formazan and the absorbance at 540nm was measured using microtitre plate reader (12). The percentage growth inhibition was calculated and the CTC_{50} value is generated from the dose-response curves for each cell line.

Virus Titration

The flask containing the monolayer was tripsinized and was seeded into a 96 well plate with an approximate 10,000 cells/well. The virus stock was serially diluted using tissue culture medium containing 2% serum. Further 100µl of each dilution was added into 6 wells each of a 96 well microtitre plates and it is incubated at 37 ºC with 5% CO_2 atmosphere and was observed for viral CPE at every 24 hour interval. 50% Tissue Culture Infect ($TCID_{50}$) was calculated using Reed and Muench method (13).

Virucidal Assay

The virus suspensions of 10 $TCID_{50}$ were incubated with test compounds of concentration 100µg/ml and 50µg/ml. (14). The solvent which was used to dissolve test compound along with virus suspension is used as virus control. After 1 hour, 100 µl of each mixture containing the test drug and virus suspension was added to the monolayer cultures which were grown in 96 well microtitre plates. CPE was observed every 24 hours to 96 hours and was compared with control and the readings are jotted down and are scored.

Results and Discussion

25g of dried and powdered stem of *T.cordifolia* was extracted using methanol and ethyl acetate in the ratio 80:20 for a total volume of 0.4L in the soxhlet apparatus. The yield of extract obtained was 0.95g which is equivalent to 3.8%.

The crude sample obtained by extraction was subjected to phytochemical analysis using the standard tests for phytochemical analysis as stated in Table 1 and was noted for the presence of Saponins, Alkaloids, Phytosterols and Triterpenoids.

The crude sample was aliquoted into a range of concentrations as represented in Table 2 to check the percentage cytotoxicity level by means of the MTT assay. The percent cytotoxicity offered by cells under varying concentration levels is as noted in Table 2 and the CTC_{50} was obtained as 315±7.5µg/ml. Fig 1 represents the Cytotoxic effect of the test drug on the Vero Cell lines under 1000µg/ml, 500µg/ml and control (Fig 2).

Virus titration was carried out in a 96 well plate with an approximate 10,000 cells/well. After further carrying out of the dilutions and incubation $TCID_{50}$ was calculated using Reed and Muench method (13).

Table 3 represents the microscopic observation of the growth of the organism and Table 4 represents the calculation of the accumulated value based on the Reed and Muench method.

The observation proves that the mortality rate in the dilution 10^{-5} is higher than 50 % and in 10^{-6} is 13%. Thus the $TCID_{50}$ was calculated using the formula as stated below:

$$TCID_{50} = \frac{(\%CPE\ at\ dilution\ next\ above\ 50\%) - 50}{(\%CPE\ at\ dilution\ next\ above\ 50\ \%) - (\%CPE\ at\ dilution\ next\ below\ 50\ \%)}$$

$$\frac{71-50}{771-13} = \frac{21}{58} = 0.36\ or\ 0.4$$

Negative logarithm of the lowest dilution = -6.0 and proportionate distance (0.4) * log dilution factor = -0.4. Thus the virus titre obtained for the virus was $10^{-6.4}$/ml

In the presence of test drug the virucidal assay measures less than or equal to 50 % reduction in viral titre when compared to the untreated cells. The inhibition is determined using end point titration which will evaluate the virucidal activity after preincubation of the virus along with the *T,cordifolia* extract. 50% end point titration is carried on confluent monolayers, infected with 10 fold serial dilutions in a 96 well titre plate. After incubation CPE was calculated and the percentage protection offered was calculated and is tabulated in Table 4 (Fig 3). It was observed that at a test concentration level of 100µg/ml and 50µg/ml the percentage protection offered is approximately 61.43 % and 23.22 % respectively.

Table.1 Preliminary Phytochemical tests for Tinospora cordifolia extract

Sl. No	Test	*Tinospora cordifolia* Extract
1	**Test for carbohydrates** a. Molisch's test	 -
2	**Test for Glycosides** a. Keller-Killiani test	 -
3	**Test for Saponins** a. Foam test	 +
4	**Test for Alkaloids** a. Mayer's test b. Dragendrodroff's test	 + +
5	**Test for Flavonoids** Alkaline reagent test	 -
6	**Test for Phenolics and Tannins** a. Ferric chloride test b. Test for Tannins	 - -
7	**Test for Phytosterols and Triterpenoids** a. Leiberman-Bucharat test b. Salkowaski test	+ +
8	**Test for fixed oils and fats** **a. Oily spot test**	 -

Table.2 Cytotoxicity testing against Vero cell lines

Sl. No	Name of Test sample	Test Conc. (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
1	T.C (Met. & EA)	1000 500 250 125 62.5	79.17±0.7 60.71±1.4 46.18±0.5 27.18±0.6 12.00±1.4	315.86±7.5

Table.3 Microscopic Observation of 96 titre plate

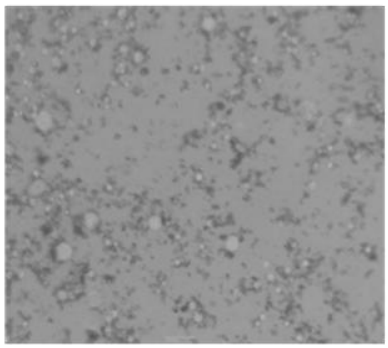
Dilutions	Observation					
10^{-1}	-	-	-	-	-	-
10^{-2}	-	-	-	-	-	--
10^{-3}	-	-	-	-	-	-
10^{-4}	-	-	-	-	-	-
10^{-5}	-	+	-	+	-	-
10^{-6}	+	-	+	+	+	+
10^{-7}	+	+	+	+	+	+
Controls	+	+	+	+	+	+

"+" = Survived, "-" Dead

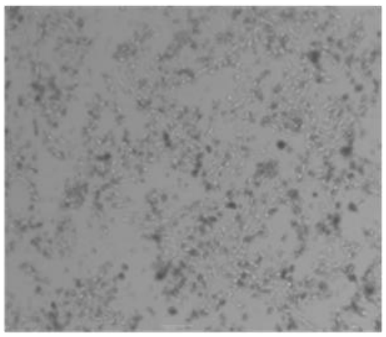
Fig.1 Dry stem of *T.cordifolia*

Fig.2 Cytotoxic effect of the sample on Vero cell lines

Control (Vero)



Vero T.C(Met & EA) (1000 µg/ml)



Vero T.C(Met & EA) (500 µg/ml)

Table 4 Arrangement of data used in computation of $TCID_{50}$ titer by Reed and Muench formula

Virus dilution	CPE ratio	Wells (+)	Wells (-)	Accumulated values			
				CPE	CPE	CPE	Percentage
10^{-1}	6/6	6	0	29	0	29/29	100
10^{-2}	6/6	6	0	22	0	22/22	100
10^{-3}	6/6	6	0	17	0	17/17	100
10^{-4}	6/6	6	0	11	0	11/11	100
10^{-5}	4/6	4	2	5	2	5/7	71
10^{-6}	1/6	1	5	1	7	1/8	13
10^{-7}	0/6	0	6	0	13	0/13	0

Table.5 Inhibitory activity of test substances against HSV-I induced cytopathic effect

Sl. No	Sample Name	CTC_{50} (µg/ml)	Test Concentration (µg/ml)	% Protection offered $10TCID_{50}$
1	T.C. (Methanolic & Ethyl acetate)	315.86±7.5	100 50	62.04±3.51 24.03±2.39

Fig.3 Inhibitory activity of test substances against HSV-1 induced cytopathic effect

The Preliminary photochemical analysis confirmed the presence of saponins, alkaloids, phytosterols and triterpinoids in the crude extract obtained from T.cordifolia using methanol and ethyl acetate. Cytotoxicity analysis as shown in Table 5 was carried out using different test concentrations ranging from 1000µg/ml to 62.5µg/ml and the CTC_{50} was calculated to be 315.86±7.5µg/ml. Virus titration was carried out by Reed and Muench

method and the value obtained was $10^{-6.4}$ / 1 ml. Virucidal assay was conducted at $10TCID_{50}$ for the test concentration 100µg/ml and 50µg/ml and it can be concluded that the percentage protection offered was observed to be 62.04±3.51 and 24.03±2.39 respectively.

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