Research Article

In Vitro Antiviral Activity of Fungal Secondary Metabolites Against Herpes Simplex Virus-2

D. Anand[1]*, S. Marimuthu[1], P. Saravanan[1], S. Rajarajan[2]
[1]P.G & Research Dept of Botany, Ramakrishna Mission Vivekananda College (Autonomous), Mylapore, Chennai, Tamilnadu, India.
[2]Vice Chancellor, SRM University, Sonepat, Haryana, India.
***Corresponding author's E-mail:** anandesingh@yahoo.co.in

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ABSTRACT

Herpes simplex virus-2 found in throughout the world which infects children's to adults especially immune compromised patients. Virus replication leads to disease and infrequently results in life threatening infection, the host virus interaction leads to latency predominant. Some of the antiviral compounds have toxicity which was tried as therapeutic use in earlier decades. Hence there is a need to search for new antiviral compounds with different mode of action. Fungal secondary metabolites are recognized as source of novel bioactive compounds. The current study was performed to isolate the secondary metabolites of endophytic fungi from a potential medicinal plant *Pongamia pinnata*. The six different concentrations of fungal compounds were used for *in vitro* cytotoxicity assay on HEp-2 cell line. The toxic free concentrations of fungal compounds were assed for antiHSV-2 activity. 40µg/ml of secondary metabolites was shown nontoxic to cell-line and exhibited the inhibitory activity against HSV-2.

Keywords: *Pongamia pinnata*; *Colletotrichum*; Cell line; Cytotoxicity; *Herpes Simplex Virus*; Antiviral.

INTRODUCTION

Herpes Simplex Virus type 2 (HSV-2) is a member of the subfamily of Alphaherpesviridae. HSV-2 is large, enveloped with double stranded DNA genome and it was linear in the virus particle. HSV-2 was found to be associated with genitial disease (infection below the belt) and usually acquired through sexual contact and the antibody to this virus is rarely found before the age of onset of sexual activity[1]. Nucleoside derivative drugs such as Acyclovir (ACV), Gancyclovir and Pencyclovir have been widely approval drugs for the treatment of HSV infections[2]. The widespread use of these drugs had shown resistant especially in immunocompromised and bone marrow transplant recipients[3-4]. In order to circumvent the problems of viral resistance and the development of new antiviral product, with the different mechanism of action are very much required. Natural products continue to be an important source of new pharmaceutical products[5]. Endophytic fungi are known to colonize internal plant tissue and they can live without causing any apparent damage or disease and isolated from different parts of plants[6]. These endophytes can be transmitted from one generation to the next through tissue of host seed[7]. Several crude extracts from different culture broths have shown antimicrobial activity against pathogenic fungi; cytotoxicity activity on human cell line, anti-herpes simplex virus type 1 activity (anti-HSV) and antimalarial activity against the protozoan *Plasmodium falciparum*[8]. Since the "gold" bioactive compound paclitaxel (taxol) discovered from the endophytic fungus *Taxomyces andreance*[9]. Plant endophytic fungi have been recognized as an important and novel resource natural product with potential application in agriculture, medicine and food industry[10-11]. Hence the present study was carried out to isolate fungal secondary metabolites from leaf of *Pongamia pinnata* and its antiviral activity against HSV-2 was performed.

MATERIALS AND METHODS

Materials Used

The cultivated plant leaf of *Pongamia pinnata* (Linn) Pierre. (Local name: Pungam, English name; Indian-beech) was collected during the month of November (Longitude 80° 17' E & Latitude 13° 04' N) and were identified and authenticated at the Department of Botany, Ramakrishna Mission, Vivekananda College, Mylapore, Chennai, India. Leaf sample was transferred to sterile, labeled polythene bags and processed within 24 hrs of collection[12].

Isolation and identification of fungi

The leaf samples were washed thoroughly in running tap water and air dried. The leaf was cut into small pieces of 0.5 cm^2, leaf pieces were taken for the study from the mid-rib portion of lamina. Then the leaf pieces were surface sterilized by immersion in 70% ethanol for 5 seconds. Followed by immersed in 4%NaOCl for 1 minute and rinsed in sterile distilled water for 10 minutes. The surface sterilized leaf segments placed with equal spaces in Petri dishes containing Potato Dextrose Agar (PDA) medium (amended with chloramphenicol 150mg/L). The Petri dishes incubated at 26 ± 1° C in a light chamber and monitored everyday for the growth of fungal colonies from the tissues of leaf segments[13]. The sterile mycelia that grew out from leaf segments were sub cultured on a PDA medium and exposed to light for induce sporulation. The isolated fungi was examined periodically and identified according to the morphology and the mechanism of spore production (Colony colour, texture,

habit and growth rate). The morphological characteristic features were confirmed and reported[14-15].

Extraction of fungal secondary metabolites

The fungus was grown in one liter Erlenmeyer flask containing 500ml of Potato Dextrose Yeast Broth (PDYB) and incubated for 21 days at 26 ± 1° C[16]. The liquid culture filtrate was collected and extracted with equal volume of ethyl acetate and was kept overnight at room temperature. Sample was evaporated under pressure using rotary evaporator at 86° C and the final dry sample was collected in sterile vials. 10mg of dry sample was resuspended in 1ml of DMSO (10% - Dimethyl sulfoxide in deionized water) and it was filter sterilized. The sterile secondary metabolites were used for cytotoxicity and antiviral studies.

Cell line

HEp-2 cell line was grown in Eagle's minimum essential medium (EMEM) with5% v/v Foetal bovine serum (FBS), Penicillin, Streptomycin, Amphotericin-B, L-Glutamine, Sodium bicarbonate and HEPES (2-[4-(2-Hydroxyethyl)1-piperzinyl ethane sulphonic acid) buffer[17]. Appropriate volume of CO_2 was passed into the medium till the pH reached the optimum range (i.e.) 7.1 - 7.5.

***In vitro* cytotoxicity assay**

After enumeration of the number of viable cells, 0.1 ml (1.4 x 10^5 cells/ml) of the cellular suspension was transferred into wells of 96 well microtitre plates[18]. The entire setup was incubated at 37°C in a 5% CO_2 atmosphere for 12 hrs. The following concentrations of fungal extracts of 60µg, 50µg, 40µg, 30ug, 20µg and 10µg were diluted with 2% FBS, MEM and 0.2ml of the each fungal extract with the medium were transferred on the monolayer of cell lines and incubated at 37°C in a 5% CO_2 atmosphere for 96 hrs (4 days). Detectable changes in the morphology of the cells, rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

Antiviral study

Virus stock

HSV - 2 (753167) strain of National Institute of Virology (NIV) Pune, was propagated in HEp-2 cell line and incubated at 37°C for 4 days. Complete cytopathic effect (CPE) in HEp-2 cell line was observed on the 4th day. Thus virus stock was used for the estimation of $TCID_{50}$ by End point dilution assay[19] and $10^{6.5}$ $TCID_{50}$/ml virus stock concentrations used for the antiviral study.

***In vitro* antiviral assay**

Monolayer of HEp-2 cells were grown in a 96 well microtitre plates and 0.1 ml viral suspension, obtained by seven consecutive ten-fold dilution in 2% FBS MEM, was added into five wells of microtitre plates (Four for antiviral, one for virus control). 0.1 ml of 2% FBS maintenance medium alone was added into cell control. The 96 well microtitre plates for HSV-2 was incubated at 37°C in 5% CO_2 atmosphere for 60 minutes to facilitate adsorption of virus to the cell line. The following concentrations of toxic free fungal metabolites of 40µg, 30ug, 20µg and 10µg were diluted with 2% FBS, MEM and 0.2ml of the each fungal extract with the medium were transferred on the virus inoculated monolayer cell lines and reincubated at 37°C temperature for 3-4 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. A gross absence of cytopathic effect was inferred by intact of cell layer, without distortion in the morphology of cells or nucleus and syncytial cells in comparison with the control rows and thus wells with or without CPE were marked. Important observations of the incubated wells, both experimental and control were recorded by microphotography through inverted tissue culture microscope. The standard antiviral agent of Acyclovir (1mg/mL) was used as positive control.

RESULTS

Identification of fungi

The fungal colony was loose with white aerial mycelium which later becomes orange colour. Several black, acervulus like masses developed on the culture plates after incubation for 10 days at 25°C with dark-orange drops of conidial masses mostly coalesce together. Conidia were aseptate, hyaline, mostly ellipsoid ranging from 12-17× 4-8µm (average 13.5×6.0µm) in size (Fig. 1 and Fig.2).

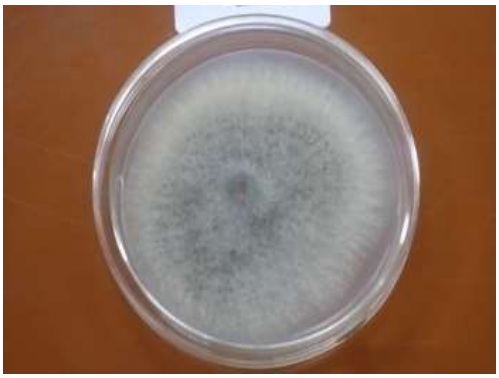

Figure 1: Growth of *Colletorichum sp.*

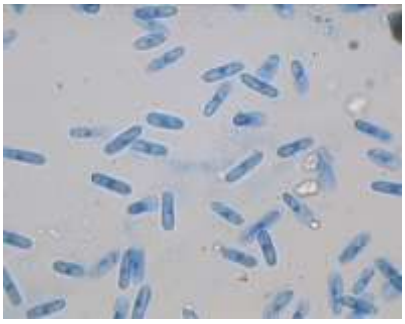

Figure 2: Conidia of the fungus

***In vitro* cytotoxicity assay**

Cytotoxic effect of fungal secondary metabolites was determined in 96 well microtitre plates. The toxic concentration and non-toxic concentrations were inferred by distortion and lysis of cells or by the intact healthy cell line. The maximal toxic free concentration of secondary metabolites was 40µg and other concentrations 30 µg, 20 µg, 10 µg did not show any cytotoxicity effects 50µg and 60µg showed toxic to HEp-2 cell line (**Table-1) (Fig. 3a, 3b)** Cell control and DMSO did not show any cytotoxicity.

***In vitro* antiviral assay**

The toxic free concentrations of secondary metabolites of 40µg, 30µg, 20µg and 10µg were used for anti HSV-2 study. The secondary metabolites of *Colletotrichum sp.* inhibited HSV-2 replication at 40 µg concentration **(Fig.3c, 3d).** The other concentrations like 30µg, 20µg and 10µg did not show any inhibitory effect on HSV-2 and the complete cytopathic effect were observed. HSV-2 infected cells treated with acyclovir indicated complete inhibition was observed at a concentration of 25µg/0.2ml. The results were tabulated (**Table-2**).

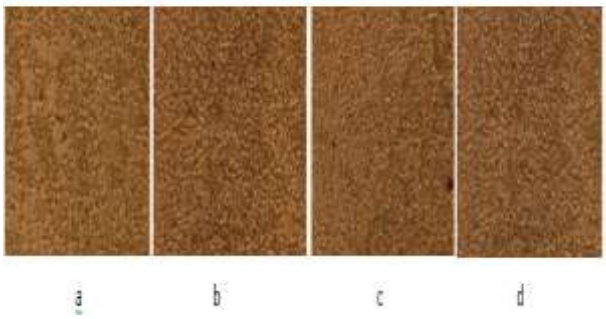

Figure 3: a. Presence of cytotoxic effect of secondary metabolites on HEp2 cell line

b. Absence of cytotoxic effect of secondary metabolites on HEp2 cell line

c. HSV-2 inoculated HEp-2 Cell line

d. Inhibitory activity of fungal secondary metabolites on HSV-2.

Table 1: Cytotoxicity activity of secondary metabolites of *Colletotrichum sp.* on HEp-2cells

S. No	Concentrations of Secondary Metabolites (µg)	Toxicity	Cell Control	DMSO Control
1.	60	+	–	–
2.	50	+	–	–
3.	40	–	–	–
4.	30	–	–	–
5.	20	–	–	–
6.	10	–	–	–

(+) → *Presence of Cytotoxicity* (–) → *Absence of Cytotoxicity*

Table 2: Inhibitory activity of secondary metabolites of *Colletotrichum sp.* against HSV-2

S. No	Concentrations of Secondary Metabolites (µg)	CPE	Virus Control	Cell Control	Acyclovir
1.	40	+	+	–	+
2.	30	+	+	–	+
3.	20	+	+	–	–
4.	10	+	+	–	–

(+) → Presence of cytopathic effect (CPE); (–) → Absence of cytopathic effect (CPE)

DISCUSSION

The emergence of drug resistant microbial pathogen is leading to the increased demand of potent antibiotics. The search of new biotic for screening large number of antimicrobial isolate cultured from nature[20]. HSV-2 is a common human pathogen which causes primary infection either in child or adult is usually mild or unnoticeable but may be severe. The recurrent episodes may or may not be associated with lesions but in either case they provide a source of infection with HSV-2, which are likely to be more frequent they cause sexual disability and mental distress. The secondary metabolites have been isolated from the endophytic fungi and its potency was proved by several drug discovery programs. The *Pongamia pinnata* is a potent medicinal plant and the antimicrobial activity of various components was proved against skin infection causing microorganisms[21-22]. The number of fungal compounds exhibited anti-tumour activity and anti-cancer activity to various cell lines[23]. Fungi secondary metabolites exhibit a large number of biological activities making Basidiomycetes an alternative target for antiviral

research[24]. Sorimachi (2001) studied that aqueous extract and fractions obtained by alcoholic extraction of *Agaricus blazei* were able to inhibit the cytopathic effect of Western equine encephalitis virus, poliovirus and HSV in cultures of Vero cells[25]. However the present study was performed to find out the toxic free concentration of secondary metabolites on HEp-2 cell lines. The secondary metabolites of *Colletotrichum* sp. showed non-toxic to cell line at 40 µg concentrations. Antiviral activity of endophytic fungi has been reported by few groups. The antibiotic produced by endophytic fungi was performed on the anti-viral, against human-cytomegalovirus (HCMV) and the two components of cytonic acid A & B showed inhibitory activity on HCMV[26]. The isolation of secondary metabolites of *Colletotrichum sp*. from leaf of *Pongamia pinnata* and its anti-HSV-1 activity was reported. The secondary metabolites of *Colletotrichum sp.* exhibited inhibitory activity against HSV-1[27]. Based on review of literature none of the work was carried out on the anti HSV-2 activity using fungal secondary metabolites.

CONCLUSION

The current study exhibits inhibitory activity of secondary metabolites against HSV-2 at 40µg concentration. Further research on the isolation of active pure compound from secondary metabolites of *Colletotrichum* sp. will help us to carry out studies on drug resistant viral strains. This will also encourage doing the research on the fungal bioactive compounds against the viral strains. The identified compounds will be reducing the frequency of recurrences of acute HSV infections.

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