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Antiviral Activity of Ethanolic Extract of *Phyllanthus vasukii sp. nov*. of the Family Phyllanthaceae

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Abstract
The genus *Phyllanthus* is well known for its medicinal importance, particularly for antiviral and hepatoprotective properties. The present study aimed to evaluate the in vitro antiviral activity of ethanolic extracts of the root and aerial parts of *Phyllanthus vasukii* sp. nov. (Family: Phyllanthaceae), an endemic species from the Southern Eastern Ghats of Tamil Nadu, India. Antiviral efficacy was assessed against herpes simplex virus type-1 (HSV-1) using the MTT colorimetric assay in Vero cell lines, along with cytotoxicity evaluation. The ethanolic extracts exhibited detectable antiviral activity, with IC_{50} values of 247.20 µg/ml for root extract and 122.26 µg/ml for aerial part extract. Both extracts showed partial inhibition of viral replication at higher concentrations and demonstrated significant cytotoxicity against Vero cells. The antiviral activity may be attributed to the presence of bioactive phytoconstituents such as flavonoids, phloroglucinol derivatives, sterols, and other secondary metabolites reported in *Phyllanthus* species. The findings suggest that *P. vasukii* possesses potential antiviral properties and supports its traditional medicinal relevance. Further studies are required to isolate, characterize, and elucidate the mechanism of action of the active compounds for possible pharmaceutical applications.

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INTRODUCTION

India has a rich sources of diversity of medicinal plants which are distributed in various geographical and ecological environment. It is well known that the traditional medicines were practiced in India in the Indian system of medicines specifically such as Ayurveda, Siddha, Homeopathy and Unani. In these system innumerable medicinal plants were employed for the preparation of drugs for specific ailments and documented in the ancient texts (Sumarta *et al.*, 2018). The genus Phyllanthus consists of 750 species and distributed in all over the world especially in China, Thailand, India, Zambia, Nigeria, Japan and Latin America. These species were used for the treatment of liver, kidney, diabetes and intestinal parasite. The plant *P.amarus* root extract inhibited Hepatitis B viral infection and Jaundice. All the species of *Phyllanthus* are enriched with specific phytoconstituents such as Phyllanthin, Hypophyllanthin, Niranthin and Geranin (Tripathi, A.K *et al*., 2006) are

responsible for biological and pharmacological activity. In the present investigation, the medicinal plant, *Phyllanthus vasukii sp.nov*. of Phyllanthceae used to study antiviral activity.

MATERIAL AND METHODS

Collection of plant material

Phyllanthus vasukii. *sp*. *nov*. (Phyllanthaceae) plant was collected at the Paramathi Vellore village, Namakkal, Southern Eastern Ghats, Tamilnadu, India. This plant was newly described one by Parthiban *et al*. (2016).The plant was identified and authenticated by Prof. Rajendran, Head and Dean of Science, Department of Botany, Bharathiar University, Coimbatore. The voucher specimen was deposited at the Department of Botany, Bharathiar University, Coimbatore, Tamilnadu.

Habitat and ecology: It is a shrub, woody and grows upto 150cms high, growing along the cultivated land, especially wetland field, in moist grassy wetland, Namakal District (11°14'N 78°10'E / 11.23°N 78.17°E), Tamil Nadu, India.

Distribution: Endemic to Southern part of Tamil Nadu.
Citation: *Phyllanthus vasukii* Parthipan *et al*., in *Int. J. Adv. Res.* 5(3), 2054-2059. 2017.

Antiviral activity

MTT antiviral assay

In vitro anti-viral evaluation procedure was based on spectrophotometrical assessment for viability of virus- infected and mock infected cells via *in situ* reduction of a tetrazolium dye MTT. Mitochondrial enzymes of viable cells convert yellow water soluble dye MTT to a soluble, purple coloured insoluble formazan. The quantification of the amount of the formazan product present in each well of the microtitre plate was then determined spectrophotometrically at 490/650 nm. The toxicity of the test compounds to host cells was measured concurrently in the same microtitre plate (Takeuchi *et al*., 1991)

Procedure

The host cells (1×105 cells ml-1) were seeded on 96-well tissue culture plates. After a 24 h period of incubation, the medium was removed and the Adeno virus type VIII was added at a dose of 10 TCID50 for 2 h to ensure the attachment of virus to the cell. After 2 h the cells were washed with PBS and replenished with 100 µL of medium containing increasing concentrations of the sample (serially diluted twofold). As cell control, 100 µL of medium was added and as virus control 100 µL of 10 TCID50 dose was added. After three days of incubation, the medium was removed and 50 ml of MTT solution (2 mg ml-1) was added to each well for 4 h at 37 ^{o}C. Then, 100 µL of iso-propanol was added to each well in order to dissolve the formazan crystals. The plates were gently shaked for 10 min to dissolve the crystals. The colour reaction was measured in an automated microplate reader at 490 nm. The untreated control was arbitrarily set as 100%. For each sample, the percentage of cell protection/virus inhibition can be calculated as follows,

$$\frac{\text{Mean OD of control group} - \text{Mean OD of treated} \times 100}{\text{Mean OD of control group}}$$

In vitro cytotoxicity assay

Determination of mitochondrial synthesis by MTT assay

Principle

The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The cleavage of MTT to a blue formazan derivative by living cells is clearly a very effective principle on which the assay is based. The principle involves the cleavage of tetrazolium salt MTT (3-(4,5 dimethyl thiazole-2 yl)- 2,5-diphenyl tetrazolium bromide) into a blue coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The numbers of cells were found to be proportional to the extent of formazan production by the cells used (Francis and Rita, 1986).

Procedure

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0x105 cells/ml using DMEM medium containing 10% FBS. Meanwhile to each well of a 96 well microtitre

plate, 100 µL of the diluted cell suspension (approximately 10,000 cells/well) was added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, the monolayer was washed once with medium and 100 µL of different test sample concentrations prepared in maintenance media were added per well to the partial monolayer in microtitre plates. The plates were then incubated at 37oC for 72 hrs in 5% CO_2 atmosphere, and microscopic examination was carried out and observations recorded every 24 hours. After 72 hours, the sample solutions in the wells were discarded and 20 µL of MTT (2mg ml^{-1}) in MEM-PR (MEM without phenol red) was added to each well. The plates were gently shaken and incubated for 3 hours at 37oC in 5% CO_2 atmosphere. The supernatant was removed and 50 µL of iso-propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540nm.The percentage growth inhibition was calculated using the following formula and concentration of drug or test samples needed to inhibit cell growth by 50% values were generated from the dose-response curves for each cell line.

$$\frac{\text{Mean OD of control group} - \text{Mean OD of treated}}{\text{Mean OD of control group}} \times 100$$

Statistical analysis

All the results were expressed as mean ± standard deviation SD and the data were analysed with the help of one –way analysis of variance (ANOVA) with Punnett's posttests. P-values ($P<0.05$) of the test data were compared with control to determine statistical significant differences. The data were analyzed by the statistical analysis system SPSS (software for windows release 10.0; SPSS Inc., Chicago IL, USA).

RESULTS

Antiviral activity

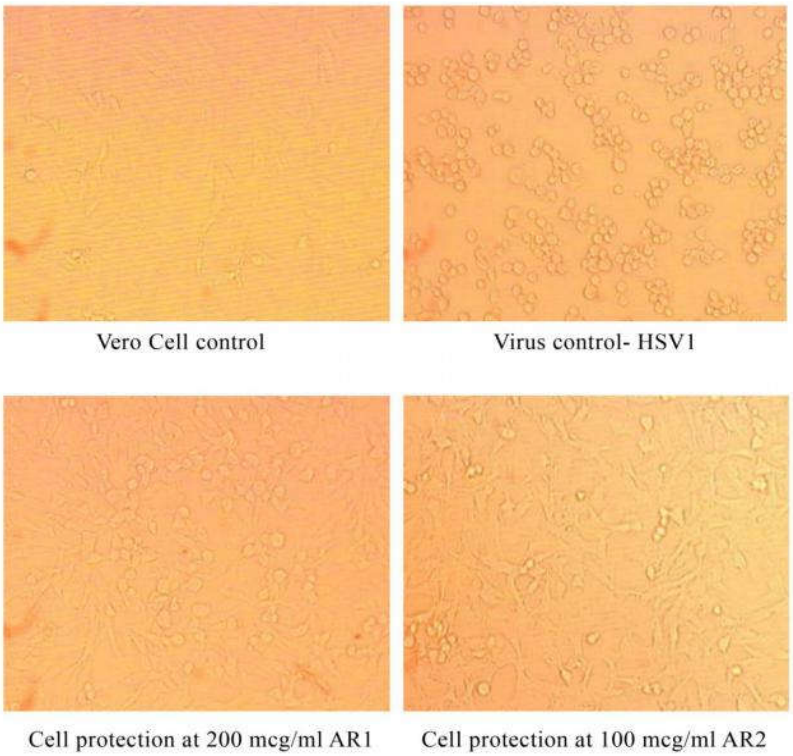
The ethanol extract of the root and aerial parts of *P. vasukii*, exhibited detectable antiviral effects towards HSV-1 with an inhibitory concentration (IC50) value of 247.20 and 122.26, respectively. Both of the extracts exhibited partial antiviral activity at the higher concentrations. The results obtained for per cent cell protection assay and virus yield assay were often comparable. Both of the extract showed significant cytotoxicity against Vero cells, and the per cent cell protection values for root extract were 11.05 and 38.05 and for aerial part extracts were 10.4 and 29.03 (Table 1 and Plate 1 a,b,c &d) respectively.

Table 1: Antiviral activity of extracts against HSV-1 virus type I at 10 TCID50 (50% Tissue culture infectivity dose) and cytotoxicity.

Plant Extract	Extract Concentration µg/ml	10 TCID50 % Cell protection	Vero cells IC50 µg/ml
Root extract	200	38.05	247.20
	100	11.05	
Aerial part extract	100	29.03	122.26
	50	10.04	

Plate - II

Antiviral activity of root and aerial parts of *P. vasukii* against HSV-1 virus type I at 10 TCID50 (50% Tissue culture infectivity dose)



Vero Cell control | Virus control- HSV1

Cell protection at 200 mcg/ml AR1 | Cell protection at 100 mcg/ml AR2

DISCUSSION

An antiviral agent, acyclovir and nucleoside analogue is widely used for the systematic treatment of HSV infections and specifically phosphorated by viral thymidine kinase in the infected cells. It is nonfunctional in the immune compromised patients. So, a new drug for viral infections is to be developed from plants sources which substitute acyclovir. Ethno pharmacology could provide an alternative principle for the discovery of antiviral agents. The polysaccharides (Marchetti *et al*., 1996), anthroquinones (Sydiskis *et al*., 1991), triterpenes (Simoes *et al*., 1998), phloroglucinol (Arisawa *et al*., 1990), flavonoids (Lin *et al*., 2000) and catechin derivatives from some medicinal plants were found to exhibit antiviral activities against replication of HSV-1. In the present study, ethanol extract of *P. vasukii* exhibited a partial antiviral activity. These extracts showed significant cytotoxicity against vero cells. In GC-MS analysis also, the presence of phytoconstituents with antiviral activity such as beta-sistosterol, phenol, amide, sulphur and nitrogen compounds, dibutylphalate and steroids were detected in the extracts of *P. vasukii*. Therefore the obtained antiviral activity of *P. vasukii* extracts against HSV-1 infection could be due to the presence of these compounds. Some plant species of the family Phyllanthaceae such as *P. amarus, P. tenella and P. vergatus* have also exhibited antiviral activity that may be due to the presence of higher content of geranin found in these plants (Bharti, 2009). Administration of leaf powder of *P. amarus* for 2-3 weeks with chronic viral hepatits B patients was found to eliminate antigen (Joseph and Raj, 2011). Similar studies were carried out on *Limonium brasilense, Psidium guava* and *P. niruri* and they exhibited high antiviral activity. The antiviral study on *P. vasukii* also correlated well with the above mentioned properties of the plant extracts.

SUMMARY AND CONCLUSION

The antiviral activity of root and aerial part extracts of *P. vasukii* exhibited detectable antiviral effect towards HSV-1. Both of the extracts have exhibited partial antiviral activity at higher concentrations. The plant extract showed significant cytotoxicity against Vero cells, and showed high percentage of cell protection. *P. vasukii* a new endemic plant possess a rich source of valuable phytoconstituents and could exhibit potential antiviral activities. These findings support the use of this plant for the treatment of folklore medicine to treat and control many diseases. The bioactive secondary metabolites may be further explored for the isolation and purification of its biologically active compound for clear understanding of the mechanism of action. These results could also be of commercial interest for the exploitation of pharmaceutical companies and research institutes for the production of new novel effective and drugs to prevent various human diseases.

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