# CHAPTER 6

## ANTI-VIRAL EFFECTS OF AQUEOUS EXTRACTS OF *HIPPOBROMUS PAUCIFLORUS* AGAINST HERPES SIMPLEX VIRUS 1 (HSV-1) AND COXSAKIE B VIRUS TYPE 6 IN CELL CULTURE

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## Abstract

*Hippobromus pauciflours* (L.f.) Radlk (Sapindaceae) is used as traditional medicine in the Eastern Cape Province of South Africa, for the treatment of eye infections. The aqueous extract from the plant was evaluated for antiviral activity against herpes simplex virus type 1(HSV-1) and coxsakie B virus type 6 *in vitro*. No antiviral activity was noted at concentrations ranging from 3.90 μg/ml to 15.60 μg/ml. Slight antiviral activity of the extract against HSV-1 was noted at concentrations of 31.25 μg/ml 24 h post infection (p.i.) as indicated by the delay in the appearance of a CPE. However, after 48 h p.i. no antiviral activity was noted. Cytotoxicity was evaluated using MTT assay in Vero cells. At concentrations ranging from 165 to 270/μg/ml, significant antiviral activity against HSV-1 as well as toxicity was observed. The extract exhibited no antiviral activity against Coxsakie virus B6.

## Introduction

Herpes simplex virus type 1 (HSV-1), a double stranded DNA containing virus, is a member of the family herpesviridae. It is the leading cause of unilateral infectious corneal blindness worldwide (Liesegang, 2001). Excluding cases of neonatal ocular herpes, which are largely the result of HSV type 2 (HSV-2), over 95% of ocular herpes infections are caused by HSV-1 (Pavan-Langston, 2000). Coxsackie viruses (CVs) are important human pathogens, causing a remarkable variety of diseases, from minor common colds to fatal myocarditis, neurological disorders, possibly acute-onset diabetes, certain chronic diseases, including diabetes, dilated cardiomyopathy, inflammatory myopathy, and chronic fatigue syndrome (Foulis *et al*., 1990; Yousef *et al.,* 1990; Kandolf *et al*., 1993; Muir *et al*., 1996; Galbraith *et al*., 1997).

Acyclovir (ACV) and other nucleoside derivatives, penciclovir, famciclovir, valaciclovir, and ganciclovir have been approved for treatment of HSV-1 and HSV-2 infections worldwide (Galasso *et al*., 1997; Leung and Sacks, 2000). Drugs with clinically relevant activity against HSV infections include interferons (IFNs), acyclovir (ACV), vidarabine (ara-A), gancyclovir (DHPG) and phosphonoformic acid (foscarnet, PFA). However, undesirable complications and the emergence of drug-resistant viruses have urged the development of new antiherpetic agents (Fritz *et al*., 2007). Screening of plant extracts for anti-viral activity has given interesting results for the search for new antiviral agents (Nagasaka *et al*., 1995; Kurokawa *et al*., 1997; Lin *et al*., 1999; Ikeda *et al*., 2000).

In our previous survey of plants used for the treatment of eye infections, traditional healers and rural dwellers of the study area listed *Hippobromus pauciflorus* as the commonest species used for the treatment of eye infections. This plant has been reported for good antimicrobial properties (Pendota *et al*., 2009). However, information on the antiviral activity of the plant has not been reported in the literature. In this study, we examined the antiviral effects of *H. pauciflorus* on HSV-1 and Coxsackie B virus type 6 (Cox B6) *in vitro.*

## MATERIALS AND METHODS

This research activity was conducted at the Department of Medical Virology, University of Pretoria in collaboration with Prof. M.B. Taylor and her co-workers.

### *Preparation of the extracts*

The leaves of the plant were air-dried at room temperature for seven days. The dried material was pulverized with an electric blender. One hundred grams (100 g) of the powder was extracted in 1000 ml of distilled water for 48 h on a mechanical shaker (Stuart Scientific Orbital Shaker, UK). The extract was filtered using a Buchner funnel and Whatman no. 1 filter paper. The resulting filtrate was freeze-dried with Savant Refrigerated Vapor Trap (RV T41404, USA) to give a yield of 10.91 g. A 2000 µg/ml (w/v) stock suspension of the extract was prepared in serum free Eagle's minimum essential medium (MEM) (Highveld Biological [Pty] Ltd, Johannesburg, South Africa). Doubling dilutions of compounds at concentrations from 3.90 µg/ml to 1000 µg/ml, in serum-free MEM, were prepared for assessment of anti-viral activity.

### *Cell cultures*

Standard cell culture techniques Grist *et al.,* (1979), were used for all procedures utilizing cell cultures. Monolayers of the Vero African Green Monkey Kidney (AGMK) cell line (ACACC No.: 84113001; European Collection of Cell Cultures) were prepared by seeding 96-well microtitre trays with $10^4$ cells per well. MEM supplemented with 5% heat inactivated foetal calf serum (FCS) (Gibco Foetal Bovine Serum, Invitrogen Life Technologies,) containing 100 U/ml penicillin and 100 µg/ml streptomycin (BioWhittaker Penicillin/Streptomycin, Lonza, Walkersville, MD) was used for the propagation of the cells. Cell cultures were incubated at $37^0$C in 5% $CO_2$ in air in a humidified atmosphere. Maintenance medium was essentially the same as the propagation medium except that it contained only 2% FCS.

***Virus stock***

A stock suspension of herpes simplex virus type 1 (HSV-1) with a titre of 2.3 X $10^7$ $TCID_{50}$/ml, was prepared from a clinical isolate of HSV-1 which was isolated and identified prior to 2002 (Diagnostics Laboratory, Dept of Medical Virology, University of Pretoria). A stock suspension of a laboratory-adapted strain of Coxsackie B virus type 6 (CV-B6), with a titre of 2.3 X $10^7$ $TCID_{50}$/ml, was prepared and titrated in cultures of the Vero cell line. The viruses was diluted in serum-free MEM and used at a final concentration of 100 $TCID_{50}$/microtitre well.

***Cytotoxicity assay***

The plant extract was tested for cytotoxicity by exposing monolayers of the Vero cell cultures to the extracts. Doubling dilutions of the extract, in serum-free MEM, from concentration of 15.60 to 250.00 µg/ml were used for testing on 24-hour old monolayers of Vero cells. The cells were monitored visually by light microscopy over a period of seven days and on the seventh day tested for cytotoxicity using tetrazolium salt reduction (MTT) assay, using the method of Hussain *et al*., (1993). Monolayers of cells exposed to serum-free MEM alone were used as control.

***Antiviral assays***

To investigate the effect of the aqueous extract of *H. pauciflorus* on viral adsorption and subsequent replication in cell culture, 96-well microtitre trays were prepared as described previously. The appropriate dilutions of the plant extract and 100 $TCID_{50}$ virus were added simultaneously to each of the six wells of the microtitre tray and the infected cell cultures incubated at $37^0$C in 5% $CO_2$ in air in a humidified atmosphere. For positive and negative controls, cells were infected with 100 µl (100 $TCID_{50}$) virus in serum-free MEM and serum-free MEM respectively.

To investigate the effect of the plant extract on the replication of the virus, 24-hour old monolayers of Vero cells in 96-well microtitre trays were starved in serum-free MEM for 1 h at $37^0$C in 5% $CO_2$ in air in a humidified atmosphere. After starvation the serum-free MEM was withdrawn and 100 µl (100 $TCID_{50}$) of virus was added to the wells and allowed to adsorb to the cell cultures for 1 h at $37^0$C 5% $CO_2$ in air in a humidified atmosphere. After adsorption for 1 h the unbound virus was withdrawn and 200 µl of the appropriate dilution of extracts in serum-free MEM was added to each of six wells and the cell cultures incubated at $37^0$C in a humidified $CO_2$ atmosphere (5% $CO_2$/95% filtered air). For positive control, cells infected with the virus were maintained in serum-free MEM, and cells mock-infected with 100 µl serum-free MEM and maintained in serum-free MEM served as negative controls. Cells were examined daily for five days, by light microscopy, for the appearance of a cytopathogenic effect (CPE). The absence of CPE at a specific concentration of the extract was considered to be indicative of antiviral activity.

**RESULTS AND DISCUSSION**

The integrity of the Vero cell monolayers, treated with concentrations from 3.9 µg/ml up to 125 µg/ml of the plant extract, was maintained and no morphological changes and/or cell death, indicative of cytotoxic effects, were observed. At extract concentrations ≥ 250 µg/ml the integrity of the Vero cell monolayers was totally destroyed, consequently no further cell proliferation assays, such as tetrazolium salt reduction (MTT) assay or alamarBlue™ assay were done.

Table 1A: Dose response pattern on the inhibition of adsorption and subsequent replication of $100TCID_{50}$ herpes simplex virus type 1 on Vero African Green Monkey kidney cells when virus was inoculated simultaneously with different concentrations of the plant extract.

| Concentration of plant extract (µg/ml) | Percentage cytopathogenic effect | | |
|---|---|---|---|
| | 24 h p.i* | 48 h p.i | 72 h p.i |
| 250.00 | T** | T | T |
| 125.00 | T***/50% | T***/95-100% | T***/100% |
| 62.50 | 50% | 100% | 100% |
| 31.25 | 50% | 100% | 100% |
| 15.60 | 95-100% | 100% | 100% |
| Positive control | 95-100% | 100% | 100% |
| Negative control | 0 | 0 | 0 |

p.i* = post-infection, T** =Toxicity noted: cell monolayer totally destroyed, no viable cells evident, T***= Partial toxicity noted: integrity of cell monolayer affected, ±50% viable cells evident.

Table 1B: Dose response pattern on the inhibition of replication of 100$TCID_{50}$ herpes simplex virus type 1 on Vero African Green Monkey kidney cells after adsorption of the virus and subsequent incubation of the infected cell cultures in different concentrations of the plant extract.

| Concentration of plant extract (μg/ml) | Percentage cytopathogenic effect | | |
|---|---|---|---|
| | 24 h p.i* | 48 h p.i | 72 h p.i |
| 250.00 | T** | T | T |
| 125.00 | T***/50% | T***/75% | T***/100% |
| 62.50 | 50-75% | 75% | 100% |
| 31.25 | 75% | 100% | 100% |
| 15.60 | 75-90% | 100% | 100% |
| Positive control | 75-90% | 100% | 100% |
| Negative control | 0 | 0 | 0 |

p.i*. = post-infection, T** =Toxicity noted: cell monolayer totally destroyed, no viable cells evident, T***= Partial toxicity noted: integrity of cell monolayer affected, ± 50% viable cells evident.

From Table 1a it is evident that when the virus was inoculated onto cell cultures simultaneously with the plant extract, partial antiviral activity against HSV-1 was noted at concentrations ≥ 31.25 μg/ml 24 h post infection (p.i.) as suggested by the delay in the appearance of a CPE. However, after 48 h p.i. no antiviral activity was noted. No antiviral activity was noted at concentrations ranging from 3.90 μg/ml to 15.60 μg/ml and at concentrations ≥ 250.00 μg/ml, the extract was toxic and totally destroyed the integrity of the cell monolayer.

From Table 1b, it is evident that after adsorption of the virus and subsequent incubation of the infected cell cultures in different concentrations of the plant extract, partial antiviral activity against HSV-1 was again noted at plant extract concentrations of ≥ 31.25 μg/ml as suggested by the delay in the appearance of a CPE. No antiviral activity was noted at concentrations ranging from 3.90 μg/ml to 15.60 μg/ml and at concentrations ≥ 250.00 μg/ml the extract was toxic and totally destroyed the integrity of the cell monolayer.

Table 2A: Dose response pattern on the inhibition of adsorption and subsequent replication of 100$TCID_{50}$ Coxsackie B virus type 6 on Vero African Green Monkey kidney cells when virus was inoculated simultaneously with different concentrations of the plant extract.

| Concentration of plant extract (μg/ml) | Percentage cytopathogenic effect | | |
|---|---|---|---|
| | 24 h p.i* | 48 h p.i | 72 h p.i* |
| 250.00 | T** | T | T |
| 125.00 | T***/0% | T***/50-90% | T***/100% |
| 62.50 | 0% | 100% | 100% |
| 31.25 | 0% | 100% | 100% |
| 15.60 | 0% | 100% | 100% |
| Positive control | 0% | 100% | 100% |
| Negative control | 0% | 0% | 0% |

p.i* = post-infection, T** =Toxicity noted: cell monolayer totally destroyed, no viable cells evident, T***= Partial toxicity noted: integrity of cell monolayer affected, ± 50% viable cells evident.

Table 2B: Dose response pattern on the inhibition of replication of 100$TCID_{50}$ Coxsackie B virus type 6 on Vero African Green Monkey kidney cells after adsorption of the virus and subsequent incubation of the infected cell cultures in different concentrations of the plant extract.

| Concentration of plant extract (μg/ml) | Percentage cytopathogenic effect | | |
|---|---|---|---|
| | 24 h p.i* | 48 h p.i | 72 h p.i |
| 250.00 | T** | T | T |
| 125.00 | T***/0% | T***/50-90% | T***/100% |
| 62.50 | 0% | 100% | 100% |
| 31.25 | 0% | 100% | 100% |
| 15.60 | 0% | 100% | 100% |
| Positive control | 0% | 100% | 100% |
| Negative control | 0% | 0% | 0% |

p.i* = post-infection, T** =Toxicity noted: cell monolayer totally destroyed, no viable cells evident, T***= Partial toxicity noted: integrity of cell monolayer affected, ± 50% viable cells evident.

From Table 2a and 2b it was evident that the plant extract had no antiviral activity at concentrations from 3.90 μg/ml to 62.50 μg/ml and at concentrations ≥ 250.00 μg/ml the compound was toxic and totally destroyed the integrity of the cell monolayer. From this preliminary data it is evident that the plant extract was highly toxic to the Vero cell cultures and that possible anti-viral activity against HSV-1 and CV-B6 was only noted at extract

concentrations where toxicity was also noted. The use of the extract from *H. pauciflorus* as a chemotherapeutic agent against HSV-1 and CV-B6 *in vivo* is therefore questionable.

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