FLAVONOIDs EFFECT ON BHV-1 AND PRV REPLICATION IN MDBK AND VERO CELL CULTURES

ABSTRACT

The inhibitory effect of three flavonoids and one phenolic acid in bovine and porcine herpes viruses 1 were investigated. Flavonoids were tested for their cytotoxic properties in concentrations of 10-250μg/mL to MDBK and VERO cells. VERO and MDBK cells were treated separately with quercetin, morin, rutin and cinnamic acid in concentrations of 30 and 20μg/mL, 25 and 30μg/mL, 30 and 30μg/mL, 30 and 30μg/mL, respectively. In virus yield reduction assay, quercetin and morin reduced viral titer in 90% (1 log) and 54% (<1 log), respectively, in VERO cells, however, rutin and cinnamic acid increased viral titer in 32% (<1 log). In MDBK cells, quercetin and morin decreased respectively 79% (<1 log) and 32% (<1 log), while rutin and cinnamic acid increased, both, 53% (<1 log). In virucidal activity, quercetin and cinnamic acid increased PRV titer while morin showed few activity and rutin decreased gradually PRV titer. Based on these results, it can be concluded that some plant metabolites present antiviral principles and, therefore, could be potential candidates to treat viral infections.

INTRODUCTION

Currently available antiviral drugs present major use restrictions, such as narrow spectrum of activity, limited therapeutic usefulness and variable degrees of toxicity. The emergence of viral resistance to drugs and drug-related side effects are among the main reasons for further refinement of antiviral drug design and development. Any new product, that could overcome some or all of those disadvantages, would be very useful and search toward this goal has been stimulated all over the world. Most research involving the development of antiviral agents has been directed toward synthetic substances. Nevertheless, recent efforts have been spent to evaluate the antiviral activity of a wide array of natural products, including plants, in order to isolate and characterize novel compounds which
could inhibit virus replication and/or treat viral infection, or even serve as models for new molecules (Hudson 1990, Che 1991, Vlietinck & Vanden Bergh 1991, Chu & Cutler 1992, Cardellina II et al. 1993, Simões et al. 1999). Plants synthesize an enormous variety of secondary metabolites via often-complex biosynthetic pathways, and many of these metabolites, as flavonoids, play important functions in interactions between plants and its environment (Dixon 1998).

Flavonoids are naturally occurring polyphenolics mainly present in fruits and vegetables but they can also be distributed in all organs of the plants (Harborne 1988). These substances consist of a three-ring structure with a diphenyl propane skeleton (C-6-C-3-C-6) (Vinson 1998). Variations on the basic structure of flavonoids yield different classes of flavonoids (Bravo 1998). In many traditional medicines, therapeutic effects of flavonoids have been described for diabetes mellitus (Ong & Khoo 1997), allergies (Inoue et al. 2002), cancer (Sarkar & Li 2003, Park & Surh 2004), viral infections (Jassim & Naji 2003, Becker 1980, Hudson et al. 1993, Mitscher et al. 1987), stomach and duodenal ulcer (Ares & Outt 1998), parodontosis (Hirasawa et al. 2002), and inflammations (Manthey et al. 2001). Although flavonoids have been used for centuries in traditional medicine, more recently they have attracted tremendous interest as possible therapeutic agents in modern medicine (Stoner & Mukhtar 1995, Sueoka et al. 2001, Albertazzi 2002). Certain flavonoids and related compounds have been investigated as inhibitors of HIV-1 reverse transcriptase, protease and integrase yonder viruses as poliovirus type 1, parainfluenza virus type 3, respiratory syncytial virus and herpes virus type 1 (Middleton 1998). Still, there is a need to find new substances with extracellular virucidal activity, since a lot of existing antiseptics and disinfectants fail to kill all pathogenic viruses after a 5min of exposure time at room temperature. Such compounds could be very useful to diminish the transfer of viable viruses from infected individual to uninfected one.

Bovine herpes virus 1 (BHV-1) and porcine herpes virus 1 (PRV) are members of Herpesviridae family and Alphaherpesvirinae subfamily. This virus subfamily comprises others important human and animal pathogens including varicella-zoster virus (VZV) and herpes human virus 1 e 2 (HSV) (Summerfield et al. 1997). Herpes virions consist of a nucleoprotein core containing the linear double-stranded DNA genome associated with proteins, an icosahedral capsid shell, an amorphous pertinacious tegument structure, and a lipid envelope (Ward & Roizman 1994), virus-encoded glycoproteins are inserted within the envelope (Summerfield et al. 1997). BHV-1 is responsible for a variety of diseases in cattle, including rhinotracheitis, pustular vaginitis, balanoposthitis, conjunctivitis, abortion, enteritis, a generalized disease of newborn calves, and possibly encephalitis. PRV is responsible for Aujeszky's disease that primarily affects swine, which serve as a virus reservoir and as a major source of natural infection for a diverse range of secondary hosts. The infection causes neurological signs in new-borns and present a fatal course, being characterized by meningoencephalitis, glangioneuritis and vasculitis, and can also occur as a generalized form affecting various organs. It is economically, the most important viral disease of swine, causing multimillion-dollar losses each year in the countries where it is found (Murphy 1999).

The aim of this study was to evaluate, in vitro, the antiviral activity of three flavonoids and one phenolic acid on BHV-1 and PRV replication.

MATERIAL AND METHODS

Flavonoids.
Flavonoids (quercetin, morin and rutin) and phenolic acid (cinnamic acid) (conceded by the Laboratório de Biofármacos, UFV - BRAZIL) were dissolved in dimethyl sulfoxide (DMSO) until final concentration of 20mg/mL and were kept at -20°C.

Cell cultures and media.
Permanent cell lines from African green monkey kidney (VERO) and Madin-Darby bovine kidney (MDBK) were cultivated in Eagle's minimal essential medium (MEM) (Sigma-Aldrich), supplemented with 10% and 5% of fetal calf serum (FCS) and equine serum (ES) respectively, antibiotics (penicillin, 500 UI and streptomycin, 100μg/mL) at 37°C in the presence of 5% CO₂ until the formation of confluent monolayers. Cells were routinely passed every two or three days.

Viruses.
Bovine herpes virus 1 (BHV-1) and porcine herpes virus 1 (PRV) were propagated in our laboratory by serial passages on MDBK and VERO cell culture, respectively. Virus stocks were stored at liq-
uid nitrogen until used and titrations were performed by the limit-dilution method. The virus titers were estimated by cytopathogenicity and expressed as 50% tissue culture infections doses/mL (Reed & Muench 1938).

**Cell toxicity assay.**

Cell toxicity was monitored by determining the effect of flavonoids on cell morphology and cell viability. Different concentrations of flavonoids and phenolic acid (10-250μg/mL) were added to confluent (MDBK) and subconfluent (VERO) cell monolayers and were cultivated at 37°C for 4 days. Cells morphology was evaluated daily and observed for microscopically detectable alterations, like loss of monolayer, rounding, shrinking of cells, granulation, and vacuolization in the cytoplasm. Cells were examined microscopically for the presence of cytotoxic effects and maximum non-toxic concentration to both cells lines determined.

**Virucidal activity.**

The direct virus inactivating effect of three flavonoids and one phenolic acid were tested by direct contact assay. Stock virus suspensions were treated, separately, with equal volumes of different substances concentrations in MEM for 1h at 37°C. The final concentration ranged from 25, 80 and 160μg/mL. The difference in the biological activities of treated and control viruses were determined based on infectivity. Surviving infectious virus titers were determined in CPE assay using the method of Reed & Muench (1938).

**Virus yield reduction assay.**

Confluent (MDBK) and subconfluent (VERO) cell monolayers in 96-well plates were overlaid with each maximum non-toxic concentration of flavonoids and phenolic acid separately (0.1mL) and 0.05mL of virus suspension (10^2-10^6 TCID₅₀/mL). The virus-induced CPE was observed on day 3 post-infection (p.i.) for both cells under inverted microscope. The reduction of virus multiplication was estimated based on viral control and viral titer was determined also by the method of Reed & Muench (1938).

**RESULTS**

The cytotoxicity of flavonoids and phenolic acid was determined by microscope examination of cell death and integrity after an incubation time of 96h. Quercetin at a concentration of 30μg/mL was toxic for VERO cells and at 20μg/mL for MDBK cells; morin was toxic to VERO cells at 25μg/mL and at 30μg/mL was toxic for MDBK cells; rutin and cinnamic acid were toxic for VERO and MDBK cells at 30μg/mL. Most of them exhibited the same maximum non-toxic concentration to both cells, 30μg/mL.

These substances were studied in respect to their inhibitory effect on BHV-1 and PRV by two separate assay methods: virucidal activity and virus

![Figure 1. Virucidal activity of flavonoids and phenolic acid at 0, 25, 80 and 160μg/mL (with 0μg/mL representing the control) on PRV in VERO cells.](image-url)
yield reduction assay. Then, the preparations ability to produce a direct inactivating effect in the contact assay was studied. The results are presented in Fig. 1.

Quercetin increased PRV titer at concentrations of 25 and 80μg/mL, but when used at 160mg/mL it didn't change the titer. Cinnamic acid also increased PRV titer in all concentrations tested. Morin showed a low inhibition of viral multiplication at 25μg/mL, but it was not able to change PRV titer in others concentrations. Although, rutin caused a gradual reduction in the PRV titer reaching 80% (<1 log) in the concentration of 160μg/mL. These experiments were performed in VERO cell culture.

In virus yield reduction assay was verified possible viral titer reduction by flavonoids and phenolic acid after treatment of MDBK and VERO cell with maximum no toxic concentration of each them immediately p.i. When VERO cells were infected and immediately treated with quercetin or morin, there was PRV titer reduction in 90% (1 log) and 54% (<1 log), respectively, while rutin and cinnamic acid increased PRV titer in 32% (<1 log) (Fig. 2).

In MDBK cells, quercetin and morin decreased BHV-1 titer in 79% (<1 log) and 32% (<1 log) respectively. Although both rutin and cinnamic acid produced increasing of 53% (<1 log) in BHV-1 titer (Fig. 3).

**DISCUSSION**

Three flavonoids and one phenolic acid were investigated for their herpes virus inhibitory effect by two separate assay methods. In virus yield reduction assay quercetin and morin showed activity against PRV and BHV-1 in VERO and MDBK cells culture, respectively. Studies in experimental models indicated that quercetin had significant influence against viral infectious (Brody 1994). However, rutin and cinnamic acid increased PRV and BHV-1 titer in VERO and MDBK cells respectively. Rutin has not shown antiviral activity for many virus and also contains a rutinose group in 3-carbon, and its hydroxylation is a pre requisite to antiviral activity (Selway 1986). Cinnamic acid is considered a precursor of a metabolic pathway to form different classes of flavonoids. In addition, there is no description in literature about possible antiviral activity of it. Based in our results, cinnamic acid does not present inhibition effect on PRV and BHV-1 multiplication.

![Figure 2. Flavonoid susceptibility of PRV virus to flavonoids and phenolic acid inhibition, determined by virus yield reduction assay in VERO cells.](image-url)
Many polyphenols are known for their antiherpes virus activities (Vanden Berghe et al. 1986). It is known that polyphenols bind to proteins to form unstable complexes (Haslam 1996). Therefore enveloped viruses and among them alphaherpesvirus may be the most vulnerable to the action of polyphenols because this class of naturally occurring substances can easily interact with the glycoproteins of the viral envelope (Serkedjieva & Ivancheva 1999). However in virucidal assay quercetin and cinnamic acid increased PRV titer, while morin showed limited activity to 25μg/mL and rutin surprisingly showed virucidal activity on PRV.

Quercetin has been reported to have virucidal activity against enveloped viruses such as herpes simplex type I, respiratory syncytial, pseudorabies, parainfluenza type 3, and Sindbis (Kaul et al. 1985, Vrijen et al. 1988). Quercetin has also been found to protect against macrophage-dependent murine cardiovirus (mengovirus) infection (Kaul et al. 1985, Vlietinck et al. 1988). The mechanisms of antiviral activity of quercetin appear to be related to its ability to bind viral protein and to interfere with viral nucleic acid synthesis (Formica & Regelson 1995). When studied at a molecular level, quercetin was found to inhibit the activity of mouse ribonucleic acid RNA polymerase II in fibroblast cell cultures (Nose 1984). Subsequent transcription studies revealed that bacterial and bacteriophage DNA and RNA polymerases were also inhibited by quercetin (Shinozuka et al. 1998). The effect of quercetin on virus replication and polymerases prompted investigators to speculate on the effect of flavonoids on reverse transcriptase (RT).

The antiviral effects detected in these experiments could have taken place in many different steps of viruses replication cycle. According to Prusoff et al. (1986) effective antiviral agents can inhibit the viral infection by affecting the virus particle itself, by interfering with the virus entry across the cell membrane and subsequent uncoating or with the integrity and transport of viral genome, or even by interfering with the replication of viral genome or other early steps of intracellular viral replication, including assembly and release of virions.

In relation to the used methodology, it can be observed that optic microscope observation cannot be sufficient to determinate details of flavonoids cytotoxicity and inhibitory effect of viruses' multiplication in the presence of these metabolites. Based on
the obtained results, it could be concluded that plant metabolites would be potential candidates as anti-

viral principles and, could be used to treat viral infec-
tions. Thus, it is important to employ others tech-
niques, as techniques targeting virus protein or en-
zymatic activity to identify which step of the virus replications are being targeted by the flavonoids.

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