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In vitro assessment of the antiviral potential of *trans*-cinnamic acid, quercetin and morin against *equid herpesvirus 1*

H.D. Gravina [a,b], N.F. Tafuri [a,b], A. Silva Júnior [a,b], J.L.R. Fietto [a], T.T. Oliveira [c], M.A.N. Diaz [d], M.R. Almeida [a,*]

[a] Molecular Animal Infectology Laboratory, Applied Biotechnology Institute (BIOAGRO), Federal University of Vicosa, Vicosa 36570-000, Brazil
[b] Animal Virology Laboratory, Department of Veterinary Sciences, Federal University of Vicosa, Vicosa 36570-000, Brazil
[c] Biofarmacos Laboratory, Department of Biochemistry and Molecular Biology, Federal University of Vicosa, Vicosa 36570-000, Brazil
[d] Biomolecular Chemistry Laboratory, Department of Biochemistry and Molecular Biology, Federal University of Vicosa, Vicosa 36570-000, Brazil

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ABSTRACT

The antiviral activity of quercetin, morin and *trans*-cinnamic acid was evaluated *in vitro* against *equid herpesvirus 1* (EHV-1) by determining the virucidal activity and using the time of addition assay to test inhibition of the viral replication cycle. The cytotoxicity of each substance was assessed using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. Quercetin showed virucidal action and inhibition of the viral replication cycle at 0 and 1 h. Morin showed potential virucidal and viral replication cycle inhibition at 0 h. *Trans*-cinnamic acid did not show virucidal activity but inhibited the viral replication cycle at −1 and 0 h. This study demonstrates the potential of these compounds as future antiviral candidates in relation to viruses of importance in veterinary medicine.

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1. Introduction

Although effective antivirals have been utilized over the past 60 years (Felipe et al., 2006), viral diseases continue to be a problem, due to the toxicity of new antivirals and the development of resistant viruses. Various secondary plant metabolites, including flavonoids, tannins, saponins and phenolic acids, exhibit promising antiviral activity (Muller et al., 2007). In particular, flavonoids have been reported to have inhibitory effects on several viruses (Choi et al., 2009; Mucsi and Pragai, 1985).

Quercetin is a water-soluble flavonoid that has been reported to have many biological actions, including antiviral activity against several viruses (Mucsi and Pragai, 1985; Douglas et al., 2003; Davis et al., 2008).

Morin is a flavonoid that displays a variety of biological actions, such as antioxidant (Kitagawa et al., 2004), anti-inflammatory (Galvez et al., 2001), anti-allergic (Kim et al., 2009) and anti-mutagenic activities (Bhattacharya and Firozi, 1988). However, antiviral activities have not been reported.

Trans-cinnamic acid occurs naturally in nature, being the precursor of flavonoids. It has great potential for a therapeutic role, showing antimicrobial, antifungal and antitumour activity (Rastogi et al., 1998; Said et al., 2004; Neves et al., 2005; Qian et al., 2010). A compound derived from *trans*-cinnamic acid, p-sulphoxy-cinnamic acid, showed antiviral activity against dengue virus, suggesting a possible antiviral potential of *trans*-cinnamic acid (Rees et al., 2008).

Equid herpesvirus 1 (EHV-1), a DNA virus member of the family *Herpesviridae* (Ostlund, 1993), is responsible for respiratory disease, abortion, neonatal death and nervous system disorders in horses (Patel and Heldens, 2005). Despite the existence and frequent use of vaccines against EHV-1, outbreaks still occur, with significant economic impact on the equine industry, because existing vaccines are not sufficiently protective (Reed and Toribio, 2004). In addition, infection control may be difficult due to the establishment of life-long latency after primary infection (Field et al., 2006).

Due to the importance of EHV-1 as a ubiquitous pathogen of horses and the absence of an antiviral treatment for the clinical disease, this study aimed to investigate the antiviral potential of *trans*-cinnamic acid and the flavonoids quercetin and morin on EHV-1 *in vitro*.

2. Materials and methods

2.1. Cells and virus

VERO cells were kept in minimum essential medium (MEM) supplemented with 10% foetal bovine serum (SBF), penicillin (1.6 mg/L) and streptomycin (0.4 mg/L). EHV-1 was derived from

* Corresponding author. Address: Molecular Animal Infectology Laboratory, Applied Biotechnology Institute (BIOAGRO), Federal University of Vicosa, Vicosa 36570-000, MG, Brazil. Tel.: +55 31 3899 29 11; fax: +55 31 3899 23 74.
E-mail addresses: marcia@ufv.br, marcia.almeida@pq.cnpq.br (M.R. Almeida).

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a standard A4/72 sample courtesy of the University of Santa Maria, RS, Brazil, and was titrated by the tissue culture infectious dose 50 ($TCID_{50}$) method, as described by Reed and Muench (1938).

2.2. Preparation of compounds

The three compounds (quercetin, morin and *trans*-cinnamic acid) were purchased from Sigma–Aldrich (Deisenhofen, Germany). The compounds were initially dissolved in dimethylsulphoxide (DMSO) at a stock concentration of 100 mg/mL at 4 °C. At the time of use, dilutions in MEM were performed so that the final concentration of DMSO did not exceed 1.2%.

2.3. Analysis of cytotoxicity

The cytotoxicity of the compounds was microscopically assessed via changes in cellular morphology and was confirmed and measured by the colorimetric method based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma–Aldrich, Deisenhofen, Germany) by mitochondrial enzymes (Mosmann, 1983). Cells were distributed in 96-well microplates and after 3 h of incubation at 37 °C in an atmosphere containing 5% CO_2 the medium was removed and the cells further incubated with different concentrations of each test compound and DMSO at 37 °C in an atmosphere containing 5% CO_2 for 72 h. After 72 h incubation, the effects on cell morphology (loss of monolayer, granulation, vacuolization in the cytoplasm, elongation and narrowing of the extension, and darkening of cell limits) were observed microscopically. The plates were then washed twice with phosphate-buffered saline (PBS), pH 7.2, and further incubated at 37 °C with 10% MTT for 4 h. Subsequently, the salt that had formed was dissolved by the addition of isopropanol–HCl 0.04 N and the absorbances read on a multiwall spectrophotometer (Bio-Tek®, Elx800) at 550 nm. The percentage of viable treated cells was calculated in relation to the untreated control (% of control cells: optical density test (OD) test/optical density control cell (OD) control cell × 100). The maximum non-toxic concentration (MNTC) of the compounds was determined.

2.4. Determination of virucidal activity

In order to assess the virucidal activity of the compounds, EHV-1 serial dilutions (10–10^7 $TCID_{50}$/mL) were incubated for 1 h with quercetin (15, 30 and 60 μg/mL), morin (30, 60 and 90 μg/mL) or *trans*-cinnamic acid (25, 50 and 100 μg/mL) at temperatures ranging from 35 to 37 °C. At specific time intervals aliquots of each virus/compound suspension were added to the already adherent and plated cells and incubated at 37 °C and 5% CO_2. After 72 h, the viral titre was calculated using the method described by Reed and Muench (1938) and compared with the control titre (untreated virus).

2.5. Time of addition studies

To investigate the mechanism by which the compounds inhibit the replication cycle of EHV-1, a study was conducted following previous methods with some modifications (Serkedjieva and Ivancheva, 1999). Cell monolayers in 96-well plates were incubated with solutions of quercetin (15, 30 and 60 μg/mL), morin (30, 60 and 90 μg/mL) or *trans*-cinnamic-acid (25, 50 and 100 μg/mL) for 1 h (1 h: pre-treatment) and then washed twice with PBS and challenged with 10–10^7 $TCID_{50}$/mL. Other cell monolayers in 96-well plates were infected with EHV-1 at 10–10^7 $TCID_{50}$/mL and, during adsorption for 1 h at 4 °C, were treated with the compounds to prevent viral internalization (0 h: effect of adsorption). One fraction of infected non-treated cells was incubated at 37 °C with MEM containing the compounds for 1 h (1 h: effect of penetration). Two hours after viral infection another aliquot of infected cells was treated with the compounds diluted in MEM (2 h: effect after virus infection). The antiviral activity of all phases was assessed by measuring the reduction in viral titre after 72 h and calculated using the method of Reed and Muench (1938).

2.6. DMSO assays

All antiviral assays were assessed under the same conditions with DMSO to evaluate the possible interference of this solvent. Two concentrations were used: 1.2%, which corresponded to the highest percentage of DMSO found in the dilutions of compounds, and 2.4%, which was used to assess with greater accuracy whether it acts as an inert compound.

2.7. Statistical analysis

Statistical analysis was performed using the Statistical Analysis System program (SAEG – Version 9.1/2007, Federal University of Viçosa, Brazil). One-way ANOVA and two-way ANOVA repeated measures were used for cytotoxicity and timing of addition assays, respectively. The Tukey test was used to compare the means ($P < 0.05$). For the results of the virucidal activity test, linear regression and non-linear fit one-phase decay was determined using the GraphPad Prism software (California, USA).

3. Results

The cytotoxic effects of the compounds were deduced from their antiviral activity by determining the MNTC of each compound using microscopic analyses and the MTT colorimetric test. Microscopically, it was observed that as the compound concentration increased, more morphological changes were visible in the cells (loss of monolayer, granulation, vacuolization in the cytoplasm, darkening of cell boundaries). Table 1 shows the MNTC of each compound

Table 1
Maximum non-toxic concentrations of quercetin, morin, *trans*-cinnamic acid and DMSO for VERO cells, assessed by optical microscopy (MNTC/OM) and assessed by MTT test (MNTC/MTT). [A1], [A2], [A3] are concentrations used in the antiviral tests.

Compounds	MNTC/OM (μg/mL)	MNTC/MTT (μg/mL)	[A1] (μg/mL)	[A2] (μg/mL)	[A3] (μg/mL)
Quercetin	70	75.1	15	30	60
Morin	100	101.3	30	60	90
Trans-cinnamic acid	110	109.3	25	50	100
DMSO	3.0 (%)	3.1 (%)	1.2 (%)	2.4 (%)	

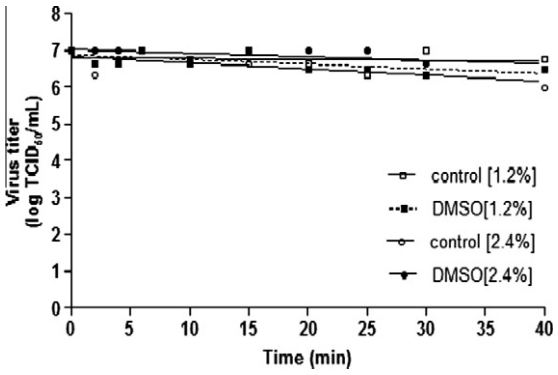

Fig. 1. Virucidal activity of DMSO [1.2% and 2.4%] tested at different times.

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