In vitro inhibition of canine distemper virus by flavonoids and phenolic acids: Implications of structural differences for antiviral design

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1. Introduction

Canine distemper is caused by a single stranded RNA virus of the genus Morbillivirus, family Paramyxoviridae. In dogs, canine distemper virus (CDV) can result in gastrointestinal and/or respiratory clinical signs, often accompanied by neurological signs (Martella et al., 2008). Although there are commercial vaccines against canine distemper, the disease remains one with the highest incidence and lethality in dogs (Rodeheffer et al., 2007). The virus has been reported in wild animals (ferrets, wild dogs, foxes, coyotes, hyenas, lions, tigers, leopards, seals, sea lions and dolphins) (Appel et al., 1994; Kennedy, 1998; Murphy et al., 1999; Moro et al., 2004). The domestic dog is most commonly infected, and although the disease has been also found in big cats, the domestic cat is not susceptible (Martella et al., 2008).

Currently, therapeutic studies for CDV have employed either synthetic inhibitors that interfere with morbillivirus replication (Scagliarini et al., 2006; White et al., 2007; Elia et al., 2008; Yoon et al., 2009; Dal Pozzo et al., 2010) or natural compounds that display anti-CDV activity in vitro at early and/or late stages of viral replication (Gallina et al., 2011; Bagla et al., 2012).

Phenolic compounds are plant secondary metabolites widely distributed among plant species and are targets of great scientific interest due to their pharmacological properties and therapeutic value (Schnitzler et al., 2008). The compounds have antioxidant, anti-allergic, anti-inflammatory, anti-mutagenic, anticarcinogenic and antibacterial effects (Shon et al., 2004; Nafisi et al., 2008; Orhan et al., 2010; Shimosaki et al., 2011). Flavonoids and phenolic acids, two major phenolic groups, have been reported to inhibit the replication of many viruses (Rees et al., 2008; Saha et al., 2009; Kim et al., 2010; Gravina et al., 2011). The antiviral activity of the flavonoid quercetin (3',4,5,7-pentahydroxyflavonol) has been reported for some viruses, including adenovirus 3 (AdV-3), herpes simplex virus (HSV) and influenza virus (Choi et al., 2009; Thapa et al., 2011). Morin (2',4,5,7-pentahydroxyflavonol), another flavonoid, had antiviral action against equine herpesvirus 1 (EHV-1) (Gravina et al., 2011). Antiviral activity of glycosidic flavonoids was shown by rutin (quercetin-3-O-rutinoside) against HSV, dengue virus 2 (DENV-2) and human immunodeficiency virus (HIV) (Tao et al., 2007; Zandi et al., 2011), and hesperidin against influenza virus (Saha et al., 2009).

Both cinnamic acid and its trans isomers are phenolic acids or intermediate compounds in the synthesis routes of flavonoids.
Phenolic acids have been reported to have antiviral effects against DENV, EHV-1 and HIV (Ichimura et al., 1999; Rees et al., 2008; Gravina et al., 2011). Some studies correlate changes in the structure of flavonoids with changes in their biological mechanisms. According to Kim et al. (2010), isoquercetin, a glycosylated form of quercetin, showed higher inhibitory activity against influenza virus in vitro and in vivo than that reported for quercetin. Another study by Thapa et al. (2011) investigated the synthesis and antiviral activity of various derivatives of quercetin with hydroxy substitutions at C-3, C-3' and C-5; synthetic analogs of quercetin demonstrated therapeutic index for influenza virus greater than that of quercetin.

Given the high impact of canine distemper disease upon the canine population as well as the lack of a specific antiviral treatment, the goal of the present work was to investigate natural compounds and their antiviral activity against CDV. We evaluated the in vitro inhibition of the CDV replicative cycle by the flavonoids quercetin, morin, rutin and hesperidin, and the phenolic acids cinnamic, trans-cinnamic and ferulic acids. Implications regarding structural differences of compounds and correlation with their antiviral activity were also considered.

2. Materials and methods

2.1. Cells and viruses

Vero cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (SBF), penicillin (1.6 mg/L) and streptomycin (0.4 mg/L) at 37 °C and 5% CO2. Canine distemper virus (strain Rockbourn, 11th passage) was titrated by determining the infective dose for 50% tissue culture (TCID50), according to the method described by Reed and Muench (1938).

2.2. Compounds

All seven phenolic compounds (quercetin, morin, rutin, hesperidin, cinnamic acid, trans-cinnamic acid and ferulic acid) were purchased from Sigma–Aldrich (Deisenhofen, Germany) (Figs. 8 and 9). Compounds were initially dissolved in dimethylsulfoxide (DMSO) to a stock concentration of 10 mg/mL and stored at 4 °C. When needed, compounds were diluted in MEM at the time of use to provide working concentrations.

2.3. Cytotoxicity assays

The cytotoxicity of the phenolic compounds was determined by microscopic evaluation searching for changes in cell viability and morphology. Non-toxic concentrations were also confirmed by a microscopic evaluation searching for changes in cell viability and treating with the compounds followed by incubation at 4 °C for 1 h to prevent virus internalisation (time 0 h: adsorption effect), after which the microplates were incubated at 37 °C. The third set of plates was treated and the compounds were then added after 1 h adsorption at 37 °C (time 1 h: penetration effect). The last set of plates was treated two hours post-infection (hpi) (time 2 h: intracellular effect). Assays were performed in triplicate. The antiviral activity of compounds at all stages of the viral replicative cycle was measured by reduction in viral yield after 72 h post treatment compared to untreated infected cells and calculated using the method of Reed and Muench (1938). All antiviral assays were assessed under the same conditions with DMSO to evaluate the possible interference of this solvent. Ribavirin (RBV), a known positive control drug was assessed in parallel to validate the test. The 50% inhibitory concentration (IC50) was defined as the compound concentration required to reduce viral CPE by 50% of the virus control. The selectivity index (SI) for the compounds was obtained by calculating the ratio of the CC50 and IC50 values for the virus replication at each time point.

2.4. Time of addition assays

The study was performed according to the method described by Serkedjieva and Ivancheva (1999) with some modifications. Briefly, 96-well microplates seeded with monolayers of Vero cells (1 × 10^5 cells/well) were individually supplemented with quercetin, morin, rutin, hesperidin, cinnamic acid, trans-cinnamic acid and ferulic acid at different times based on time of infection. The first set of plates was treated for 1 h prior to viral infection (time – 1 h: pre-treatment effect). Cells were then washed twice with PBS followed by infection with serial dilutions of CDV (10–10^-5 TCID50/mL). The second set of microplates were both infected and treated with the compounds followed by incubation at 4 °C for 1 h to prevent virus internalisation (time 0 h: adsorption effect), after which the microplates were incubated at 37 °C. The third set of plates was infected and the compounds were then added after 1 h adsorption at 37 °C (time 1 h: penetration effect). The last set of plates was treated two hours post-infection (hpi) (time 2 h: intracellular effect). Assays were performed in triplicate. The antiviral activity of compounds at all stages of the viral replicative cycle was measured by reduction in viral yield after 72 h post treatment compared to untreated infected cells and calculated using the method of Reed and Muench (1938). All antiviral assays were assessed under the same conditions with DMSO to evaluate the possible interference of this solvent. Ribavirin (RBV), a known positive control drug was assessed in parallel to validate the test. The 50% inhibitory concentration (IC50) was defined as the compound concentration required to reduce viral CPE by 50% of the virus control. The selectivity index (SI) for the compounds was obtained by calculating the ratio of the CC50 and IC50 values for the virus replication at each time point.

2.5. Evaluation of compounds structures

Compounds were divided in two groups, flavonoids and phenolic acids, based on their characteristics and chemical properties. For each group we established the common concentration used in the time of addition assays in order to analyze their structural differences and correlated it to their antiviral activity. The following flavonoids were compared to each other at a common concentration of 30 μg/mL: (i) quercetin and morin, (ii) quercetin and rutin, (iii) quercetin and hesperidin, and (iv) rutin and hesperidin. For phenolic acids, a comparative analysis between (i) cinnamic and trans-cinnamic acids, and (ii) trans-cinnamic and ferulic acids was performed at a concentration of 10 μg/mL.

2.6. Data analyses

Statistical analyses were performed using the program Statistical Analysis and Design of Experiments – SISVAR (Sisvar 5.1 Build 72, Federal University of Lavras, MG, Brazil). Cytotoxicity and time of addition assays were analyzed by ANOVA one-way and two-way ANOVA, respectively. CC50 and IC50 were calculated from a linear regression equation. The Tukey test was used to compare means. Values of p less than 0.05 were considered significant.

3. Results

3.1. Cytotoxic effect

The cytotoxicity of the evaluated compounds was determined by microscopic observation of cell morphology associated with
the measurement of cell viability by the colorimetric method MTT. We observed that intensity and variety of cellular morphological changes (loss of monolayer, granularity, cytoplasmic vacuolation, stretching and narrowing of the extensions as well as darkening of the cell edges) became more apparent with increasing concentrations of the compounds. Table 1 shows CC50 values obtained from the cytotoxicity assays.

DMSO CC50 was higher than the DMSO% used to dissolve the compounds, proving the absence of DMSO cellular toxicity against Vero cells at volumes used to dissolve the compounds. The phenolic compounds showed CC50 values ranging from 115.88 ± 11.19 (quercetin) to 195.90 ± 7.84 μg/mL (morin).

### 3.2. Time of addition assays

Antiviral activity of compounds was determined by time of addition assays. These tests had the purpose of investigating which step of the viral replicative cycle the compounds might be affecting. IC50 and SI (CC50/IC50) values obtained from the cytotoxicity and antiviral assays are shown in Table 1.

No significant differences were observed in viral titres with DMSO at the highest concentration (0.8%) as a solvent for the compounds in time of addition assays (not shown data). DMSO concentrations did not interfere with antiviral activity exhibited by flavonoids and phenolic acids. A positive control with ribavirin (RBV), which affects the intracellular phase of the CDV replicative cycle, was also performed (Supplementary Fig. 1).

The IC50 and SI values of phenolic compounds exhibiting activity ranged from 3.56 ± 0.13 (ferulic acid, time 0 h) to 40.52 ± 1.69 μg/mL (morin, time 1 h) and from 4.44 (trans-cinnamic acid, time −1 h) to 41.6 (ferulic acid, time 0 h), respectively (Table 1).

Our results showed that all flavonoids displayed antiviral activity against CDV. As demonstrated in Fig. 1, quercetin reduced viral yield at times 0 h (EC50 11.72 μg/mL and SI 9.88), 1 h (EC50 12.66 μg/mL and SI 9.15), and 2 h (EC50 19.57 μg/mL and SI 9.31). Fig. 2 demonstrated that morin had antiviral effect at times 0 h (EC50 34.02 μg/mL and SI 5.76) and 1 h (EC50 40.82 μg/mL and SI 4.83). Reduction in viral titres was most significant at the concentration of 80 μg/mL. Rutin and hesperidin (Figs. 3 and 4) showed a significant reduction in viral infectivity at times 0 h (rutin: EC50 10.41 μg/mL and SI 15.01; hesperidin: EC50 11.09 μg/mL and SI 14.78) and 1 h (rutin: EC50 13.45 μg/mL and SI 11.62; hesperidin: EC50 13.92 μg/mL and SI 11.78). On the other hand, hesperidin had also shown antiviral effect at time 2 h (EC50 21.63 μg/mL and SI 7.58) at the concentration of 30 μg/mL. Antiviral activity exhibited by rutin and hesperidin at time 0 h was greater than others affecting the times 1 and 2 h.

The three phenolic acids tested also showed inhibitory effects against CDV replication. As shown in Fig. 5, antiviral effect of cinnamic acid was demonstrated at times 0 h (EC50 4.46 μg/mL and SI 30.12) and 2 h (EC50 6.18 μg/mL and SI 21.74), but cinnamic acid only showed a reduction in viral yield at 2 h only at a concentration of 10 μg/mL. Addition of trans-cinnamic acid (Fig. 6) led to reduction of viral titres at times −1 h (EC50 37.27 μg/mL and SI 4.44) and 0 h (EC50 23.11 μg/mL and SI 7.16) at the concentration of 60 μg/mL. Fig. 7 shows the antiviral action of ferulic acid at times 0 h (EC50 3.56 μg/mL and SI 41.6) and 1 h (EC50 3.63 μg/mL and SI 40.8), with no statistical differences between them.

### 3.3. Analyses of antiviral activity versus chemical structure

To facilitate structural evaluation of compounds related to their antiviral effects, we chose equal working concentrations for the flavonoids (30 μg/mL) and phenolic acids (10 μg/mL) in the time of addition assays. Establishment of efficient relationships between phenolic compounds was important to understand the variation of the inhibitory effects based on changes in their chemical structures. According to data presented in Table 2, flavonoids that exhibited greater antiviral activity were rutin and hesperidin, affecting the stage of adsorption; there was no significant difference between them. Quercetin and hesperidin showed inhibitory effect at almost all viral replicative cycle stages, except in the stage prior to infection. Quercetin and hesperidin were also the only flavonoids that displayed similar antiviral activity in the intracellular stage.

Among phenolic acids evaluated ferulic acid had the highest inhibitory activity against CDV in the stages of adsorption and penetration with no significant differences between them (Table 3). Cinnamic acid, on the other hand, was the only phenolic that showed inhibition in the intracellular viral stage. Trans-cinnamic acid did not show antiviral activity against CDV at the same working concentration as the other compounds.

### 4. Discussion

In the present study several flavonoids (quercetin, morin, rutin, and hesperidin) and phenolic acids (cinnamic, trans-cinnamic, and ferulic acids) were evaluated regarding their in vitro ability to inhibit stages of the CDV replicative cycle. All flavonoids and phenolic acids demonstrated antiviral action against CDV. The observed inhibitory effects of flavonoids in the stages of adsorption and penetration suggest a direct inactivation of the virus, perhaps by binding to viral particles in an irreversible manner or by destabilizing viral ligands that are essential to the infectious process, such as envelope glycoproteins. Consequently, the role of flavonoids in

### Table 1

Cytotoxicity, anti-CDV activity and selectivity indices of the tested compounds.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>CC50 (μg/mL)</th>
<th>IC50 (μg/mL)</th>
<th>SI</th>
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<tbody>
<tr>
<td></td>
<td>Time (h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quercetin</td>
<td>115.88 ± 11.19</td>
<td>11.72 ± 0.85</td>
<td>12.66 ± 1.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Morin</td>
<td>195.90 ± 7.84</td>
<td>34.02</td>
<td>40.52 ± 1.69</td>
</tr>
<tr>
<td>Rutin</td>
<td>156.33 ± 7.12</td>
<td>10.41</td>
<td>13.45 ± 1.65</td>
</tr>
<tr>
<td>Hesperidin</td>
<td>161.99 ± 21.09</td>
<td>11.09 ± 0.45</td>
<td>13.92 ± 2.2</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>134.37 ± 19.08</td>
<td>4.46 ± 0.78</td>
<td>6.18 ± 1.28</td>
</tr>
<tr>
<td>Trans-cinnamic acid</td>
<td>165.45 ± 13.73</td>
<td>37.27 ± 8.02</td>
<td>23.11 ± 4.48</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>148.11 ± 13.47</td>
<td>3.56 ± 0.13</td>
<td>3.63 ± 0.08</td>
</tr>
<tr>
<td>DMSO (%)</td>
<td>2.11 ± 0.11</td>
<td></td>
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</tr>
</tbody>
</table>

a CC50 and IC50 values represent mean ± standard error of the mean values.

b Selectivity index (CC50/IC50).

c Time of addition assay. Stages of CDV replicative cycle: Pre-treatment (−1 h), adsorption (0 h), penetration (1 h), and intracellular (2 h).
Fig. 1. Inhibitory effects of quercetin at different stages of CDV replicative cycle. Concentrations of 15 or 30 µg/mL were added at varying times during virus infection: Pretreatment (-1 h), adsorption (0 h), penetration (1 h), and intracellular (2 h). **Concentration is statistically different compared to untreated control (p < 0.01).

Fig. 2. Inhibitory effects of morin at different stages of CDV replicative cycle. Concentrations of 30 or 80 µg/mL were added at varying times during virus infection: Pretreatment (-1 h), adsorption (0 h), penetration (1 h), and intracellular (2 h). **Concentration is statistically different compared to untreated control (p < 0.01).

Fig. 3. Inhibitory effects of rutin at different stages of CDV replicative cycle. Concentrations of 15 or 30 µg/mL were added at varying times during virus infection: Pretreatment (-1 h), adsorption (0 h), penetration (1 h), and intracellular (2 h). **Concentration is statistically different compared to untreated control (p < 0.01).
Fig. 4. Inhibitory effects of hesperidin at different stages of CDV replicative cycle. Concentrations of 15 or 30 μg/mL were added at varying times during virus infection: Pre-treatment (−1 h), adsorption (0 h), penetration (1 h), and intracellular (2 h). **Concentration is statistically different compared to untreated control (p < 0.01).

Fig. 5. Inhibitory effects of cinnamic acid at different stages of CDV replicative cycle. Concentrations of 5 or 10 μg/mL were added at varying times during virus infection: Pre-treatment (−1 h), adsorption (0 h), penetration (1 h), and intracellular (2 h). **Concentration is statistically different compared to untreated control (p < 0.01).

Fig. 6. Inhibitory effects of trans-cinnamic acid at different stages of CDV replicative cycle. Concentrations of 10 or 60 μg/mL were added at varying times during virus infection: Pre-treatment (−1 h), adsorption (0 h), penetration (1 h), and intracellular (2 h). **Concentration is statistically different compared to untreated control (p < 0.01).
the early stages of viral replication would reduce the number of infected cells and the formation of viral progeny. Furthermore, selectivity index (SI) greater than 3 indicates potential antiviral activity for compounds derived from plants (Chattopadhyay et al., 2009). All phenolic compounds evaluated in the present work exhibited SI values equal or greater than 3 (Table 1).

Quercetin and hesperidin inhibited the intracellular phase of CDV replication cycle. It has been demonstrated that quercetin and some other flavonoids can inhibit viral polymerase and interfere with the synthesis of viral nucleic acid (Formica and Regelson, 1995; Cushnie and Lamb, 2005). Interference by such phenolic compounds in the CDV post-infection stage has also been demonstrated by Gallina et al. (2011); the phenolic dimer proanthocyanadin A2 resulted in both reduction of viral RNA synthesis and viral progeny, suggesting that the replicative complex may be affected.

On the other hand, Formica and Regelson (1995) correlated the antiviral property of quercetin with its ability to bind to envelope glycoproteins and the viral capsid. Moreover, Schnitzler et al. (2008) showed that quercetin bound to glycoproteins of the viral envelope which blocked the host cell–virus interaction. According to Kim et al. (2010) quercetin showed a reduction of influenza virus A replication in the early stages of infection. Other studies with flavonoids and tannins showed similar results at the stages of adsorption and penetration of different viruses (Kuo et al., 2009; Gravina et al., 2011). The structural similarities among flavonoids might imply similar inhibitory mechanisms.

All phenolic acids evaluated in this study also demonstrated antiviral properties against CDV. Cinnamic acid derivatives have been reported to have antiviral effects against DENV (Rees et al., 2008) as well as EHV-1 in stages of pre-treatment and adsorption

Fig. 7. Inhibitory effects of ferulic acid at different stages of CDV replicative cycle. Concentrations of 5 or 10 μg/ml were added at varying times during virus infection: Pre-treatment (−1 h), adsorption (0 h), penetration (1 h), and intracellular (2 h). **Concentration is statistically different compared to untreated control (p < 0.01).

<table>
<thead>
<tr>
<th>Flavonoids</th>
<th>Log virus titer C+/Log virus titer [C]</th>
<th>F value</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time (h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>−1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Quercetin</td>
<td>1.02ab</td>
<td>1.20ab</td>
<td>1.16ab</td>
</tr>
<tr>
<td>Morin</td>
<td>1.02abc</td>
<td>1.10ab</td>
<td>1.05abc</td>
</tr>
<tr>
<td>Rutin</td>
<td>0.99abc</td>
<td>1.42ab</td>
<td>1.18abc</td>
</tr>
<tr>
<td>Hesperidin</td>
<td>1.00abc</td>
<td>1.32ab</td>
<td>1.16abc</td>
</tr>
<tr>
<td>F value</td>
<td>0.97 NS</td>
<td>35.01</td>
<td>5.93</td>
</tr>
<tr>
<td>CV (%)</td>
<td>2.78</td>
<td>3.31</td>
<td>3.94</td>
</tr>
</tbody>
</table>

Values in bold indicate significant reduction in virus titer compared to the virus control (p < 0.01).

Averages represented by the same lower case letter (column) and upper case letter (row) did not differ by Turkey test. C+ = untreated infected cells (positive control); [C] = common working concentration (30 μg/ml); −1 h: pre-treatment; 0 h: adsorption; 1 h: penetration; 2 h: intracellular; NS: no significant.

Antiviral action is statistically significant by Tukey test, p < 0.05.

Antiviral action is statistically significant by Tukey test, p < 0.01.

Fig. 8. Quercetin, morin, rutin and hesperidin chemical structures. R: sugar residue.

Fig. 9. Cis-cinnamic, trans-cinnamic and ferulic acids chemical structures.
hydroxyl at C-2 in ring A (Fig. 8). Although the structural similarity explains the antiviral action to inactivate viral particles and/or cellular receptors, per-

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