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Inhibition of viral RNA synthesis in canine distemper virus infection by proanthocyanidin A2

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ABSTRACT

Canine distemper virus (CDV) is a contagious and multisystemic viral disease that affects domestic and wild canines as well as other terrestrial and aquatic carnivores. The disease in dogs is often fatal and no specific antiviral therapy is currently available.

In this study, we evaluated the *in vitro* antiviral activity against CDV of proanthocyanidin A2 (PA2), a phenolic dimer belonging to the class of condensed tannins present in plants. Our results showed that PA2 exerted *in vitro* antiviral activity against CDV with a higher selectivity index compared to ribavirin, included in our study for the previously tested anti-CDV activity. The time of addition assay led us to observe that PA2 was able to decrease the viral RNA synthesis and to reduce progeny virus liberation, at different times post infection suggesting multiple mechanisms of action including inhibition of viral replicative complex and modulation of the redox milieu. These data suggest that PA2, isolated from the bark of *Aesculus hippocastanum*, has potential usefulness as an anti-CDV compound inhibiting viral replication.

1. Introduction

Canine distemper virus (CDV) is a highly infectious and an acknowledged lethal pathogen of wild and domestic carnivores. CDV belongs to the genus *Morbillivirus* of the family *Paramyxoviridae*, has a lipid envelope that contains non segmented single stranded RNA genome of negative polarity which encodes for two surface glycoproteins (the hemoagglutinin H and the fusion protein F), the viral matrix protein (M), the nucleocapsid protein (N), the phosphoprotein (P) and the large RNA-dependent RNA polymerase (L) (Sidhu et al., 1993). The replicative complex of paramyxoviruses, critical for both transcription and replication, is tripartite being formed by N–P–L proteins and is considered a promising target for the establishment of an effective antiviral chemotherapy (Bourhis et al., 2006). CDV is characterised by a systemic infection with high lethality rates in hosts without protective immunity. The common manifestations of CDV are pyrexia, anorexia, nasal discharge, conjunctivitis, diarrhoea and skin pustules with hyperkeratosis (Martella et al., 2008). In a high percentage of animals the central nervous system is infected causing severe complications with infiltrations of inflammatory cells and demyelination (Summers and Appel, 1994; Vandevelde and Zurbriggen, 1995).

The incidence of CDV in canine population seems to have increased in the past decades worldwide and even though the disease is controlled by vaccination, an increasing number of distemper cases has been recorded also in vaccinated dogs (Decaro et al., 2004; Ek-Kommonen et al., 1997; Scagliarini et al., 2003). Treatment consists of supportive care and antibiotics to prevent secondary bacterial infections but no antiviral therapy is available for the treatment of CDV. The nucleoside analogue 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin, RBV) is the only commercially available molecule with a well known antiviral activity against several members of the *Paramyxoviridae* family (De Clercq et al., 1991; Leyssen et al., 2005; Shigeta et al., 1992). It has demonstrated antiviral activity towards CDV *in vitro* (Dal Pozzo et al., 2010; Elia et al., 2008; Scagliarini et al., 2006).

Despite the identification of a growing number of synthetic compounds with high anti-paramyxovirus activity (White et al., 2007; Yoon et al., 2008), it is still worth to explore and better characterise the activity of plant-derived compounds. Plants can be a source of candidate drugs because of their rich content of a wide variety of secondary metabolites with antimicrobial properties. Phytochemicals

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represent an alternative to synthetic compounds in the treatment of infectious diseases and provide substitutes or first choice molecules in case of the development of resistant viruses.

Although no plant-derived drug is currently in clinical use to treat viral diseases, promising activity has been shown by some natural product/natural product derived candidates of diverse class particularly the phenolics, coumarins, flavonoids and alkaloids (Chattopadhyay and Naik, 2007). In particular, flavonoids are synthesized in response to microbial infections, hence have broad spectrum of antimicrobial activity and are well known inhibitors of many essential enzymes of viral replication and form complex with extracellular and soluble proteins (Chattopadhyay and Naik, 2007). Proanthocyanidins, also known as condensed tannins, being phenolic polymers, belong to the flavonoid group. These molecules are naturally occurring plant metabolites widely available in fruits, vegetables, nuts, seeds, flowers and bark. In particular, proanthocyanidin A2 (PA2) is a dimeric procyanidine (Fig. 1) resulting from the condensation of monomeric flavanols, this polyphenol has promising structural features since it possesses two catechol functions on the C and D rings (Husain et al., 1987; Fine, 2000).

Several authors have investigated the antiviral activity of PA2 and the relationship between flavonoid structure and inhibitory activity against DNA and RNA viruses (Brinkworth et al., 1992; Fesen et al., 1993; Iwasawa et al., 2009). Selway (1986) proposed that the antiviral mechanism of action can be based on both inhibition of viral polymerase and reduced binding of viral nucleic acid or viral capsid proteins. In addition, three proanthocyanidins from *Pavetta owariensis* (with structural similarity to PA2) have been shown to have activity against herpes simplex virus and Coxsackie B virus (Baldé et al., 1990; Yamada, 1991). Among the pharmacological properties of PA2, an immunomodulatory and an antioxidant activity have been also described (Lin et al., 2002; Liu et al., 2010; Zhang et al., 2005).

The aim of this study was to evaluate the *in vitro* antiviral activity against CDV of PA2 extracted from bark of *Aesculus hippocastanum* and to investigate its possible mechanism(s) of action.

2. Materials and methods

2.1. Cells and virus

VERO cells (culture collection ATCC® number CCL-81™) were used for the in *vitro* growth of CDV. Cells were cultured at 37 °C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen Corporation) supplemented with 10% fetal calf serum (FCS), 2 mmol/L of L-glutamine, 1 mmol/L of sodium pyruvate and 7.5% sodium bicarbonate. Bussell-CDV strain (Bussell and Karzon, 1965) has been propagated in cells using a 2% FCS medium and were subsequently titrated. The viral titer was expressed as the 50% infectious dose in the cell culture ($TCID_{50}$/mL).

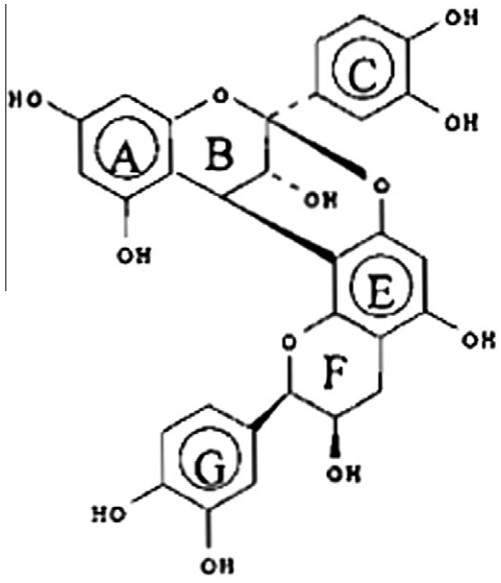

Fig. 1. Chemical structure of proanthocyanidin A2.

2.2. Proanthocyanidin A2

PA2 was extracted from bark of *A. hippocastanum* (provided by Indena S.p.A. Milan, Italy. Patent No. US6429202) with 98.76% purity determined by HPLC. PA2 in form of powder, was dissolved in a solution of dimethyl sulfoxide (DMSO) and phosphate-buffered saline (PBS) (GIBCO, Invitrogen, UK) in the ratio 1:1 (v/v) and then diluted in DMEM 2% foetal calf serum immediately before testing in the cell culture assay. Due to the reported poor stability of PA2 in cell culture medium (Lu et al., 2011) the working solutions were prepared immediately before testing.

2.3. Cytotoxicity assay

The cell toxicity of PA2, DMSO and RBV (ICN Biomedicals, Inc., Aurora, OH) were tested on growing cells using a colorimetric assay based on the mitochondria metabolization of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MI) as previously described (Denizot and Lang, 1986). The 50% cytotoxic concentration (CC_{50}) was defined as the compound concentration required to reduce the number of viable cells by 50% of the cell control. The CC_{50} values were expressed as the mean ± the standard deviation of at least three independent experiments.

2.4. Intracellular antiviral assay

The intracellular activity of PA2 against CDV was evaluated by the cytopathic effect (CPE) reduction assay (CPE-reduction assay) on confluent cells, using the protocol previously described (Scagliarini et al., 2006). Briefly, VERO cells seeded in 96-well microtiter plates were infected with 10^4 $TCID_{50}$/well for 2 h at 37 °C in 5% CO_2. After infection fresh medium containing several twofold dilutions in duplicate of PA2 were added. The antiviral activity was assessed when 100% CPE was reached in the virus control and expressed as the 50% inhibitory concentration (IC_{50}), defined as the minimum compound concentration required to reduce viral CPE by 50% compared to the virus control. The IC_{50} was calculated as the mean of three independent experiments ± the standard deviation. The selectivity index (SI) for the compound was obtained by calculating the ratio of the CC_{50} for cell viability and IC_{50} for the virus replication. During the CPE-reduction assay, RBV was used as a reference compound able to reduce the *in vitro* viral growth.

2.5. Extracellular antiviral assay

In order to evaluate the extracellular antiviral activity, VERO cells were cultured in 96-well microtiter plates and the confluent monolayer was infected with a viral inoculum of 10^4 $TCID_{50}$/100 μL/well and in the same time cells were treated with serial dilutions of PA2 and incubated at 37 °C and 5% CO_2. After 2 h, the medium containing the inoculum plus the compound was removed and replaced by DMEM 2% FCS.

The IC_{50} was calculated as the mean of three independent experiments ± the standard deviation.

2.6. Study of CDV replication cycle

2.6.1. Virus titration of samples collected during CDV replication cycle

The features of the CDV life cycle in VERO cells were studied, in order to better characterize the antiviral activity of PA2. 6-well microtiter plates were used and confluent VERO cells were infected with 10^5 $TCID_{50}$/well Bussell-CDV. After 2 h of incubation at 37 °C and 5% CO_2, the viral inoculum was removed and 3 mL of 2% FCS culture medium were added. After 8, 16, 24, 48 and 72 h, the supernatants and the cell monolayers were collected separately and stored at −80 °C. These samples were used to measure the viral growth by titration assay and the viral nucleic acid amount by SYBR Green Real Time PCR. In order to titrate the extracellular virus, the supernatants were thawed and the virus yield was evaluated by virus titration in 96-well microtiter plates. To collect the intracellular virus, the 6-well microtiter plates were thawed and the disrupted cells contained in PBS were collected from the bottom of the microplates and then centrifuged at 1200 rpm for 10 min. The supernatants, with the intracellular virus, were used for the virus titration. The titration was performed with serial 10-fold dilutions of the samples and the viral titer was expressed in $TCID_{50}$/mL.

2.6.2. Kinetics of viral RNA accumulation during CDV replication cycle

A two steps Real Time PCR technique was used to quantify the viral nucleic acid in the supernatants and in the cell monolayers collected from the study of the CDV replication cycle. Viral RNA was purified using the NucleoSpin® RNA II (Macherey–Nagel GmbH & Co. KG, Germany) and was stored at −80 °C. The target for the Real Time assay was within the CDV P gene encoding the phosphoprotein. To distinguish viral mRNA from viral genomic and antigenomic RNA, the purified nucleic acid was reverse transcribed using oligo (dT), forward and reverse primers. The nucleotide sequences of the forward and the reverse primers were 5′-GTCGGTAATCGAGGATTCGAGAGAG-3′(qCDVF4) and 5′-GCCGAAAGAATATCCCCAGTTAG-3′(qCDVR3), respectively. The reverse transcription reaction was performed with GeneAmp® RNA PCR (Applied Biosystems, USA) in a final volume of 20 μL, using 4 μL of Buffer 5×, 2 μL of $MgCl_2$ solution, 2 μL of DTT, 2 μL of dNTPs, 0.5 μL of oligo (dT) (for mRNA), or primer forward or reverse of the P gene (for genomic and antigenomic RNA, respectively), 0.5 μL of RNase inhibitor, 0.3 μL of reverse transcriptase, 3.7 μL of water and 5 μL of RNA. The reverse transcription was carried out in one step at 45 °C for 15 min, using the forward and reverse primers, while using the oligo (dT) a pre-incubation at 25 °C for 10 min was necessary, followed by 15 min at 45 °C. The nucleic acid quantification was obtained using the SYBR® Premix Ex Taq™II (Takara Bio Inc., Japan) with Rotor-Gene™3000 (Corbett Research, Australia). The reaction was performed in a final volume of 25 μL using 12.5 μL of SYBR® Premix Ex Taq™ 2×, 0.5 μL of the forward and reverse primers, 9.5 μL of water and 2 μL of cDNA. The reaction included a first denaturation step at 95 °C for 30 s, followed by 40 cycles at 95 °C for 20 s, 60 °C for 20 s and 72 °C for 20 s.

To create the standard curve, a fragment of the P gene amplified with the DMV1 and DMV2 primers pair (Barrett et al., 1993) was cloned into the pCR® 4-TOPO vector (TOPO TA Cloning® Kit, Invitrogen, Netherlands). Ten-fold dilutions of the plasmid were used in each reaction to create the calibration curve for absolute quantification of the viral samples. Each sample and each plasmid dilution were repeated in duplicate during the same reaction and the nucleic acid concentration was calculated as the mean of the two measurements.

2.7. Virus yield assay

The effect of three different concentration of PA2 was evaluated on the virus yield of Bussell-CDV in VERO cells. Confluent monolayers of VERO cells grown in 6-well microtitre plates were infected with CDV with 10^5 $TCID_{50}$/well. After 2 h of incubation at 37 °C and 5% CO_2, any residual virus was removed and replaced by medium containing 150, 100 and 75 μg/mL of PA2. Infected non-treated and mock infected controls were included at each time point: 24, 48 and 72 h post infection (p.i.). Cell monolayer and supernatant were collected separately and stored at −80 °C for further quantification. In particular, the supernatants were used in a titration assay for the quantification of extracellular viruses. The titration assay was performed with serial 10-fold dilutions of the samples and the viral titre, expressed as $TCID_{50}$/mL, was calculated using the Reed and Muench method for the determination of the 50% endpoint.

The viral nucleic acid amount in the cells monolayers and the supernatants was quantified by SYBR Green Real Time PCR targeting the P gene after RNA retrotranscription (RT PCR) using random primers as described in the Section 2.6.1. In each quantification assay five 10-fold dilutions of a recombinant plasmid were amplified to generate a standard curve, while the viral RNA samples were tested in duplicate. The inhibition of CDV replication was expressed as RNA copies number/μL at each time point.

2.8. Time of addition assay

To investigate the PA2 possible target, a time of addition assay was carried out using three different concentrations (150, 100 and 75 μg/mL) of PA2, each added at several times p.i. VERO cells grown in 24-well microplates were infected with Bussel-CDV strain at a m.o.i. of approximately 0.5. After an incubation time of 2 h at 37 °C and 5% CO_2 the viral inoculum was removed and replaced by 2% FCS culture medium. At different times p.i. (2, 8, 12, 14, 16, 24), the three concentrations of PA2 were added. At each time point, virus and cell controls were included in the assay. In the presence of 100% CPE in the virus controls (approximately 3 days p.i.), cells were collected in correspondence of each tested condition. The viral load was assessed by virus titration and the titer was expressed in $TCID_{50}$/mL. The viral nucleic acid amount was also quantified by SYBR Green Real Time PCR targeting the P gene after RNA retro-transcription using random primers as described in the previous paragraph. The inhibition of CDV replication was expressed as RNA copies number/μL at each time point.

2.9. Statistical analysis

All data were analysed by Prism GraphPad software 4.0 version. The data were expressed as the mean ± SD and statistical differences were examined independently using the Student's *t*-test. A p value of less than 0.05 was considered significant.

3. Results

3.1. Cytotoxicity and antiviral assays

The results obtained from the cytotoxicity and antiviral assay are showed in Table 1. The CC_{50} value of DMSO was higher than the DMSO % used to dissolve PA2, confirming that the volume of DMSO used to dissolve PA2 was not toxic for the cell monolayer. PA2 showed to be active against CDV and demonstrated a lower

Table 1
Results obtained from the cytotoxicity and the intracellular antiviral assays.

Compound	Cytotoxicity CC_{50} (μg/mL)	Antiviral activity IC_{50} (μg/mL)	Selectivity index SI
PA2	254.22 ± 31.71	89.81 ± 4	2.83
RBV	28.16 ± 2.58	26.08 ± 5.37	1.079
DMSO	1.37%	/	/

cytotoxicity compared to RBV. PA2 was also more selective than RBV. Neither RBV nor PA2 demonstrated to have extracellular antiviral activity.

3.2. CDV replication cycle

3.2.1. Virus titration of samples collected during CDV replication cycle

The features of CDV replication in VERO cells were explored via viral titration and viral RNA quantification applied to the supernatants and the cell monolayers collected separately at different times p.i. The results obtained after viral titration are presented in Fig. 2A where the viral titer was expressed as $\log_{10}$ of the $TCID_{50}$ value/mL. No viral particles were detected after 8 and 16 h p.i. in the supernatants, while CDV replication was evident after 16 h p.i. in the cell monolayer. Only starting from 24 h p.i. CDV infectious particles were detectable in the supernatant assay where the viral titer increased 3 $\log_{10}$ between 24 and 48 h. In the cell monolayer, the viral titre was increasing of 1.5 $\log_{10}$ between 16 and 24 h and 1 $\log_{10}$ between 24 and 48 h p.i., being constantly higher than the viral titer measured in the supernatants at the same time points.

3.2.2. Kinetics of viral RNA accumulation during CDV replication cycle

The kinetics of CDV RNA accumulation were also investigated in the course of the CDV replication cycle (Fig. 2B). Using different reverse transcription strategies, the growing kinetics of the mRNA and the genomic–antigenomic RNA referred to the P gene were monitored. In the infected cells between 8 and 24 h p.i. mRNA accumulated exponentially before reaching a plateau between 48 and 72 h p.i. In particular, between 8 and 24 h, P mRNA increased by 2 $\log_{10}$, showing a high viral transcription activity (Fig. 2B). The kinetics of the genome–antigenome was strikingly different (Fig. 2B). In the host cells between 8 and 72 h p.i., the genome–antigenome RNA accumulation followed a constant gradient with a rising of 0.5–1 $\log_{10}$ cDNA copy number/μL at each time point.

3.3. Virus yield

The inhibition of viral growth, in the presence of three different concentrations of PA2 and at different time p.i., was evaluated through viral titration and RNA quantification. The virus titration showed that at 24 h p.i. 150 μg/mL of PA2 completely inhibited the CDV infectivity, while the concentrations of 100 and 75 μg/mL produced a 30% virus yield reduction compared to the virus control ($p < 0.05$). After 48 and 72 h p.i., only 150 μg/mL of PA2 was able to significantly inhibit viral replication, with an extracellular virus yield reduced by 3 log compared to the virus untreated control (Fig. 3A). Quantitative RT-PCR revealed that viral RNA failed to be detected in the supernatant of infected cell monolayers treated with the highest concentration of PA2 at 24 h p.i., while a significant RNA reduction ($p < 0.05$) was observed at 48 and 72 h p.i. only with 150 μg/mL (Fig. 3B).

3.4. Time of addition

To investigate the PA2 mechanism of action a time of addition assay was carried out by adding three different concentrations (150, 100 and 75 μg/mL) of PA2 at 2, 8, 12, 14, 16 and 24 h p.i.

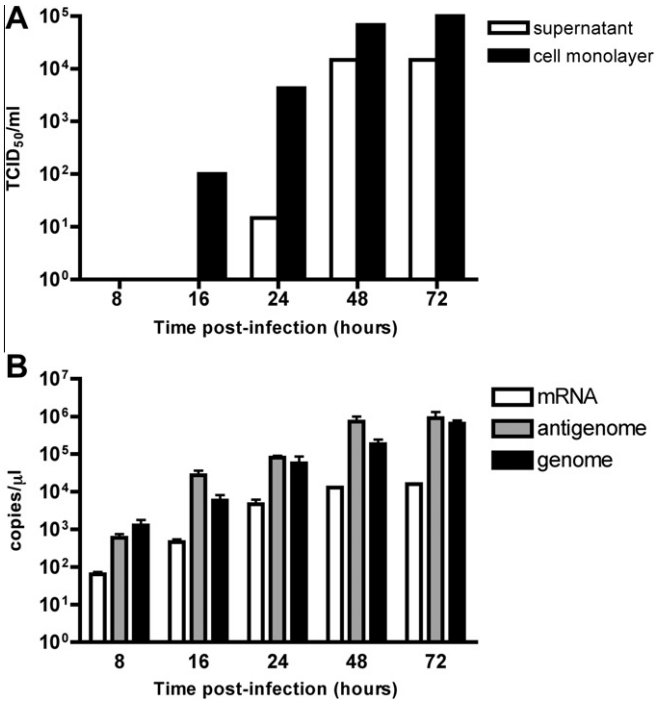

Fig. 2. CDV replication cycle. (A) Viral titration from supernatants (white columns) and the cell monolayers (black columns) collected during CDV replication cycle. The viral titer was expressed in $TCID_{50}$/mL. CDV infectious particles have been detected after 24 h post-infection in the supernatant and after 16 h p.i. in the cell monolayers. (B) Kinetics of viral nucleic acid accumulation in the infected cells during CDV replication *in vitro*. CDV P gene was used as target and the growth kinetics of P mRNA (white columns), viral genome RNA (black columns) and viral antigenome RNA (grey columns) were studied. The quantification of the viral nucleic acid is expressed in copies/μL and subsequently reported in a log scale.

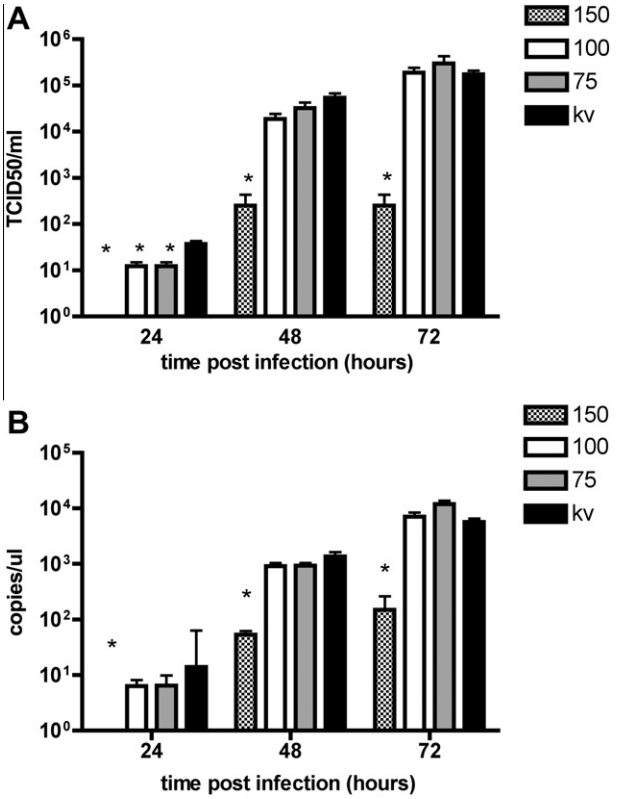

Fig. 3. Virus yield. (A) Viral growth was measured in the presence of different concentrations of PA2 after 24, 48 and 72 h post infection. CDV infectivity was measured in the supernatants by virus titration. (B) Viral RNA in the supernatants was quantified by SYBR Green real time PCR, the total RNA is expressed in copies/μL and represented in a log scale. At each time point, an untreated control has been tested (kV). Stars indicate data significantly different from the respective untreated control ($P < 0.05$, Student's *t*-test).

Among the three tested concentrations, 150 μg/mL was the only one able to inhibit completely the viral infectivity at each time point. A reduction ranging from 25% to 40% and from 16% to 22% in the viral titer was observed respectively after the treatment with 100 and 75 μg/mL compared to the untreated control (Fig. 4A).

The Real Time PCR showed that the addition of the three concentrations of PA2 produced a significant dose-dependent decrease of the viral RNA ($P < 0.05$) between 8 and 16 h p.i. In particular, after the treatment with 150 μg/mL, the inhibition in the viral RNA synthesis ranged from 45% to 61% (Fig. 4B).

4. Discussion

At present, antivirals have only few applications in veterinary medicine despite their potential impact in reducing the socio-economic consequences of disease outbreaks and in curing diseased pets. In the veterinary field vaccination is still considered as the main tool to control viral diseases but the possibility to treat clinically diseased animals in a rapid and specific way should be part of an integrated disease control strategy. Recently the use of antivirals has been proposed for foot-and-mouth disease as a prophylactic and therapeutic tool (Grubman, 2005; Goris et al., 2008). Similarly for classical swine fever virus *in vivo* experiments suggested the possible application of selected and potent antivirals as a control tool combined to vaccination in order to bridge the "immunity gap" in case of an outbreak (Vrancken et al., 2009).

The development of viral resistance towards antiviral agents enhances the need for new effective compounds against viral infections (Jassim and Naji, 2003). Natural products are promising sources for novel antivirals, in particular flavonoids, including proanthocyanidin, have been extensively studied, showing that in addition to their antioxidant activity, they possess antibacterial and antiviral efficacy (Cheng et al., 2002; Iwasawa et al., 2009; Takeshita et al., 2009; Xu et al., 2010).

In this study, we investigated the effect of the condensed tannin PA2 on the *in vitro* replication of CDV. Our results showed that PA2 exerted *in vitro* antiviral activity against CDV with a twofold higher selectivity index compared to RBV. The virus yield assay confirmed the antiviral activity of PA2 showing a time dependent reduction in the virus viability. The highest concentration of PA2 (150 μg/mL) was able to reduce the production of viral progeny at any time p.i., while lower concentration of PA2 were able to significantly reduce the virus yield only within 24 h p.i. The reduced production of infectious particles observed in the 150 μg/mL treated sample was associated with a significant decreased viral RNA amount. This finding suggested a first possible mechanism of PA2, thus the reduction of viral RNA synthesis.

To date, the molecular mechanisms of transcription and replication of CDV is still poorly understood; this complicates detailed studies on the mechanism of (novel) inhibitors. Our observations revealed that the release of infectious CDV particles from an infected culture started after 24 h p.i. Cell-associated virus and cell-free virus were characterised by an exponential growing phase until 48 h p.i. We concluded that a viral replication cycle was lasting approximately 24 h and that the eclipse period was extended around 16 h p.i. To understand the molecular mechanisms of CDV replication, we further investigated the dynamics of viral RNA synthesis during CDV infection. Our data, with an exponential growth of P mRNA and a linear growth of genome–antigenome RNA, suggested that during the first 24 h of infection viral transcription represented the main enzymatic activity of the viral RNA-dependent RNA polymerase (RdRp). This finding is in agreement with the observation of Plumet et al. (2005) about the dynamics of viral RNA synthesis during MV infection. In this study the authors showed that MV mRNA had an exponential accumulation until 24 h post-infection and they assumed that RdRp had a prevalent transcriptase activity during this stage of the infection (Plumet et al., 2005).

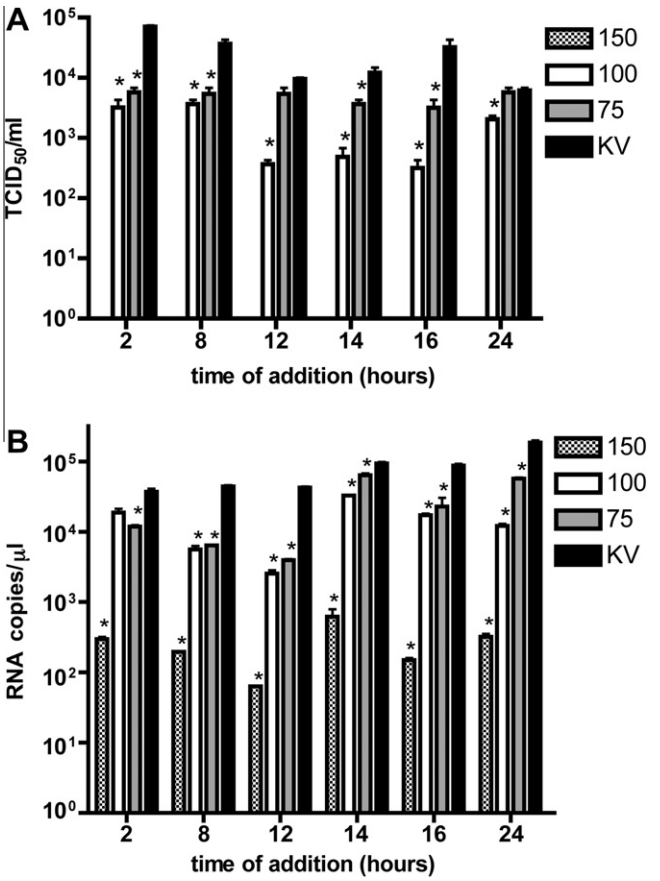

Fig. 4. Time of addition. (A) The viral load was assessed by virus titration, an inhibitory effect on virus replication has been shown after the addition of 100 and 75 μg/mL of PA2 at different times p.i. (B) Quantification of viral RNA, after the addition of 150, 100 and 75 μg/mL of PA2 at different times p.i. At each time point, an untreated control has been tested (kV). Stars indicate data significantly different from the respective untreated control ($P < 0.05$, Student's *t*-test).

To better identify the moment of the major anti CDV activity of PA2, we performed a time of addition assay which confirmed the initial finding of the interference of PA2 with viral RNA synthesis. However, the results obtained with PA2 were striking different from the ones obtained previously with RBV and its analogue EICAR, which clearly exhibited an anti-CDV activity limited to the first 10 h p.i. (Dal Pozzo et al., 2010). The addition of PA2 at each time point induced a reduced but constant presence of viral RNA accompanied by a contemporary inhibition of the viral replication. These results lead us to speculate that PA2 could exert its activity during the early and the late stages of CDV cycle, suggesting the existence of additional mechanisms of action explaining the anti CDV activity of PA2. PA2 inhibition of virion assembly was already observed for other viruses such as HSV-1 (Cheng et al., 2002). Ho et al. (2009) showed recently that the antioxidant capacity of tea polyphenols was able to reduce EV 71-induced oxidative stress with a consequent reduction of viral replication and progeny virus production. Since it is widely known that PA2 exerts a potent antioxidant activity *in vitro* and *in vivo* (Iwasawa et al., 2009) it is plausible to speculate that its antiviral activity may be also attributed to a modulation of the cellular redox milieu. The oxidative stress is able to affect interactions between host and viruses influencing pathogenesis and ROS (Reactive Oxygen Species) generation plays a fundamental role in the pathogenesis of CDV demyelination (Stein et al., 2004).

In conclusion, our *in vitro* observations suggest that PA2 exerts an anti-CDV activity during the early and the late stages of the viral replication cycle. This finding supports the existence of multiple mechanisms of actions which would need further investigations and future studies.

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