



In-Vitro Antiviral Activities of Extracts of Plants of The Brazilian Cerrado against the Avian Metapneumovirus (aMPV)

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ABSTRACT

Avian metapneumovirus (aMPV) is a negative-sense single-stranded RNA enveloped virus of the *Metapneumovirus* genus belonging to the *Paramyxoviridae* family. This virus may cause significant economic losses to the poultry industry, despite vaccination, which is the main tool for controlling and preventing aMPV. The aim of this study was to evaluate the antiviral activity of extracts of four different native plants of the Brazilian Cerrado against aMPV. The antiviral activity against aMPV was determined by titration. This technique measures the ability of plant extract dilutions (25 to 2.5 µg mL⁻¹) to inhibit the cytopathic effect (CPE) of the virus, expressed as inhibition percentage (IP). The maximum nontoxic concentration (MNTC) of the extracts used in antiviral assay was 25 µg mL⁻¹ for *Aspidosperma tomentosum* and *Gaylussacia brasiliensis*, and 2.5 µg mL⁻¹ for *Arrabidaea chica* and *Virola sebifera*. Twelve different extracts derived from four plant species collected from the Brazilian Cerrado were screened for antiviral activity against aMPV. *G. brasiliensis*, *A. chica*, and *V. sebifera* extracts presented inhibition rates of 99% in the early viral replication stages, suggesting that these extracts act during the adsorption phase. On the other hand, *A. tomentosum* inhibited 99% virus replication after the virus entered the cell. The biomonitoring fractioning of extracts active against aMPV may be a tool to identify the active compounds of plant extracts and to determine their precise mode of action.

INTRODUCTION

Avian metapneumovirus (aMPV) is a negative-sense single-stranded RNA enveloped virus of the *Metapneumovirus* genus belonging to the *Paramyxoviridae* family. This family includes viruses responsible for causing respiratory diseases in turkeys and in humans. In chickens, the aMPV is involved in the etiology of a multi-factorial disease also known as the Swollen Head Syndrome (SHS). Another member of the *Paramyxoviridae* family that is relevant to the poultry industry is the Newcastle disease virus. Avian metapneumovirus may cause significant economic losses to the poultry industry. Although vaccination is the main tool for the control and prevention of diseases caused by aMPV, pharmaceutical intervention is required under some circumstances. Furthermore, vaccinated subjects may acquire incomplete protection and/or there may be a reversion of the vaccine virus to pathogenicity (Catelli *et al.*, 2006).

Currently, at least 40 antiviral chemotherapeutic compounds are approved for the treatment of individuals infected with a variety of viruses (De Clercq, 2004). Most of the approved drugs and at least half of them are used for the treatment of human immunodeficiency virus (HIV) infection, as well as for the treatment of infections by herpes



virus (e.g., herpes simplex virus, varicella zoster virus, and cytomegalovirus), hepatitis B virus, hepatitis C virus, or influenza virus. Most of the approved antiviral agents are nucleoside analogs, which inhibit viral DNA synthesis (herpes virus) or viral reverse transcription (HIV) (Mukhtar *et al.*, 2008)

The use of different anti-microbial drugs derived from plants is currently limited to human diseases, with very few applications in veterinary medicine (Pieroni *et al.*, 2006). However, considering the evidence accumulated to date, it is expected that their utilization will become increasingly important both in terms of limiting the environmental dissemination of pathogens and of animal health maintenance.

One of the most critical issues in the development of new antiviral drugs is the model to be used. The cell substrate requirements for virus replication limits the screening of new compounds. For this reason, few drugs have emerged as efficient antivirals (Abou-Karam & Shier, 1990 and Jassim & Naji, 2003). New technologies aimed at increasing the throughput of the antiviral screening process and the significant advances in viral molecular biology are opening new frontiers; however, no considerable success in therapeutics has been achieved yet.

A new antiviral screening technique has been used to optimize the discovery of new substances. The technique is widely used in the discovery of drugs against cancer in order to evaluate the pharmacological properties of the compounds. Additionally, experimental models should mimic the physiological properties of the processes in the human body, thus reducing the number of false positives (Smee *et al.*, 2002 and Cos *et al.*, 2006).

The screening of antiviral compounds requires the development of cell-culture systems that allow optimal viral replication and the selection of the most appropriate method to evaluate the action of these compounds against viruses (Smee *et al.*, 2002).

The cytopathic effect assay is one of the most reliable and robust assays for the screening of large compound libraries, and it has been successfully adapted into a high throughput format for the identification of novel antiviral agents against a number of viruses. The influenza virus (Noah *et al.*, 2007 and Severson *et al.*, 2008), the severe acute respiratory syndrome coronavirus (SARS-CoV) (Severson *et al.*, 2007), the arenavirus (Bolken *et al.*, 2006), and the bluetongue virus (Li *et al.*, 2009) were identified using this technique.

This study aimed at evaluating the antiviral activity of four different Brazilian Cerrado plants against aMPV. In addition, aMPV could be a surrogate to evaluate other members of the *Paramyxoviridae* family with similar replication characteristics.

MATERIALS AND METHODS

Extraction of plant material

Plants were obtained from the Cerrado biome (the second largest biome in South America) and deposited at the University of Campinas, Campinas, SP, Brazil (Table 1).

Table 1 – Cerrado plant and description of each extracts used for aMPV screening.

Items	Plants/ Voucher (registration number)	Description of Extracts
01	<i>Aspidosperma tomentosum</i> Mart. UEC-IB-267*	Extraction at room temperature with ethyl acetate
02		Extraction at room temperature with dichloromethane
03		Extraction at room temperature with acetone
04	<i>Gaylussacia brasiliensis</i> (Spreng.) Meisn. UEC-IB-266*	Extraction at room temperature with dichloromethane
05		Extraction at room temperature with acetone
06		Extraction at room temperature with ethyl acetate
07		Extraction at room temperature with ethanol 70%
08		Extraction at room temperature with ethanol 60%
9	<i>Virola sebifera</i>	Soxhlet extraction with dichloromethane
10	UEC-IB-258*	Soxhlet extraction with ethanol
11	<i>Arrabidaea chica</i> CPQBA-1348**	Ethanol/ 0.3 % citric acid extraction with no enzymatic treatment
12		Ethanol/ 0.3 % citric acid extraction with enzymatic treatment

* UEC-IB-267; UEC-IB-266 and UEC-IB-258: Deposited at the Institute of Biology, State University of Campinas - Campinas/SP, Brazil.

** CPQBA-1348: Deposited at the Center for Chemical, Biological and Agricultural (CQBA) – State University of Campinas, Campinas/SP, Brazil

Extracts from the leaves of *Aspidosperma tomentosum* Mart., known as “peroba-do-cerrado” (1); *Virola sebifera* L., known as “ucuúba-do-cerrado or mucuíba” (2); and *Gaylussacia brasiliensis* Meisn, known as “camarinha” (3) were obtained by using dichloromethane, and the resulting compound was further extracted with 70% ethanol in combination with maceration at room temperature. The plant *Arrabidaea chica* Verlot, known as “carajuru and guajuru-piranga” (4), was submitted to enzymatic treatment with xylase, followed by extraction with methanol supplemented



with 3.0g of citric acid, resulting in an enzyme crude extract (CE). A crude extract of *A. chica* (4) with no enzymatic treatment was also obtained (SE).

Virus and cell line

The CER cells (chicken embryo related cell) were propagated as monolayer cultures using minimal essential medium (MEM) with Earle's salts and supplemented with 10% fetal bovine serum (FBS). Cells were serially diluted from 1:2 to 1:10, according to conventional procedures using 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA).

The aMPV strain SHS/669/03 was isolated by D'Arce *et al.* in 2005 and belongs to aMPV subtype A. When the virus was incubated with the cells, the MEM medium was not supplemented with FBS.

Cell cytotoxic effect

Maximum nontoxic concentration (MNTC) was determined microscopically by the observation of morphological changes in the cells at 24, 48 and 72 hours of incubation. Cell suspensions were seeded at 100 μL /well in a 96-well culture plate at a density of 1×10^5 cells/mL. The plates containing cells were pre-incubated for 24 h at 37 °C to allow stabilizations prior to the addition of samples (100 μL) at four concentrations (0.25, 2.5, 25, and 250 $\mu\text{g}\cdot\text{mL}^{-1}$) (Kohn *et al.*, 2007).

Cytotoxicity after 72 h was measured using the sulforhodamine B (SRB) assay, performed as described by Kohn *et al.* (2007). Briefly, the cells were fixed using 50% Trichloroacetic Acid (TCA) at 4°C (50 μL per well, final concentration 10%) for 1h. The supernatant was discarded and the plates were washed five times with filtered water. The cells were stained for 30 min with 0.4% SRB in 1% acetic acid (50 μL per well) and subsequently washed four times with 1% acetic acid to remove any unbound dye. The plates were air-dried, and protein-bound dye was solubilized with 150 μL (100 mM) of Trizma buffer. The resulting optical density (OD) was read in a multi-well plate under a spectrophotometer at 540 nm.

Virus titration

The cells were seeded in 96-well culture plates at a density of 1×10^5 cells/mL and then incubated at 37 °C in a humidified atmosphere containing CO₂ for 24 h. Serial dilutions of virus stocks were prepared and cells were infected accordingly. After an additional incubation period (1-2 days), the cytopathic effect was recorded. The 50% tissue-culture infective dose

(TCID₅₀) per mL was calculated as previously described by Reed & Munch (1938).

Antiviral activity

The determination of the antiviral activity of evaluated plant extracts was based on cytopathic effect inhibition. All experiments were performed in triplicate. For the evaluation of inhibition, cells were seeded in 96-well culture plates. After 24h of incubation, the medium was replaced with 100 μL Dulbecco's Modified Eagle's Medium (DMEM), containing the plant extracts at MNTC, and 50 μL of logarithmic dilutions of the virus were added in quadruplicate; the plates were incubated for 3 days. Controls consisted of untreated infected (virus titer), treated non-infected (extract control), and untreated non-infected (cell control) cells. Viral titers were calculated as previously described by Reed and Munch (1938), and determined as 50% of the infective dose in tissue culture (TCID₅₀/mL). Antiviral activity of each extract was determined as the logarithm reduction factor (\log_{10}) of the viral titer compared with untreated infected controls. Values were expressed as titer (TCID₅₀/mL) and inhibition percentage (IP), as described in Koseki *et al.* (1990). The inhibition percentage was calculated according to the formula: $(IP) = (1 - T/C) \times 100$, where T is the antilog of the extract-treated viral titers and C is the antilog of the control (without extract) viral titers. IP was considered positive if greater than or equal to 98%.

The antiviral activity was initially evaluated with a single dose at MNTC against different viral concentrations. The extract was considered positive if there was a 1.5 log decrease in the viral titer. In order to confirm antiviral activity, a concentration response curve with different extract concentrations in the presence of 100 TCID₅₀/mL was calculated using the MTT¹ assay to establish the half maximal effective concentration (EC50). Briefly, an MTT solution at 5 mg·mL⁻¹ in PBS was added to the 96-well culture plates at 20 μL /well at each time point. Following 4h of incubation, 100 μL of DMSO was added to each well and mixed thoroughly to dissolve the dark-blue formazan crystals from surviving cells. The resulting optical density (OD) was read in a multiwell plate in a spectrophotometer at 540 nm (Mosmann, 1983 and Scudiero *et al.*, 1988).

¹ MTT is a cell viability assay. According to Mossmann (1983), the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was the first homogeneous cell viability assay developed for a 96-well format that was suitable for high throughput screening (HTS).



Statistical analysis

The 50% cytotoxic (CC_{50}) and 50% inhibition (IC_{50}) concentrations were calculated from concentration-effect curves. The results were obtained from triplicate assays with at least five extract concentrations. The percentage of cytotoxicity was calculated as $[(A - B)/A] \times 100$, where A and B are the OD 540 nm of untreated and of treated cells, respectively. The percentages of protection were calculated as $[(A - B) \times 100 / (C - B)]$, where A, B and C indicate the absorbance of the extracts or fractions, virus and cell controls, respectively. Each obtained EC_{50} value was defined as the effective concentration that reduced the absorbance of infected cells to 50% when compared with cell and virus controls. The CC_{50} and IC_{50} of each compound were obtained from dose-effect curves (not shown). The CC_{50} and IC_{50} are the average of three assays with five concentrations within the inhibitory range of the compounds. The selective index was defined as CC_{50}/IC_{50} .

Potential stage of the viral infection cycle

Cells and viruses were incubated with the active plant extracts at different stages during the viral infection cycle in order to assess different modes of antiviral action (virus inactivation before infection or during the virus adsorption and replication phases). Cells were pretreated with the extracts prior to viral infection (virus inactivation); viruses were incubated with the extracts before infecting the cells (adsorption phase); the cells were infected with the virus and incubated with the plant extracts (replication phase). Each extract was used at its maximum noncytotoxic concentration.

Statistical analysis

The selectivity index (SI) was determined as the EC_{50} to IC_{50} ratio. All experiments were performed in triplicate, and three independent experiments were conducted. Data are presented as mean \pm SD. The Student's t-test was used to evaluate the difference between the test and control samples. Differences were considered statistically significant when p-value was < 0.05 .

RESULTS AND DISCUSSION

Medicinal plants have been traditionally used for the treatment of different health conditions, including infectious diseases (Severson *et al.*, 2008; Li *et al.*, 2009). According to Cragg's 2009 report, approximately 60% of the anti-tumor and anti-infective agents that are

commercially available or in late stages of clinical trials today are of natural product origin. Therefore, there is no doubt that traditional medicinal plants may serve as potential sources for the development of new antiviral agents in the future.

Developing new antiviral drugs is a difficult task due to the poor selective toxicity and fast selection of resistant viral variants that naturally arise given a selective pressure. The frequency of viral resistance to antiviral drugs is increasing and consequently, viral diseases remain difficult to treat.

The screening of plants as possible sources of antiviral agents has led to the discovery of potent inhibitors of *in-vitro* viral replication, increasing the probability of identifying new bioactive plant compounds (Severson *et al.*, 2008; Li *et al.*, 2009).

In-vitro assays usually rely on the virus ability to infect and replicate in specific cell lines in cell culture systems. Cell culture systems provide a rapid and reliable method to grow viruses at higher titers, to apply reverse genetics, and to test antiviral compounds.

A total of 12 extracts derived from four plant species collected from the Brazilian Cerrado were screened for antiviral activity against aMPV. Table 1 shows the plant names and extraction procedures. The results suggests that there is an increasing activity of the extract *A. tomentosum* acetone and *G. brasiliensis* dichloromethane with SI higher than 1.5. However, the most active extracts against AMPV were from *A. chica* and *V. sebifera* with an inhibition percentage of 99%, both extracted with acetone. The four extracts were tested to determine in which phase of the virus replication cycle they were most active.

The first stage of the antiviral assay is necessary to determine the maximum concentration (MNTC) of the extract that is not toxic to the cells. After the MNTC of the extracts was determined, 25 $\mu\text{g mL}^{-1}$ of *Aspidosperma tomentosum* and *Gaylussacia brasiliensis*, and 2.5 $\mu\text{g mL}^{-1}$ of *Arrabidaea chica* and *Viola sebifera* were applied. The concentration range used to assess the activity of the extracts did not induce significant toxicity to the host cells and the 50% cytotoxic concentration (CC_{50}) determined for each extract were well tolerated by the CER cells. The CC_{50} ranged from 25 to 0.25 $\mu\text{g mL}^{-1}$ and none of the extracts tested induced any visible changes in cell morphology and cell density.

In order to identify the step of the viral cycle where replication was prevented, cells were infected with viruses after pre-incubating the cells with each evaluated extract. The viruses were pretreated with the



extracts prior to infection and were added during or after the adsorption phase or during the intracellular replication period. Three different treatments were applied. The cells were infected with the virus, followed by addition of the extracts after 1 h in order to evaluate the viral replication phase, viruses were pretreated with the extract before infecting the cells (virus inactivation), while the extract were added to the cells before viral infection to evaluate any effects during viral adsorption. In all experiments, the extracts were used at their MNTC, and the results are shown in Figures 1 and 2.

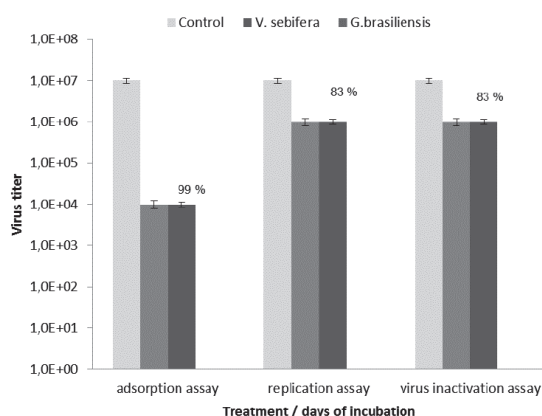


Figure 1 – Antiviral activity, as determined by inhibition percentage (IP), using virus adsorption, replication and inactivation assays of *Virola sebifera* and *Gaylussacia brasiliensis* extracts. Virus titer (TCID₅₀) was determined three days after infection and compared with untreated control cells. Virus and CER cells were pretreated with each extract at MNTC. Experiments were independently repeated and data presented here represent the average of three experiments.

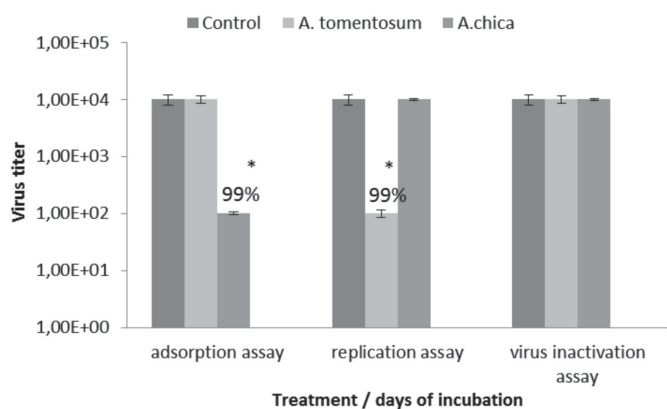


Figure 2 – Antiviral activity, as determined by inhibition percentage (IP), using virus adsorption, replication and inactivation assays of *Aspidosperma tomentosum* and *Arrabidaea chica*. Virus titer (TCID₅₀) was determined three days after infection and compared with untreated control cells. Virus and CER cells were pretreated with each extract at MNTC. Experiments were independently repeated and data presented here represent the average of three experiments.

The analyses of the results (Figures 1 and 2) suggest that the *G. brasiliensis*, *A. chica*, and *V. sebifera* extracts inhibit 99% of the virus during the early replication stage and that these extracts act during the viral adsorption phase. The fact that the extracts maintained their activity under these conditions suggests that they

establish stable bonds with virus receptors on the cell surface. However, this may also be due to other inhibitory mechanisms acting during the adsorption or penetration of the virus into the cell. Further studies should be conducted to determine the precise mode of action of these extracts. The 50% cytotoxic (CC₅₀) and 50% inhibition (IC₅₀) concentrations, as well as the selective index (SI) of the extracts active against aMPV were calculated and are listed in Table 2.

Table 2 – Inhibition percentage (IP), 50% cytotoxic (CC₅₀) and 50% inhibition (IC₅₀) concentrations, and selective index (SI) of the four extracts against aMPV in CER cell line.

Crude Extract	IP (%)	CC ₅₀ (µg.mL ⁻¹ ± SD)	IC ₅₀ (µg.mL ⁻¹ ± SD)	SI
<i>Aspidosperma tomentosum</i>	97	64.9 ± 0.05	45.86 ± 0.02	1.5
<i>Gaylussacia brasiliensis</i>	99	41.23 ± 0.03	22.33 ± 0.12	1.8
<i>Virola sebifera</i>	99	2.7 ± 0.08	202 ± 0.05	0.1
<i>Arrabidaea chica</i>	99	117.1 ± 0.017	412.7 ± 0.018	0.3

CONCLUSION

Despite continuous advances made in antiviral therapy, viral diseases are still the leading cause of death globally. Being obligate intracellular parasites, the replication of viruses is dependent on the metabolic pathways of the host cell. Since viruses and hosts are intimately connected, the design of effective antiviral agents that attack viral enzymes or virus replication, without affecting the host cell, has proven to be difficult.

The results of the present investigation provide further evidence of the potential use of medicinal plants, which possibly represent a reservoir of pharmacologically active substances. The study showed that *A. tomentosum*, *V. sebifera*, *G. brasiliensis*, and *A. chica* crude extracts are capable of inhibiting 99% of aMPV *in vitro*. However, further studies on plant fractions, aiming at isolating and identifying their active compounds and at determining their precise mode of action are required.

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