



## In vitro effects of bufotenine against RNA and DNA viruses

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### Abstract

Bufotenine, an alkaloid that can be found in plant extracts and skin secretions of amphibians, is reported to have potential antiviral activity. The present study evaluated the antiviral activity of bufotenine against different genetic lineages of rabies virus (RABV, a single-stranded, negative-sense RNA virus), canine coronavirus (CCoV, a positive-sense RNA virus) and two double-stranded DNA viruses (two strains of herpes simplex virus type 1/HSV-1 [KOS and the acyclovir-resistant HSV-1 strain 29R] and canine adenovirus 2, CAV-2). The maximal non-toxic bufotenine concentrations in Vero and BHK-21 cells were determined by MTT assays. The antiviral activity of bufotenine against each virus was assessed by examination of reductions in infectious virus titres and plaque assays. All experiments were performed with and without bufotenine, and the results were compared. Bufotenine demonstrated significant RABV inhibitory activity. No antiviral action was observed against CCoV, CAV-2 or HSV-1. These findings indicate that the antiviral activity of bufotenine is somewhat linked to the particular infectious dose used and the genetic lineage of the virus, although the mechanisms of its effects remain undetermined.

**Keywords** Rabies · Herpesvirus · Adenovirus · Coronavirus · Alkaloid · Bufotenine

### Introduction

Viruses infect all types of organisms, from archaea to unicellular bacteria and animals; currently, more than 6.500 species of viruses have been described [1]. Most viruses are apathogenic to their hosts. On occasion, disease may occur as unwanted collateral damage from viral infections;

however, in their ideal form, parasitism, viral infections should bring no harm to either the virus or its host. Because of inadequate adaptation, particular disease conditions eventually become associated with defined viral infections. Illnesses can be caused by RNA viruses such as rabies virus (*Rabies lyssavirus*, RABV) and canine coronavirus (*Alphacoronavirus 1*, CCoV) or DNA viruses such as human herpesvirus type 1 (*Human alphaherpesvirus 1*, HSV-1) and canine adenovirus type 2 (*Canine mastadenovirus A*, CAV-2). Many other viruses have been identified as aetiological agents of diseases; in the last year, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was identified as responsible for COVID-19, a globally pandemic respiratory disease [2]. The dissemination of SARS-CoV-2 sparked studies seeking to develop vaccines, antivirals or identify new potential antiviral actions of different agents. Unfortunately, to date, antiviral agents have been successfully developed for only a few virus, such as herpesvirus, hepatitis B virus, hepatitis C virus and influenza virus [3]. As such, there is still an urgent need for molecules that present antiviral potential [4].

Antiviral agents can be found in different sources, such as marine organisms, in which the first agent against human immunodeficiency virus (HIV), AZT, was identified [5, 6].

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Another potential source of antiviral agents is plants such as *Maytenus ilicifolia*, *Ilex theezans* and *Ilex brevicuspis*, native species from South Brazil that show antiviral activity against HSV-1 [7]. Among these, alkaloids have been evaluated as antivirals on a number of occasions, such as berberine, which reduces chikungunya virus, human cytomegalovirus, human papillomavirus, HSV and HIV infection [8, 9]. Alkaloids are secondary compounds that have been identified predominantly in plants and less frequently from fungi and animals. Alkaloids have an extensive range of biological activities, including, in some cases, antiviral properties [10]. Here, we focused on the potential antiviral activity of bufotenine, an indole alkaloid that occurs naturally in plants and amphibians. Indole alkaloids can interact with cellular receptors to act as agonists or antagonists. The antiviral activity of bufotenine on RABV was first described by Vigerelli [11–14]. However, here, a study was conducted to determine the antiviral activity of bufotenine against viruses with different mechanisms of replication, including negative- and positive-sense RNA viruses (RABV and CCoV) and double-stranded DNA viruses (HSV-1 and CAV-2).

## Materials and methods

### Bufotenine

The alkaloid was purified from *Anadenanthera colubrina* seeds as described by Vigerelli et al. (2014) [11] and initially diluted in Eagle's minimal essential medium (E-MEM) containing 10% heat-inactivated foetal bovine serum (FBS) to a 9 mg/mL concentration. The preparation was stored at 4 °C until use.

### Viruses

All viruses described below were propagated in the respective cell lines as described in the Cell lines section, collected and stored at –80 °C for subsequent experiments.

### RNA viruses

Four genetic lineages of rabies virus (RABV) were used: a genetic lineage from the insectivorous bat *Eptesicus furinalis* (964/06), a genetic lineage from the haematophagous bat *Desmodus rotundus* (4005/10), a genetic lineage from the wild dog *Cerdocyon thous* (4871/11) and a genetic lineage from the domestic dog *Canis lupus familiaris* (3629/11). In addition, strain MAV #795 of the RNA canine coronavirus (CCoV) was used.

### DNA viruses

The following DNA viruses were used: the herpesvirus type 1 (HSV-1) strains KOS (University of Rennes, France) and acyclovir-resistant HSV-1 strain 29R and the canine adenovirus type 2 (CAV-2) strain Toronto A26/61.

### Cell lines

Baby hamster kidney cells (BHK-21, originally ATCC CCL-100) were used to propagate RABV, while African green monkey kidney cells (Vero, originally ATCC CCL-81) were used to propagate CCoV, HSV-1 and CAV-2. The cells were cultured in E-MEM containing 10% heat-inactivated FBS and maintained at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere.

### Cytotoxicity assay

The cytotoxicity of bufotenine towards uninfected cells was evaluated with an MTT (3–4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide) assay. Different concentrations of bufotenine (4.0, 2.0, 1.0 and 0.5 mg/mL) were added to 96-well microplates seeded with  $5 \times 10^5$  cells/well of either BHK-21 or Vero cells. For a positive cytotoxicity control, 20% dimethyl sulfoxide (DMSO) was added to the culture medium. The negative controls included cells overlaid with cell culture medium only. After 24 h at 37 °C, the medium was removed, and 50 µL of MTT (5 mg/mL in E-MEM without FBS) was added to the plates, which were then incubated at 37 °C for 4 h. After incubation, the MTT solution was removed, and 100 µL of DMSO was added to each well to solubilize the formazan crystals. After gently shaking the plates, the crystals were completely dissolved, and the absorbances were measured in a spectrophotometer (Abs 540 nm).

### Antiviral activity

The antiviral activity of bufotenine for each virus was assessed on the basis of the reductions in infectious virus titres (Reductions in infectious virus titres section) and plaque counts (Plaque assay section). All experiments were performed in quadruplicate with and without bufotenine according to concentration previously determined by the MTT assay (Cytotoxicity assay section), and the results were compared. Bufotenine was prepared according to the previously determined concentration described in [Cytotoxicity assay](#) section.

## Reductions in infectious virus titres

Virus titres were determined by limiting dilution and are expressed as the 50% tissue culture infection dose per 50  $\mu\text{L}$  ( $\text{TCID}_{50}$ ) according to Reed and Muench, 1938 [15]. Briefly, virus stocks were prepared in tenfold dilutions ( $10^{-1}$  to  $10^{-7}$ ) in E-MEM, and 50  $\mu\text{L}$  of stock was added to the wells of 96-well microplates in quadruplicate. Subsequently, 50  $\mu\text{L}$  of a cell suspension containing  $5.0 \times 10^5$  cells/mL was added. The plates were then incubated at 37 °C in a humidified 5%  $\text{CO}_2$  atmosphere for 72 to 120 h. For visualization of RABV antigens, cells were stained as described below (section Direct fluorescent antibody (dFA) test for detection of RABV antigens). The plates were examined daily in search of evidence of cytopathic effects of CCoV, CAV-2 and HSV-1 with the aid of an optical microscope.

**Direct fluorescent antibody (dFA) test for the detection of RABV antigens** After 72 h, the medium was removed by aspiration, and the BHK-21 cells were fixed by adding cold 80% acetone (200  $\mu\text{L}$ ). After 30 min, the acetone was discarded by inversion, and the microplates were dried. Next, an anti-rabies IgG/fluorescein isothiocyanate conjugate (Pasteur Institute) was added as previously described [16]. After this period, the plates were washed three times with phosphate-buffered saline (PBS) and then three more times with distilled water. Finally, 50  $\mu\text{L}$  of 10% glycerine was added to each well, and the plates were examined under an inverted fluorescence microscope.

## Plaque assay

Briefly, confluent cell monolayers prepared on 96-well plates were infected with 0.1, 1, 10 and 100 plaque-forming units (PFU) of RABV, CCoV and CAV-2 in 50  $\mu\text{L}$  of inoculum with and without bufotenine and grown for 72 to 120 h. For HSV-1, the assay was performed in 6-well plates. Vero cells were seeded 24 h before infection, and then 0.1, 1, 10 and 100 PFU, bufotenine and 0.3% agarose

in E-MEM supplemented with FBS were added. Plaques were stained with Coomassie blue. For visualization of RABV antigens, the cells were stained as described above (section Direct fluorescent antibody (dFA) test for detection of RABV antigens). The plates were examined daily in search of evidence of cytopathic effects of CCoV and CAV-2 with the aid of an optical microscope.

## Statistical analysis

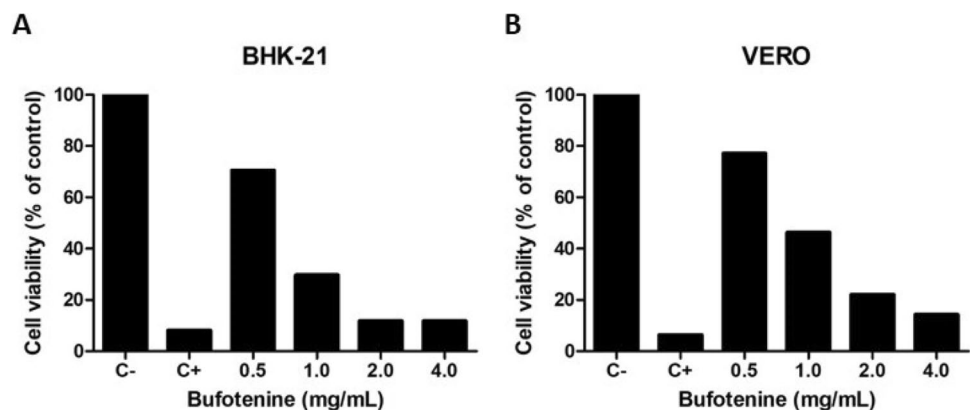
The 50% cytotoxic concentration ( $\text{CC}_{50}$ ) was estimated by a non-linear regression analysis of the concentration–effect curves. Multiple comparisons were performed using one-way ANOVA. Comparisons between the “with bufotenine” and “without bufotenine” groups were performed using Student’s *t* test. Statistical significance was accepted at  $p < 0.05$ , and the analyses were performed using GraphPad Prism version 5.0 software for Windows.

## Results

### Cytotoxicity assay

The MTT method was used to measure the cytotoxicity of bufotenine in BHK-21 and Vero cells. As shown in Fig. 1, when bufotenine was used at 0.5 mg/mL, the cell viability was 71% and 77% for BHK-21 and Vero cells, respectively. As this was the highest concentration of the drug that induced low cytotoxicity, it was used for all subsequent assays. The  $\text{CC}_{50}$  of bufotenine were 0.64 mg/mL and 0.82 mg/mL for BHK-21 and Vero cells, respectively.

**Fig. 1** Cytotoxicity assay of bufotenine in BHK-21 (A) and Vero (B) cells, as determined by MTT assay. C-, negative control (no bufotenine added); C+, positive cytotoxicity control (E-MEM plus 20% DMSO)



## Antiviral activity

### Reductions in infectious virus titres

**Effect of bufotenine on RABV** When evaluating the effect of bufotenine on cells infected with different RABV strains, a statistically significant difference in infectious titres was detected with one lineage only (strain 964/06, originating from a non-haematophagous bat, *Eptesicus furinalis*). There was a 1.7 log<sub>10</sub> reduction in infectious titre following bufotenine addition. The infectious titres registered with other RABV strains were not significantly affected by the addition of bufotenine (Fig. 2).

**Effect of bufotenine on CCoV, HSV-1 and CAV-2** Bufotenine did not inhibit CCoV, HSV-1 or CAV-2. The viral titres with and without bufotenine were similar for these viruses, and no significant reductions in titres were identified.

### Plaque assay

**Effect of bufotenine on RABV** As shown in Fig. 3, bufotenine had significant effects on all strains of RABV tested when 100 PFU was used. However, when 10 PFU was used, it was also possible to observe significant reductions except in the strain with a genetic lineage from the domestic dog, 3629/11 (Fig. 3B).

**Effect of bufotenine on HSV-1** The results regarding the action of bufotenine against the two strains of HSV-1 are shown in Fig. 4. Figure 4A shows the effect of bufotenine against the HSV-1 standard strain KOS. Despite the difference observed between 10 and 100 PFU, with a 23.5% and 12.9% reduction, these values were not considered statistically significant. At 1 PFU, no reduction in titres were identified with bufotenine. For acyclovir-resistant HSV-1 strain

29R, no significant reductions were identified with any of the PFUs evaluated (Fig. 4B).

**Effects of bufotenine on CCoV and CAV-2** Bufotenine did not inhibit CCoV or CAV-2. The numbers of viral plaques with and without bufotenine were similar for these viruses for all PFUs used for the experiments.

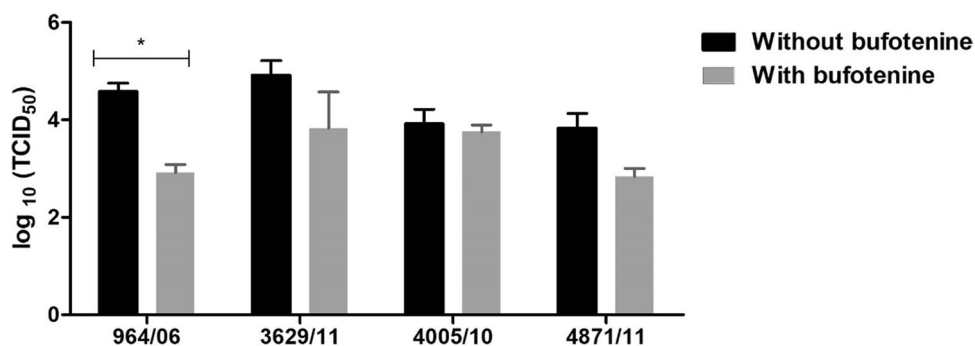
In Table 1, the action of bufotenine against each studied virus is summarized as determined with the two methods employed.

## Discussion

Viral infections remain major health issues worldwide. In order to combat viruses that have devastating effects on humans and animals, efforts to discover new antiviral compounds have been made. However, the current selection of antivirals approved for clinical use is limited. The majority of antiviral agents currently available have been developed for the treatment of human immunodeficiency virus (HIV) [3].

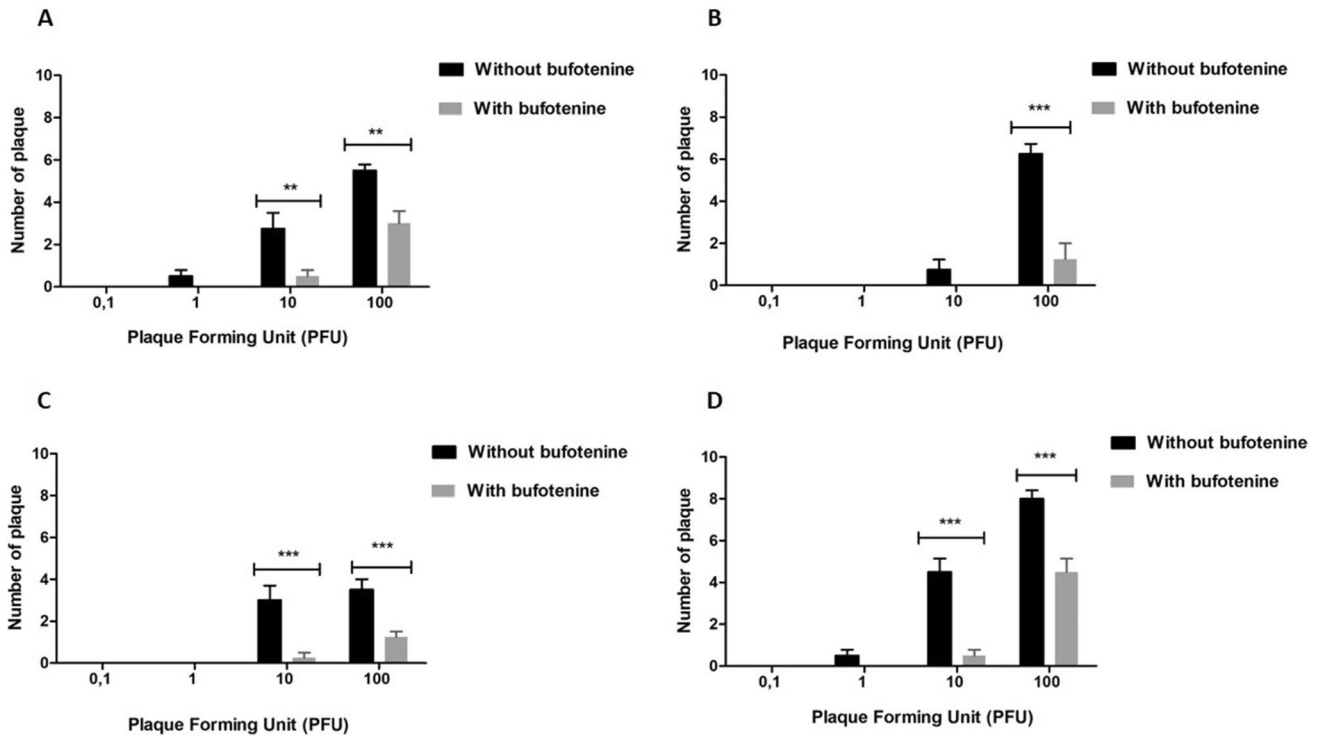
Bufotenine is an alkaloid whose antiviral activity against RABV has already been identified [11–14]. In the present study, we evaluated the antiviral potential of bufotenine against DNA and RNA viruses that infect humans and animals.

CAV-2 is an important dog pathogen from the *Adenoviridae* family that is responsible for respiratory disease and is related to secondary infections. The treatment is only symptomatic and supportive [17]. Similarly, CCoV is another pathogenic agent related to gastroenteritis that in some cases can be fatal, mostly in young dogs. No treatment is available [18]. Thus, there is a great need for the development of antiviral drugs against viral diseases for which no treatments are currently available.



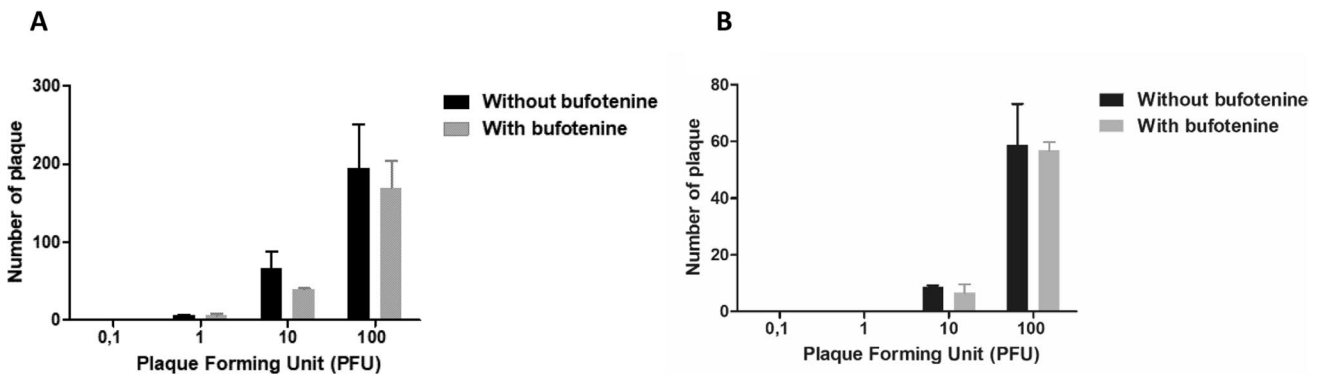
**Fig. 2** Effect of bufotenine (0.5 mg/mL) on infectious titres after 72 h of different strains of rabies virus (RABV) (964/06, genetic lineage from the insectivorous bat *Eptesicus furinalis*; 4005/10, genetic lineage from the haematophagous bat *Desmodus rotundus*; 4871/11,

genetic lineage from the wild dog *Cerdocyon thous*; and 3629/11, genetic lineage from the domestic dog, *Canis lupus familiaris*) multiplied in BHK-21 cells (\*)  $P < 0.05$



**Fig. 3** Effect of bufotenine against different plaque-forming units (0.1, 1, 10 and 100 PFU) of rabies virus (RABV) with and without bufotenine after 72 h. **A** 964/06, genetic lineage from insectivorous bat *Eptesicus furi-nalis*, (\*\*\*)  $P < 0.001$ . **B** 3629/11, genetic line-

age from domestic dog *Canis lupus familiaris* (\*\*\*)  $P < 0.001$ . **C** 4005/10, genetic lineage from haematophagous bat *Desmodus rotun-dus*, (\*\*\*)  $P < 0.001$ . **D** 4871/11, genetic lineage from wild dog *Cer-docyon thous* (\*\*\*)  $P < 0.001$



**Fig. 4** Effect of bufotenine against different plaque-forming units (100, 10, 1 and 0.1 PFU) of herpesvirus type 1 (HSV-1) with and without bufotenine after 72 h. **A** KOS strain and **B** 29-R/acyclovir-resistant strain

Before evaluation of its antiviral activity, the cytotoxic effects of bufotenine were investigated in Vero and BHK-21 cells to determine the maximum non-toxic concentration of bufotenine. For that, an MTT colorimetric assay was used. In this assay, a concentration of 0.5 mg/mL bufotenine was chosen because it is a concentration that maintains high viability of cells.

Contrary to the findings of Vigerelli et al. (2014) [11], our findings revealed anti-rabies action through difference

in viral titration and plaque assays with distinct doses of the virus from distinct genetic lineages of RABV. In titration, compared with the control, bufotenine was able to significantly reduce the non-haematophagous bat (*Eptesicus furi-nalis*) titre, although when comparing different doses of the virus, we observed reductions in the numbers of plaques for all lineages of RABV. This finding demonstrates that the action of bufotenine depends on the number of infectious particles. At 10 PFU, bufotenine presented the maximum

**Table 1** Summary of the effects of bufotenine against RNA and DNA viruses. The table summarizes all results obtained in antiviral assays (viral titration and plaque assays) for each virus. +, antiviral activity of bufotenine; –, no antiviral activity of bufotenine. \*EF, *Eptesicus furi*; CLF, *Canis lupus familiaris*; DR, *Desmodus rotundus*; CT, *Cercopithecus thomasi*

Virus		Characteristic	Sample	Antiviral activity viral titration	Antiviral activity number of plaques
RNA	CCoV	Enveloped	A26/61	-	-
	RABV		964/06 (EF)*	+	+
			3629/11 (CLF)*	-	+
			4005/10 (DR)*	-	+
			4871/11 (CT)*	-	+
DNA	CAV-2	Nonenveloped	MAV#795	-	-
	HSV-1	Enveloped	KOS	-	-
			29R, acyclovir-resistant (ACV)	-	-

effects in almost all lineages, except the domestic dog lineage, with greater than 80% reductions in the numbers of plaques. Here, were tested two different methods to identify an antiviral action of bufotenine, viral titration and plaque assay; however, the results were different according to the method used. Perhaps the difference could be explained by the stage of viral replication that bufotenine acts.

The adopted methodologies in this study to evaluate the antiviral action were viral titration and plaque assay. Both are indirect techniques used to quantify infectious particles in unknown samples [19, 20]. In the viral titration, susceptible cells were inoculated with serial dilutions of the virus with and without bufotenine. In this case, the initial concentration of the virus was unknown. On the other hand, the plaque assay was performed with known concentrations based on the titre obtained previously. Besides that, the plaque assay can demonstrate a macroscopic effect of the interest compound.

Another possible hypothesis is that bufotenine acts extracellularly through competition with viral particles for the cellular receptors used for RABV, as suggested by Vigerelli et al. (2014) [11]. For RABV, some receptors are proposed to be responsible for binding the virus and allowing it into cells. Some of these receptors may be nicotinic acetylcholine receptor (nAChR), neuronal cell adhesion molecule (NCAM) and p75 neurotrophin receptor (p75NTR) [21]. On the other hand, Vigerelli et al. (2020) [13] demonstrated that nAChR does not seem to interact with alkaloids. New investigations are necessary to clarify the relationship between alkaloids and cells.

Although BHK-21 has some neuronal receptors as NCAM, more research is needed to identify the exact mechanism of action of bufotenine in RABV [22, 23]. For other viruses, bufotenine was not able to suppress infection; nevertheless, for the HSV-1 standard strain (KOS), we could observe an effect at 10 PFU, however, with no statistical significance. It is important to highlight the fact that this study only investigated one specie from the *Herpesviridae* family; future studies could consider the

antiviral potential of bufotenine against other members of this family. In addition, associations of bufotenine with other compounds or medications could be explored to potentialize the antiviral effect. Numerous data indicate that the use of combined compounds can be effective in antiviral effects [24–26]. Among the drugs that exhibit inhibitory action against herpesvirus replication, the most commonly used in human medicine are nucleoside analogues; unfortunately, numerous resistance mechanisms have been demonstrated for some of these drugs [27, 28]. Thus, the search for new compounds with alternative mechanisms of action has become necessary.

HSV-1, like RABV, may have neurotropic potential, which could justify the action of bufotenine against these two viruses. Most likely, these two viruses share common molecules or receptors to which bufotenine binds. Vigerelli et al. (2020) indicate that bufotenine probably can interact with other cell structures beyond receptors. Ion channels, proteins and endocytosis can have an important role in the mechanism of action of bufotenine [13]; however, the exact mechanism remains unknown.

Another debatable aspect of bufotenine is the hallucinogenic effects of bufotenine due to similarities to other bioactive molecules such as LSD [29]. However, it has been suggested that the bufotenine cannot cross the blood–brain barrier, due to poor lipid solubility. On the other hand, a study conducted in 2018 by Vigerelli et al. [12] showed the effects in mice of different doses by subcutaneous inoculation. The results demonstrated the alkaloid was found in some organs, including the brain, although without significant effects. The same study associated the use of bufotenine with an increased survival rate of rabid mice from 15 to 40% [13] being a good candidate as a prototype, once there is no antiviral treatment available for rabies.

Although HSV-1 therapy is available, the treatment consists of blocking viral replication and consequently decreasing the oral lesion. The therapy is not effective on the latency stage, and even with some antiviral agents approved, the resistance phenomenon occurs [30]. Although the rates of

resistance are low, new alternatives or combined therapy can be an alternative strategy for the patients.

Bufotenine was not effective against CAV-2 or CCoV in the titration or in the plaque assay. A few studies have investigated antiviral action against these viruses, such as an in vitro study conducted by Cueto et al. (2011) [31] on the effect of an extract of propolis against CAV-2, bovine viral diarrhoea virus (BVDV) and feline calicivirus (FCV). The extracts proved to be effective against CAV-2 and BVDV but relatively less effective against FCV.

Similarly, Silva et al. [32] used titration to demonstrate the antiviral activity of P34 peptide isolated from *Bacillus* against the animal viruses CCoV, CAV-2, canine distemper virus (CDV), canine parvovirus type 2 (CPV-2), equine arteritis virus (EAV), equine influenza virus (EIV), feline calicivirus (FCV) and feline herpesvirus type 1 (FHV-1). The peptide was able to reduce the titres of the DNA and RNA viruses FHV-1 and EAV, respectively, but had no effects on CAV-2 and CCoV.

Interestingly, treatment with bufotenine did not affect all viruses equally. Stronger effects were observed against RABV, while weaker effects were observed against HSV-1. This heterogeneous activity might indicate some specificity against distinct types of viruses. Clear differences in activity against DNA versus RNA viruses and enveloped versus non-enveloped viruses were not observed. Therefore, additional experiments might provide further explanations.

## Conclusion

Despite continuous developments in antiviral therapy, viral diseases are still important causes of human and animal death. Since viruses and hosts are intimately related, the design of effective, virus-specific antiviral agents that do not affect host cells has proven to be difficult.

The results of the present investigation provide further evidence of the potential use of alkaloids. The study showed that bufotenine can inhibit RNA (RABV) viruses, depending on the viral load.

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**Author contribution** All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Camila Mosca Barboza, Daniel Carvalho Pimenta, Hugo Vigerelli de Barros, Andréa de Cássia Rodrigues da Silva, Jaíne Gonçalves Garcia, Raphaela Mello Zamudio, Juliana Galera Castilho Kawai, Jarbas Alves Montanha, Paulo Michel Roehle and Helena Beatriz de Carvalho Ruthner Batista. The first draft of the manuscript was written by Camila Mosca Barboza, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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**Data availability** The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

**Code availability** Not applicable.

## Declarations

**Ethics approval** Ethical approval was obtained from the animal ethics committee of the Instituto Pasteur of Sao Paulo, Brazil (16.2018).

**Consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Conflict of interest** The authors declare no competing interests.

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