Polish Journal of Veterinary Sciences Vol. 23, No. 2 (2020), 177–184

DOI 10.24425/pjvs.2020.132763

Original article

An in vitro assessment of antiviral activity for ethanol extract of *Desmodium canadense* against bovine herpesvirus type 1

R. Lelešius[1,2], P. Girdauskaitė[2], A. Karpovaitė[2], R. Mickienė[3], T. Drevinskas[3], N. Tiso[3], O. Ragažinskienė[4], L. Kubilienė[5], A. Maruška[3], A. Šalomskas[2]

[1] Institute of Microbiology and Virology, Veterinary Faculty,
Lithuanian University of Health Sciences, Tilzes 18, 47181, Kaunas, Lithuania
[2] Department of Veterinary Pathobiology, Veterinary Faculty,
Lithuanian University of Health Sciences, Tilzes 18, 47181, Kaunas, Lithuania
[3] Instrumental Analysis Open Access Centre,
Vytautas Magnus University, Vileikos 8, 44404, Kaunas, Lithuania
[4] Medicinal Plant Sector, Kaunas Botanical Garden of Vytautas Magnus University,
Zilibero 6, 46324, Kaunas, Lithuania
[5] Faculty of Pharmacy, Lithuanian University of Health Sciences, Sukileliu 13, 50162, Kaunas, Lithuania

Abstract

Herpesviruses (HV) are pathogens causing infections in humans and animals worldwide. Since it shares many common features with other HV, bovine HV type 1 (BoHV-1) was selected as a model to test the anti-herpesviral activity of medicinal plants.

Fifteen plants were chosen in this study for their medical, antibacterial and antiviral properties. The aim was to investigate ethanolic extracts from the selected medicinal plants for anti-BoHV-1 activity. The virucidal activities were evaluated by comparing the effect of noncytotoxic concentrations of extracts on BoHV-1 strain 1640 replication in Madin-Darby bovine kidney (MDBK) cells. Virucidal activity was determined by means of virus titration after exposure to the extracts. The extract of *Desmodium canadense* was found to be the most effective virucide – the 50% tissue culture infective dose ($TCID_{50}$) after exposure was 3.75 $\log_{10}$ and the virus reduction factor was ≥5.0±0.25 $\log_{10}$. The extract of *D. canadense* was therefore chosen for further studies. Virus yield reduction assays showed that *D. canadense* extract had time-dependent and dose-dependent effects. It effectively reduced virus titre from 8.33 $\log_{10}$ to 4.67 $\log_{10}$ ($p<0.01$). The virucidal activity was also confirmed by real-time polymerase chain reaction (real-time PCR), where the number of threshold cycles (Ct) was inversely proportional to the virus titre in $TCID_{50}$ The virucidal activity was also confirmed by real-time polymerase chain reaction (real-time PCR). This method showed that the number of threshold cycles (Ct) was inversely proportional to the virus titre (direct correlation with exposure time R=0.9321). The extract of *D. canadense* showed a high virus reduction capacity. In future, such active substances should be identified for the development of effective antivirals.

Key words: bovine herpesvirus 1, plant extracts, *Desmodium canadense*

Correspondence to: R. Lelešius, e-mail: raimundas.lelesius@lsmuni.lt

Introduction

The medicinal properties of plants have long been investigated and plants that have a curative effect are used in folk medicine (Hudson 2012). Biologically active substances of medicinal plants are currently being studied (Tilburt and Kaptchuk 2018). The efficacy of plant extracts is becoming important not only for the treatment of people but of animals as well (Bisset and Wichtl 2001). Medicinal plants are used mainly in human medicine (Drevinskas et al. 2018), while the use and efficacy of substances of plant origin in veterinary medicine has not yet been studied in depth.

Herpesviridae is a large family of enveloped DNA viruses. HV are pathogens that have spread worldwide and cause health problems in humans and animals (Azab et al. 2018). HV cause serious latent, recurring infections in mammalians and therefore it is very difficult to control HV infections in different species.

In veterinary medicine animals can be protected by being vaccinated against HV, but there are no safe and sufficiently efficient prophylactic measures and the choice of methods is very limited (Thomasy and Maggs 2016, Villa et al. 2017).

BoHV-1 is one of the most important pathogens in the cattle industry. This bovine pathogen is mainly associated with respiratory and reproductive symptoms such as rhinotracheitis, pustular vulvovaginitis, balanoposthitis, abortion, infertility, conjunctivitis and encephalitis (Tikoo et al. 1995, Azab et al. 2018). BoHV-1 is a member of the *Alphaherpesvirinae* subfamily and shares certain biological properties with the herpes simplex virus (HSV) type 1 (HSV-1) and type 2 (HSV-2) (Jones 2013). BoHV-1 establishes lifelong latency in ganglionic neurons in the peripheral nervous system after initial replication in the mucosal epithelium (Jones 1998). Corticosteroid-induced stress consistently leads to reactivation from latency and virus transmission (Jones et al. 2011).

The search for new methods to control HV infections is underway, therefore medicinal plants or substances derived from them are being tested for possible future measures in the prevention and treatment of HV infections. Since it shares many common features with other herpesviruses, BoHV-1 was chosen as a model to test the anti-herpesviral activity of medicinal plants.

The plants in this study were selected for their medical, antibacterial and antiviral properties. Medicinal plants or substances derived from them are being tested in many countries as a tool in the prevention of infections such as BoHV-1, but the anti-BoHV-1 viral properties of the selected plants have not so far been studied. The aim of this study was to investigate extracts from 15 selected medicinal plants for anti-BoHV-1 activity.

Materials and Methods

Plant extracts

Sixteen 40% (vol.) ethanol extracts of medicinal plants were prepared. The plants were grown in the Kaunas Botanical Garden of Vytautas Magnus University (Lithuania) and were selected according to their antimicrobial activity and ethnomedical or traditional uses against infectious diseases (Cos et al. 2006). Herbs, leaves and roots (Table 1) were selected for extraction depending on the accumulation of active compounds.

Preparation of plant extracts

The solvent ethanol was diluted with sterile bidistilled water to 40% (vol.) concentration. Dried materials of the plants (0.5 g) were extracted with 10 ml solvent. The extraction was performed in an orbital shaker for 24 hours at room temperature (20°C) and the extracts were filtrated using a paper filter and then a polyvinyl difluoride membrane filter with a 0.22 µm pore size. The concentration of the extracts was 50 mg/ml with reference to the starting material. The plant extracts were stored at 4°C in a refrigerator until the experiments.

Cells and virus

MDBK cells and MDBK-adapted BoHV-1 strain 4016 (Lisov et al. 2015) were kindly provided by Dr. I. Jacevičienė of the Department of Virus Research at the National Food and Veterinary Risk Assessment Institute in Lithuania. The cells were cultivated in Dulbecco's modified Eagle's medium (DMEM:F-12, Lonza, Switzerland) supplemented with 10% foetal bovine serum (FBS, Biochrom GmbH, Germany) at 37°C in a 5% CO_2 incubator. Penicillin (100 U/ml) and streptomycin (100 µg/ml) were used for the prevention of microbial contamination.

MDBK cells were cultured in T25 flasks and infected with BoHV-1 at a multiplicity of infection (MOI) of 1. After 48 hours of incubation in maintenance medium with 2% FBS and antibiotics, the flasks were microscopically examined for a cytopathogenic effect (CPE). After examination, the flasks with infected cells were frozen and thawed three times. The aliquots of the virus were prepared by centrifugation for 15 min at 2000 x g and stored at -80°C until use.

Virus titration

Determination of $TCID_{50}$ of the control and the treated BoHV-1were performed in 96-well plates with MDBK cells. Serial dilutions of BoHV-1 in tenfold steps were prepared. Each sample was tested in sextu-

Table 1. List of plants.

No.	Latin name	English name	Family	Material
1.	*Agastache foeniculum* (Pursh) Kuntze	Blue giant hyssop	*Lamiaceae*	Herb
2.	*Angelica archangelica*	Garden Angelica	*Apiaceae*	Leaves
3.	*Angelica archangelica*	Garden Angelica	*Apiaceae*	Roots
4.	*Chamaemelum nobile* (L.) All.	Chamomille	*Asteraceae*	Herb
5.	*Desmodium canadense* (L.) DC.	Canada tickclover	*Fabaceae*	Herb
6.	*Echinacea purpurea* (L.) Moench	Eastern purple coneflower	*Asteraceae*	Herb
7.	*Geranium macrorrhizum* L.	Bigroot geranium	*Geraniaceae*	Herb
8.	*Hyssopus officinalis* L.	Hyssop	*Lamiaceae*	Herb
9.	*Melissa officinalis* L.	Lemon balm	*Lamiaceae*	Herb
10.	*Mentha piperita* L.	Peppermint	*Lamiaceae*	Herb
11.	*Nepeta cataria* L.	Catnip	*Lamiaceae*	Herb
12.	*Origanum vulgare* L.	Oregano	*Lamiaceae*	Herb
13.	*Perilla frutescens* (L.) Britton.	Perilla	*Lamiaceae*	Herb
14.	*Salvia officinalis* L.	Common sage	*Lamiaceae*	Herb
15.	*Satureja montana* L.	Winter savory	*Lamiaceae*	Herb
16	*Thymus vulgaris* L.	Common thyme	*Lamiaceae*	Herb

plicates and the experiments repeated twice. CPE was evaluated after 96 hours. Virus titres and standard deviations were calculated using the Kärber method (Kärber 1931) and the virus reduction capacity was evaluated (Ruppach 2014).

Cytotoxicity assay

The cytotoxicity assay was performed to choose the noncytotoxic concentration of plant extracts for screening and determination of virucidal activity.

The 50% cytotoxic concentration (CC_{50}) was determined for each extract on MDBK cells using MTT assay (Mosmann 1983). First, cells were seeded at a concentration of 1×10^4 cells/well in a 96-well plate and grown at 37°C for 1 day. The assay was performed in octuplicate for each extract. After 72 hours, MTT reagent (10 µl, 5 mg/ml, Sigma-Aldrich, USA) was added and incubated for 4 h at 37°C. 0.1 ml dimethyl sulfoxide (DMSO, Carl Roth, Germany) was then added to each well and the plates placed on the shaker for 5 min. The absorbance of each well was measured at 620 nm in a microplate reader (Multiskan™ FC Microplate Photometer, USA) and the percentage of cell survival was calculated. Finally, dose-response curves were plotted to enable the calculation of CC_{50} that causes lysis and death of 50% of cells.

Determination of virucidal activity

Testing solutions were prepared by mixing BoHV-1 (from $10^{8.0}$ to $10^{8.75}$ $TCID_{50}$/ml) suspensions in DMEM:F12 with 2% FBS with previously established noncytotoxic extract volumes. The virus and extract mixtures were incubated at 20°C for 1 hour and titrated as described above. Controls of cells, the virus and extracts were included. After 96 h of incubation, the plates were examined using an inverted microscope (Leica, Germany) to detect CPE.

Sodium hypochlorite (NaOCl – Sigma-Aldrich, USA) in a final concentration of 0.05-0.1% was used as the positive control. NaOCl was diluted in water of standard hardness to a stock concentration. Sodium thiosulphate (Sigma-Aldrich, USA) in distilled water was used to neutralise the activity of NaOCl.

Based on the results of screening for virucidal activity, the extract of *D. canadense* was chosen for further investigation.

Virucidal assay for real-time PCR

Residual virus activity after treatment was evaluated by means of virus titration and real-time PCR.

The cells were seeded in 24 well plates and incubated for 48 hours. Testing solutions were prepared by mixing 0.1 ml of BoHV-1 with 0.9 ml of DMEM:F12 with 2% FBS, and appropriate volumes of plant extract of *D. canadense*. The mixtures were incubated at 20±2°C for 1 hour. Prior to inoculation, the cell culture medium was decanted and the cells inoculated with a mixture of the test materials and virus at a MOI of 1. After 1 hour of contact, test mixtures were aspirated and used for further determination of unbound extracellular virus residual infectivity in $TCID_{50}$ and real-time PCR. For determination of the intracellular virus, the cells were washed three times with PBS and then maintained with 0.6 ml of DMEM:F12 with 2%

Table 2. BoHV-1 primers and probe for real-time PCR at the gB locus.

Oligonucleotide	Sequence (from 5' to 3'_)	Product length (base pairs)
BoHV-1 forward	TGT GGA CCT AAA CCT CAC GGT	97
BoHV-1 probe [a]	AGG ACC GCG AGT TCT TGC CGC	
BoHV-1 reverse	GTA GTC GAG CAG ACC CGT GTC	

[a] MGB labeled

FBS. After 24 hours of incubation, the cells were evaluated microscopically for CPE and the plates frozen and thawed three times. Aliquots of the test mixtures and virus were prepared by centrifugation for 10 min at 2000 x g. Finally, the prepared aliquots were stored at -80°C. The prepared mixtures were used for quantification of both the treated and untreated virus and viral nucleic acids by means of $TCID_{50}$ assay and quantitative real-time PCR respectively.

Real-time PCR assay

Real-time PCR was performed as described by Abril et al. (2004).

DNA isolation was carried out using the Genomic DNA purification kit (Thermo Fisher Scientific, USA, K0721) A protocol. Briefly, 20 µl supernatants of cell culture treated with BoHV-1 were resuspended in 180 µl of the digestion solution. 20 µl of proteinase K solution was then added and mixed by vortexing to obtain a uniform suspension. The samples were then incubated at 56°C for 10 min. After incubation, 20 µl RNase A solution was added and mixed. The samples were incubated for 10 min at room temperature. The samples were then mixed with 0.2 ml of lysis solution and vortexed for 15 s to obtain a homogenous mixture. 0.4 ml of 50% ethanol was then added and vortexed. The prepared lysates were transferred to GeneJET Genomic DNA purification columns and centrifuged for 1 min at 6000xg. After centrifugation, the GeneJET Genomic DNA purification columns were placed into new 2-ml collection tubes. Wash Buffer I (0.5 ml) was added and centrifuged for 1 min at 8000 x g. The samples were then washed with Wash Buffer II (0.5 ml) and centrifuged for 3 min at 12000 x g. Finally, 0.2 ml elution buffer was added and incubated for 2 min to elute DNA. The samples were centrifuged for 1 min at 8000 x g. The purified DNA was used immediately or stored at -20°C.

Primers and probes for quantitative real-time PCR (TaqMan) were designed with Primer Express software (version 1.0; Applied Biosystems, USA) to amplify sequences within the open reading frames of the glycoprotein B genes of BoHV-1 (Abril et al. 2004). Oligonucleotide primers and MGB-labelled probes (Table 2) were synthesised by Invitrogen (USA). Amplifications were performed using a TaqMan Universal master mix II (catalogue #4440038, Applied biosystems, USA). Briefly, quantitative real-time PCR amplifications were carried out in a volume of 25.0 µl containing 12.5 µl of mastermix, 1.6 µl of a mixture of primers and probe, and 10.9 µl of the DNA sample. The final concentrations of primers and probes were as follows: 240 nM each of BoHV-1 forward primer and BoHV-1 reverse primer and 160 nM of BoHV-1 probe. The real-time PCR conditions for the reactions were set as follows: 2 min at 50°C, 10 min at 95°C, and then 40 cycles consisting of a denaturation step at 95°C for 15 s and an annealing-elongation step at 60°C for 1 min. Amplification plots were recorded and analysed, and Ct was determined using a Mastercycler (Eppendorf). The real-time PCR was repeated four times and Ct values were recorded. The means, confidence interval (CI) and standard deviations (sd) were then calculated.

Statistical and data analysis

The differences between the methods and extracts were evaluated using Fisher's criteria and Student's t-test. Pearson's correlation coefficient (R) was calculated. The data were regarded as significant when $p<0.05$.

Results

Screening for virucidal activity

In this study, the anti-BoHV-1 activities of 16 ethanol extracts from medicinal plants that belong to 15 different species were analysed. It was shown that some, but not all, of the 16 plant extracts directly contacting the virus were able to inhibit virus replication. The extract of *D. canadense* was the most effective. The titre of BoHV-1 after exposure to ethanol extract of *D. canadense* decreased to 3.75 $\log_{10}$ and the virus reduction factor was $\geq 5.0 \pm 0.25$ $\log_{10}$ in the $TCID_{50}$ test.

The extracts of *Satureja montana* and *Origanum vulgare* had a moderate effect on inhibition of BoHV-1 replication. The titres after exposure with *S. montana* extract were 5.75 $\log_{10}$ (virus reduction factor 3.00 ± 0.21 $\log_{10}$ $TCID_{50}$) and with *O. vulgare* they were

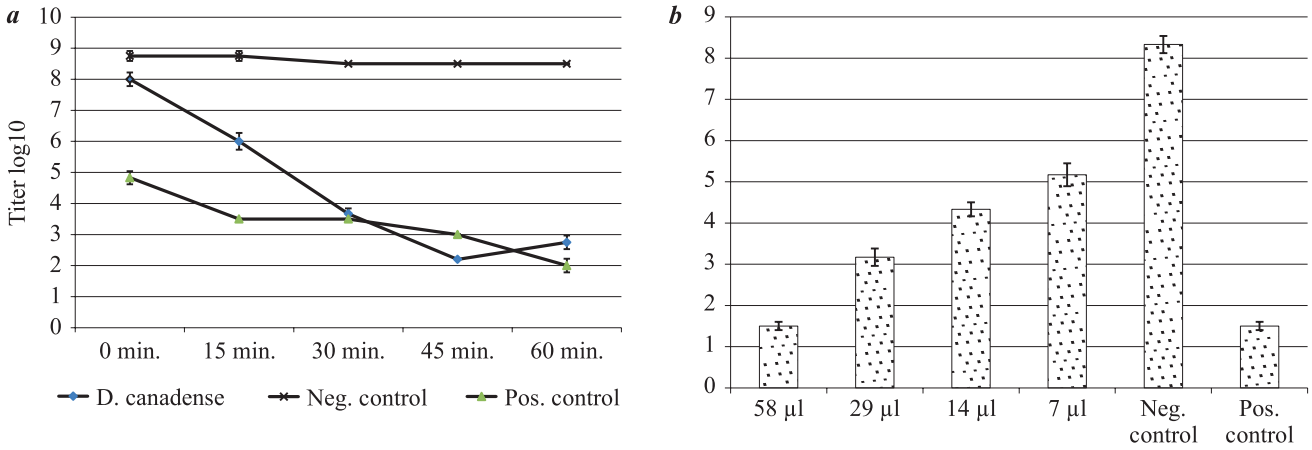

Fig. 1. *a*. Time-dependent efficacy of *D. canadense* (29 µl/ml) extract against BoHV-1. Negative control represents the untreated virus and NaOCl was used as a positive control at a concentration of 0.1% active chlorine. Data are from two independent experiments.
b. Dose-dependent activity of *D. canadense* extract against BoHV-1 in $TCID_{50}$ assay. Virus titre is expressed in log_{10}. Negative and positive controls are included. The detection limit was 1.5 log_{10}. Data are from two independent experiments.

Table 3. Evaluation of the virucidal activity of *D. canadense* extract by virus titration and real-time PCR.

Test material	Amount of BoHV-1 evaluated by $TCID_{50}$ and real-time PCR					
	24 hours after adsorbtion and incubation			Non adsorbed virus		
	$TCID_{50}$	Real-time PCR		$TCID_{50}$	Real-time PCR	
		Ct±sd[1]	CI[2]		Ct±sd[1]	CI[2]
D. canadense extract, 29 µl/ml	4.67±0.17	33.87±2.47	30.5–35.3	3.50±0.45	33.24±0.94	32.3–34.2
Virus + ethanol control	8.33±0.17	22.45±1.18	21.3–23.6	4.83±0.21	33.95±1.40	32.6–35.3
NaOCl, 0.1%	≤1.5[3]	≥40[4]	–	NT	NT	NT
NaOCl, 0.05%	3.83±0.21	29.92±0.67	29.2–30.7	NT	NT	NT
Virus control	8.75±0.16	20.98±0.43	20.5–21.5	NT	NT	NT

[1] Ct±sd – mean number of threshold cycles ± standard deviation; [2]CI – confidence interval; [3] 1.5 log_{10} – detection limit; [4] maximal number of cycles. Data are from four independent experiments

5.75 log_{10} (virus reduction factor 2.25±0.27 log_{10} $TCID_{50}$). The virus reduction potential of thirteen other extracts was indicative or not significant (virus reduction factor ≤2.0 log_{10}).

Virucidal effect of *D. canadense*

Therefore, the extract of *D. canadense* was chosen for further studies. Since CC_{50} of ethanolic extract of *D. canadense* ≥ 58.0 µl/ml for MDBK cells, 1:2 CC_{50} (29.0 µl/ml) was used for further studies.

The study of dynamics of virus inactivation showed that NaOCl immediately and effectively inactivated BoHV-1 (0 min – virus reduction factor was 3.92 log_{10}), and after 15 min the virus reduction factor was 5.25 log_{10} in $TCID_{50}$ test (Fig. 1). The highest effect of NaOCl was observed after 60 min of exposure ($R = 0.9529$, $p = 0.012$).

The potential for virucidal activity of *D. canadense* extract was observed after 15 min (virus reduction factor 2.75 log_{10} in $TCID_{50}$ test, $p<0.01$), and effective inactivation was found after 30 min (virus reduction factor 4.83 log_{10} in $TCID_{50}$ test, $p<0.01$; Fig. 1).

The *D. canadense* extract exhibited dose-dependent virucidal activity (Fig. 1). It was determined that 7 µl/ml and 14 µl/ml of extract decreased the titre of BoHV-1 by 3 to 4 log_{10} and 29 µl/ml and 58 µl/ml – >5 log_{10} respectively, and 0.1% NaOCl totally inactivated BoHV-1, while 0.05% reduced the virus titre by 4.5 log_{10}.

The virus yield reduction assay showed that *D. canadense* extract effectively reduced virus titre from 8.33 log_{10} to 4.67 log_{10} ($p<0.01$). The virucidal activity was also confirmed by real-time PCR. The number of Ct in real-time PCR was inversely proportional to the virus titre in $TCID_{50}$ (direct correlation with exposure time ($R=0.9321$). Real-time PCR showed that the amount of non-adsorbed live and/or inactivated BoHV-1 DNA was similar when compared to the control (33.95±1.40) and those treated with *D. canadense* extract (33.24±0.94), while titration of the virus showed a difference in $TCID_{50}$ (Table 3).

Discussion

A wide range of traditional medicinal plants and herbs have been reported to show antiviral activities against various viruses. Viruses of veterinary importance are still used as animal models in the development of human therapeutics (Dal Pozzo and Thiry 2014). In the present research on plant extract antiviral properties, a laboratory strain of BoHV-1 4016 was used for the tests (Lisov 2015). The strain of this virus is notable for being adapted to the MDBK cell culture, with the virus titre reaching a maximum of $10^{8,75}$ $TCID_{50}$/ml after replication. It is recommended that virus strains that have a high replication titre in cell cultures are used to evaluate the antiviral properties, and especially the virucidal properties, of various substances (Ruppach 2014).

Previous tests have shown that the tested plant extracts have an antiviral effect and that BoHV-1 can be affected by plant extracts. Yamasaki et al. (1998) found that a preparation containing *S. montana* has an effect on the human immunodeficiency virus (HIV) type 1. The authors of that study concluded that the water-soluble polar substances of *S. montana* extract are active ones.

In the present test, *M. piperita* and *T. vulgaris* extracts were not effective against BoHV-1, although other tests have shown that the hydroalcoholic and aqueous extracts of *M. piperita* and *T. vulgaris* have a significant effect against human HV – HSV-1 and HSV-2 (Nolkemper et al. 2006).

The *D. canadense* herb contains flavonoids such as apigenin, apigenin-7-O-glucoside, luteolin, rutin, 2-vicenin, vitexin, isovitexin, vitexin rhamnoside, orientin, homoorientin, quercetin, hyperoside, astragalin and kaempherol (Puodžiūnienė et al. 2010). It also contains saponins and phenolic acids (chlorogenic acid, vanillic, 4- hydroxycinnamic, ferulic and caffeic). The *Desmodium* herb exhibits antioxidant, antibacterial, anti-inflammatory, hepatoprotective, diuretic and analgesic activities. C-glycosides of flavonoids are known to exhibit antioxidant, hepatoprotective, anti-inflammatory and antiviral effects (Puodžiūnienė et al. 2011).

It is possible that the essential oil of *O. vulgare* has an effect on BoHV-1. The study with relative HSV-1 has shown that the envelope of this virus is damaged during treatment with *O. vulgare* essential oil (Siddiqui et al. 1996). Other tests have also been undertaken with various other plant extracts, for example *Prunus myrtifolia* and *Symphyopappus compressus*, during which an effective impact against BoHV-1 was determined when the largest non-cytotoxic concentration reached 1000 μg/ml (Fernandes et al. 2012). Furthermore, Brazilian plants (*Maytenus ilicifolia* and *Aniba rosaeodora*) have antiviral activity against BoHV-5 (Kohn et al. 2012).

There is little information about studies of *D. canadense* to determine its antiviral or virucidal properties. It is known that the polyphenols in *D. canadense* inhibit the replication of human herpes zoster and HSV (Mezenthev et al. 2016). It has been established that *D. canadense* contains a large amount of flavonoids that are effective against HSV (Puodziuniene et al. 2013). The present study with different number of *D. canadense* extract shows a direct correlation between the concentration of the extract and the virucidal effect (Fig. 1). The study of the aqueous extract of *Phyllanthus orbicularis* on BoHV-1 showed that its efficacy was also dependent on the dose.

While testing the efficacy of the *D. canadense* extract in comparison with NaOCl (positive control) at different time intervals, it was determined that the virucidal effect of the extract was significantly effective after 30 min, but more slowly than NaOCl (Fig. 1). In the authors' opinion, it is most likely that the active pharmacological substances contained in *D. canadense*, such as phenolic compounds, are responsible for its virucidal properties. Before the test, it was expected that the NaOCl would be far more effective than the plant extracts, but the comparative evaluation demonstrated that the difference between their effects was not significant. The protein of the bovine serum in the cell culture medium could be significant in the results since the medium with 2% FBS was used to dilute the virus and in the mixtures of the tested plant extracts. This effect has been observed in other studies, where it has been found that the impact of NaOCl against HIV can be reduced by the proteins of the blood serum (Van Bueren et al. 1995).

The comparison of *D. canadense* and NaOCl (0.05-0.1%) showed that the virus-inactivating effect was similar, except that the extract acted more slowly. BoHV-1 titre was reduced to 3.8 $\log_{10}$ after the treatment with NaOCl at a concentration of 0.05% and this concentration was not completely significant, as compared to the 0.1% NaOCl concentration ($\leq$1.5 $\log_{10}$). It was considered that NaOCl at a concentration of 0.1% had completely inactivated BoHV-1 in 1 hour (the time of the contact). The results of the virus yield reduction and the real-time PCR assays correlated. The real-time PCR showed a higher number of cycles the less viral DNA there was in the sample.

Evaluation of the virucidal activity of *D. canadense* extract by virus titration and real-time PCR (Table 3) showed that the extract had a virucidal effect, but had no effect on the cycle of virus reproduction. This explained why the amount of DNA of non-

-adsorbed virus treated with extract or control was similar when measured by real-time PCR, while the virus yield reduction showed that the amount of the control virus was 4.83±0.21 $TCID_{50}$ compared with the treated one of 3.50±0.45 $TCID_{50}$.

During testing, it was determined that the majority of plant extracts had virucidal properties. Ethanol extracts of plants contain various biologically active substances or compounds, which is why the antiviral effect is most likely caused by combinations of several compounds. In future, it would be of value to carry out further research to allow the mechanism of the antiviral effect of plant extracts to be determined. It would also be useful to identify the active substances or compounds with virucidal and/or antiviral effects. Of course, while applying the classical virological method, the identification of tens, perhaps even hundreds of biologically active components would be overly complicated work. Therefore, when performing multiple data analysis, it would be helpful to apply the recently developed technique of hierarchical clustering (Smusz et al. 2013) or chemical analysis, machine-learning methods and antiviral tests (Drevinskas et al. 2018).

In conclusion, this research has shown that the ethanol extract of *D. canadense* effectively reduced virus activity and replication in infected cells. In future, active substances should be identified for the development of effective antivirals for curing HV infections.

Acknowledgements

The research was granted by the Research Council of Lithuania, project No. MIP-065/2015.

References

Abril C, Engels M, Liman A, Hilbe M, Albini S, Franchini M, Suter M, Ackermann M (**2004**) Both viral and host factors contribute to neurovirulence of bovine herpesviruses 1 and 5 in interferon receptor-deficient mice. J Virol 78: 3644-3653.

Azab W, Dayaram A, Greenwood AD, Osterrieder N (**2018**) How host specific are herpesviruses? Lessons from herpesviruses infecting wild and endangered mammals. Annu Rev Virol 5: 53-68.

Bisset NG, Wichtl M (**2001**) Herbal drugs and phytopharmaceuticals. In: A handbook for practice on a scientific basis. Transl 2nd German ed., CRC Press, Washington, pp 566.

Cos P, Vlietinck AJ, Berghe DV, Maes L (**2006**) Anti-infective potential of natural products: how to develop a stronger in vitro 'proof-of-concept'. J Ethnopharmacol 106: 290-302.

Dal Pozzo F, Thiry E (**2014**) Antiviral chemotherapy in veterinary medicine: current applications and perspectives. Rev Sci Tech 33: 791-801.

Drevinskas T, Mickienė R, Maruška A, Stankevičius M, Tiso N, Šalomskas A, Lelešius R, Karpovaitė A, Ragažinskienė O (**2018**) Confirmation of the antiviral properties of medicinal plants via chemical analysis, machine learning methods and antiviral tests: a methodological approach. Anal Methods 10: 1875-1885.

Fernandes MJ, Barros AV, Melo MS, Simoni IC (**2012**) Screening of Brazilian plants for antiviral activity against animal herpesviruses. J Med Plant Res 6: 2261-2265.

Jones C (**1998**) Alphaherpesvirus latency: its role in disease and survival of the virus in nature. Adv Virus Res 51: 81-133.

Jones C (**2013**) Bovine herpes virus 1 (BHV-1) and herpes simplex virus type 1 (HSV-1) promote survival of latently infected sensory neurons, in part by inhibiting apoptosis. J Cell Death 6: 1-16.

Jones C, da Silva LF, Sinani D (**2011**) Regulation of the latency--reactivation cycle by products encoded by the bovine herpesvirus 1 (BHV-1) latency-related gene. J Neurovirol 17: 535-545.

Hudson JB (**2012**) Applications of the phytomedicine *Echinacea purpurea* (purple coneflower) in infectious diseases. J Biomed Biotechnol 2012: 769896..

Kohn LK, Queiroga CL, Martini MC, Barata LE, Porto PS, Souza L, Arns CW (**2012**) *In vitro* antiviral activity of Brazilian plants (*Maytenus ilicifolia* and *Aniba rosaeodora*) against bovine herpesvirus type 5 and avian metapneumovirus. Pharm Biol 50: 1269-1275.

Lisov A, Vrublevskaya V, Lisova Z, Leontievsky A, Morenkov O (**2015**) A 2,5-dihydroxybenzoic acid-gelatin conjugate: the synthesis, antiviral activity and mechanism of antiviral action against two alphaherpesviruses. Viruses 7: 5343-5360.

Mezenthev D, Dababneh MF, Kyslychenko V, Burda N (**2016**) Identification and determination of quantitative content of flavonoids in *Desmodium canadense* (L) DC herb of Persei variety. J Chem Pharm Res 8: 164-166.

Mosmann T (**1983**) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55-63.

Nolkemper S, Reichling J, Stintzing FC, Carle R, Schnitzler P (**2006**) Antiviral effect of aqueous extracts from species of the *Lamiaceae* family against Herpes simplex virus type 1 and type 2 in vitro. Planta Med 72: 378-382.

Puodžiūnienė G, Buzaitė L, Barsteigienė Z, Ivanauskas L, Ragažinskienė O (**2010**) The dynamics of accumulation of phenolic compounds in the herb of showy tick trefoil (*Desmodium canadense* (L.) DC.) along phenological phases of development. Medicinos teorija ir praktika 16: 81-85.

Puodziuniene G, Kairyte V, Janulis V, Razukas A, Barsteigiene Z, Ragazinskiene O (**2011**) Quantitative HPLC estimation of flavonoids in showy tick trefoil (*Desmodium canadense*) herbs. Pharm Chem J 45: 88-90.

Ruppach H (**2014**) Log_{10} reduction factors in viral clearance studies. BioProcess J 12: 24-30.

Siddiqui YM, Ettayebi M, Haddad AM, Al-Ahdal MN (**1996**) Effect of essential oils on the enveloped viruses: antiviral activity of oregano and clove oils on herpes simplex virus type 1 and Newcastle disease virus. Med Sci Res 24: 185-186.

Smusz S, Kurczab R, Bojarski AJ (**2013**) A multidimensional analysis of machine learning methods performance

in the classification of bioactive compounds. Chemometr Intell Lab Syst 128: 89-100.

Thomasy SM, Maggs DJ (**2016**) A review of antiviral drugs and other compounds with activity against feline herpesvirus type 1. Vet Ophthalmol 19: 119-130.

Tikoo SK, Campos M, Babiuk LA (**1995**) Bovine herpesvirus 1 (BHV-1): biology, pathogenesis, and control. Adv Virus Res 45: 191-223.

Tilburt JC, Kaptchuk TJ (**2008**) Herbal medicine research and global health: an ethical analysis. Bull World Health Organ 86: 594-599.

Van Bueren J, Simpson RA, Salman H, Farrelly HD, Cookson BD (**1995**) Inactivation of HIV-1 by chemical disinfectants: sodium hypochlorite. Epidemiol Infect 115: 567-579.

Villa TG, Feijoo-Siota L, Rama JL, Ageitos JM (**2017**) Antivirals against animal viruses. Biochem Pharmacol 133: 97-116.

Yamasaki K, Nakano M, Kawahata T, Mori H, Otake T, Ueba N, Oishi I, Inami R, Yamane M, Nakamura M, Murata H, Nakanishi T (**1998**) Anti-HIV-1 activity of herbs in *Labiatae*. Biol Pharm Bull 21: 829-833.