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MICROBIOLOGY

Potent *in vitro* antiviral activity of the extract of *Echinacea angustifolia* against bovine herpesvirus 1

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ABSTRACT: *Echinacea angustifolia* is a perennial plant that has been traditionally used to treat various microbial diseases. This study evaluated the *in vitro* antiviral properties of an ethanolic extract of *E. angustifolia* against bovine *alphaherpesvirus* 1 (BoHV-1). BoHV-1 infection is associated with respiratory, reproductive and neurological disease in cattle, resulting in major economic losses. When cells were treated with *E. angustifolia* extract at a non-cytotoxic concentration at different times (3, 6 or 24 hours) before BoHV-1 inoculation, no viral detection was possible after 72h, while in untreated cells the virus reached a titer of 105.5TCID50 /25µl (median). Incubating the extract with BoHV-1 24 hours before cell inoculation did not result in loss of viral infectivity. Cells infected with 103 TCID50 of BoHV-1 and not treated with *E. angustifolia* showed an average of 45.8% viability after 48 hours of infection, while 91.1% remained viable when treated 6 hours after or 84.1% 24 hours after infection, demonstrating a significant reduction in the cytopathic effect. In addition, *E. angustifolia* significantly reduced the relative mRNA expression of the antiviral cytokines IFNα and IFNβ in the treated cells, suggesting that the antiviral activity is not due to immunomodulation. The obtained data indicated that the ethanolic extract of *E. angustifolia* may directly interfere with virus attachment, entry, and/or egress from infected cells. Taken together, the presented data emphasized the promising antiviral activity of *E. angustifolia* against BoHV-1.
Key words: antimicrobial, bohv-1, medicinal plants, phytotherapy.

Potente atividade antiviral *in vitro* do extrato de *Echinacea angustifólia* contra o herpesvírus bovino tipo 1

RESUMO: *Echinacea angustifolia* é uma planta perene que vem sendo utilizada para tratar diversas enfermidades de origem microbiana. Neste estudo foram avaliadas propriedades antivirais, *in vitro*, de um extrato etanólico de *E. angustifolia* frente ao alphaherpesvírus bovino 1 (BoHV-1). BoHV-1 causa enfermidades que afetam principalmente o trato respiratório e genital de bovinos, e que resultam em grandes perdas econômicas. Quando células foram tratadas com o extrato de *E. angustifolia* em concentração não citotóxica em diferentes tempos (3, 6, ou 24h) antes da inoculação do BoHV-1, não foi possível detectar o vírus por até 72 horas após, enquanto que nas células não tratadas o vírus alcançou o título de 105.5TCID50/25µl (média). A incubação do extrato com BoHV-1 24 horas antes da inoculação celular não resultou em inativação da infectividade viral. Células infectadas com 103 TCID50 de BoHV-1 não tratadas com *E. angustifolia* apresentaram em média 45,8% de viabilidade após 48 horas da infecção, enquanto que 91,1% permaneceram viáveis quando tratadas após 6 horas ou 84,1% após 24 horas da infecção, demonstrando uma significativa redução no desenvolvimento de efeito citopático. Além disso, *E. angustifolia* reduziu significativamente a expressão relativa de mRNA das citocinas antivirais IFNα e IFNβ nas células tratadas, sugerindo que a atividade antiviral não ocorre devido a imunomodulação. *E. angustifolia* reduziu significativamente a expressão relativa de mRNA das citocinas antivirais IFNα e IFNβ nas células tratadas, indicando que a atividade antiviral não ocorre devido a imunomodulação. Os dados obtidos indicam que o extrato etanólico da *E. angustifolia* pode interferir com a adsorção, penetração e/ou o egresso viral das células infectadas. Tomados em conjunto, os dados apresentados enfatizam uma promissora atividade antiviral da *E. angustifolia* contra o BoHV-1.
Palavras-chave: antimicrobiano, BHV-1, plantas medicinais, fitoterapia.

INTRODUCTION

Echinacea spp. is a genus of perennial herbaceous plant, belonging to the Asteraceae family, consisting of nine species already described (FLAGEL et al., 2008). *Echinacea* spp. preparations have been extensively used as an alternative for preventing and/or treating common cold, coughs, bronchitis and other upper respiratory infections and urinary tract infections in humans (OGAL et al., 2021), as well as for treating wounds and bacterial infections (BURLOU-NAGY et al., 2022). The use of *Echinacea* spp. for medicinal purposes has a long-standing tradition. This tradition is primarily associated with three species: *Echinacea purpurea*, *Echinacea pallida*, and *Echinacea angustifolia* (BARNES et al.,

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2005). Additionally, some *Echinacea* preparations are known to exert antioxidant and anti-inflammatory activity espousing to its potential immunomodulatory activities (DOBRANGE et al., 2019).

Bovine *alphaherpesvirus* 1 (BoHV-1) is a member of the Herpesviridae family, Alphaherpesvirinae subfamily, Varicellovirus genus. It is a significant etiological agent associated mainly with reproductive and respiratory diseases in cattle (MUYLKENS et al., 2007). The World Organization for Animal Health (OIE) has classified BoHV-1 in category B for infectious diseases due to its high worldwide prevalence and significant impact on beef and dairy cattle farming (MUYLKENS et al., 2007). Besides, it is important to note that evaluating compounds with antiviral activity that are promising against BoHV-1 could provide valuable insights into their antiviral and/or therapeutic potential against human herpes simplex virus (HSV-1 and HSV-2) due to significant similarities in genomic organization and viral replication characteristics.

Some studies investigating the antiviral activity of *Echinacea* spp. against various viral agents have shown promising results. CHEMINAT et al. (1988) detected activity of *E. pallida*, atributted to a cichoric acid and echinacoside, as well as caffeic acid, against the replication of vesicular stomatitis virus, a virus that affects several species and which, in pigs and ruminants, is clinically confused with foot-and-mouth disease.

BINNS et al. (2002) examined extracts from a variety of different species and plant parts from *Echinacea* spp. for antiviral activity against HSV-1. Assays were designed to test virucidal activity or viral growth inhibition, and they also incorporated exposure to light in case photosensitizers were involved. The hexane root extract of *E. purpurea* and the ethanol inflorescence extract of *E. sanguinea* showed more substantial antiviral activity.

According to HUDSON et al. (2005) aqueous extracts of *E. purpurea* contain a relatively potent activity against HSV and influenza virus, but not against rhinovirus. In contrast, *E. angustifolia* gave no water-soluble antiviral activity, but the ethanolic fractions contained significant activity against all three viruses. This activity was correlated with the presence of alkamides. In this study, *E. pallida* gave no antiviral activity in any of the fractions. Another study evaluated an extract of *E. purpurea* against the influenza virus H5N1, H7N7 and swine-origin H1N1, and demonstrated interference with the virus's entry into host cells by blocking its cellular receptor binding activity (PLESCHKA et al., 2009). Furthermore, a controlled clinical study showed that *Echinacea* can reduce the use of antibiotics in children with respiratory disease (OGAL et al., 2021).

More recently, a mixture of Hypericum perforatum and *E. purpurea* or *E. angustifolia* in a 1:1 ratio was found to have significant antiviral and virucidal effects against SARS-CoV-2, in a study conducted by BAJRAI et al. (2022). It also described the discovery that small molecules of *E. angustifolia* interact with the RNA polymerase of the Japanese encephalitis virus, making it a promising natural compound inhibiting viral RNA polymerase to be considered in future antiviral drug development studies (YADAV et al., 2022).

At present, there is no specific drug licensed for the treatment of BoHV-1 infections. Although *Echinacea* spp. is commonly used medicinally in humans, there are no records of investigations into the use of *E. angustifolia* against BoHV-1. This study evaluated the antiviral and virucidal, prophylactic, and therapeutic capacity of an ethanolic extract of *E. angustifolia* against BoHV-1 *in vitro*.

MATERIALS AND METHODS

Equinacea angustifolia extract

A tincture of *E. angustifolia* obtained from the whole plant was used, in the form of a 20% ethanolic extract. The extract was produced in accordance with the standards of the Brazilian Pharmacopoeia (ANVISA, 2022).

Cells and virus

The MDBK (Mardin-Darby bovine kidney) cell line and the BoHV-1 Los Angeles (LA) strain, from the biorrepository of the Laboratório de Virologia e Imunologia da Universidade Federal de Pelotas (UFPEL), were used for all the tests. The cells were grown in EMEM (Sigma-Aldrich, USA) supplemented with fetal bovine serum, 200 I.U./mL of streptomycin and penicillin (Sigma-Aldrich, USA) and 2.5 µg/mL of amphotericin B (Fungizone, Cristália®, Brazil), at 37 °C and 5% CO2. The virus was multiplied in cell culture, quantified, and stored at -80 °C until use in the experiments. The viral titer was expressed as the infective dose for 50% of the cell culture (TCID50). All assays were always conducted in triplicate in three independent experiments.

Cytotoxicity assay

The extract's cytotoxicity was evaluated using the neutral red (NR) assay, following the methodology previously described (FERNANDES

et al., 2013). NR is a vital dye that incorporates into the vacuoles of viable cells and can be detected spectrophotometrically. Serial dilutions of the *E. angustifolia* extract were added to the MDBK cells and kept for 72 hours, after the NR assay was carried out. The percentage of cell viability was determined using the equation: AT/AC X 100, in which AT is the absorbance of the treated cells and AC the absorbance of the control (untreated cells).

Virucidal activity assay

The direct inactivation capacity was investigated by incubating the BoHV-1 suspension (106.5TCID50/mL titer) with 0.5% *E. angustifolia* extract for 24 hours at 37 °C, after which its residual infectivity was titrated using the limiting dilution method, as described by KÄRBER (1931). Viral inoculum treated with EMEM without extract was used as a control.

Antiviral assays

Prophylactic activity assay

In order to assess the ability of the *E. angustifolia* extract to prevent or reduce viral replication in treated cells (antiviral activity), confluent MDBK cell cultures had the supernatant medium removed and 0.5% *E. angustifolia* extract added, which was kept in contact with the cells for 1, 3, 6 or 24 hours. After these different periods, the cell supernatant was removed and BoHV-1 was inoculated and titrated on these cells. The titer was read after 72 hours of incubation.

Activity therapeutic assay

To determine the existence of post-infection activity and to detect a significant reduction in replication, MDBK cells were infected with 103 TCID50 of BoHV-1 and after 6 or 24 hours were treated with 0.5% *E. angustifolia* extract. After 48 hours of infection, the percentage of cell protection was determined by NR assay, as described.

Expression of mRNA for interferon alpha (IFNα) and beta (IFNβ) by qPCR

Expression of mRNA for interferon alpha (IFNα) and beta (IFNβ) was assessed by qPCR after 106 MDBK cells had been exposed to 0.5% *E. angustifolia* for 3, 6 and 24 hours. After treatment, the cells were collected with TRI Reagent® (Sigma-Aldrich, USA) and stored at -80 °C until RNA extraction, according to the manufacturer's recommendations. Only samples with a purity ratio (OD260/280) of between 1.8 and 2, determined using a spectrophotometer (NanoDrop Lite, Thermo Fisher Scientific Inc., USA), were used. The cDNA was synthesized using the iScript™ cDNA Synthesis Kit (BIO RAD). The synthesized cDNA was standardized at 500ng for a final reaction volume of 20µL. The samples were stored at -20 °C until qPCR was performed.

In a final volume of 10 µL, 5 µL of GoTaq® qPCR Master Mix (Promega, Madison, WI, USA), 0.25 µM of forward primer, 0.25 µM of reverse primer, 2.4 µL of DNAse and RNAse free water, 0.1 µL of CxR Reference Dye and 2 µL of sample (1 ng of cDNA) were added. The primers sequences to genes IFNα and IFNβ were described by WANG et al. (2021) The qPCR was carried out in a StepOnePlus™ Real-Time PCR System thermal cycler (Applied Biosystems™). The following conditions were used for the detection and quantification of mRNA for IFNα and IFNβ: 95 °C for 30 seconds, followed by 40 cycles of 95 °C for 15 seconds, 62 °C for 30 seconds and finally 72 °C for 3 minutes. The beta-actin (ACTB) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes (LEUTENEGGER et al., 2000) were used as endogenous controls under the following reaction conditions: denaturation at 95 °C for 2 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. For the melting curve, it was 95 °C for 15 seconds, 55 °C for 1 minute and 95 °C for 15 seconds. The expression of each target in each sample in relation to the expression in the reference cDNA was calculated using the 2-ΔΔ CT method (LIVAK & SCHMITTGEN, 2001), standardized in relation to the geometric mean CT of the two reference genes for each sample.

Statistical analysis

To evaluate the qPCR results, Dunnett's post-test was applied to make multiple comparisons between the treatments and the control. The endogenous controls also underwent analysis of variance; however, the post-test used in this case was Tukey's, for multiple comparisons of the all-against-all type. Student's t-test was used for all statistical analyses of antiviral activity. All statistical analyses were carried out using GraphPad Prism software version 7. P-values of less than 0.05 ($P < 0.05$) were considered significant.

RESULTS AND DISCUSSION

Although, *Echinacea* spp. has 9 described species (FLAGEL et al., 2008), preparations of *E. purpurea* are the most widely studied and

used (BARRET et al., 2003), and there is little information *on E. angustifolia*. The ethanolic extract of *E. angustifolia* in the present study showed low cytotoxicity, being non-cytotoxic from dilutions ≤ 1% after three days of incubation on MDBK cells. However, in order to ensure a wide safety margin, the tests to assess virucidal, antiviral and therapeutic activities were carried out with 0.5% *E. angustifolia*.

The *E. angustifolia* extract showed a significant anti-BoHV-1 effect. To determine the mode of antiviral action, experiments were conducted at different stages of the viral replication cycle. Initially, cells were treated with 0.5% *E. angustifolia* for 3, 6 or 24 hours prior to infection, to check for protective activity. BoHV-1 could not be detected 72 hours after the viral suspension had been added to the cell culture. Even when the cells were treated only for 1 hour before viral inoculation, there was almost no virus (100.5 TCID50/25µl), being in untreated cells BoHV-1 reached a titer of 105.5TCID50/25µl (average). Interestingly, when the *Echinacea* extract was added at the same time as the viral inoculation (time 0), no antiviral effect was detected, a fact confirmed in experiments carried out at three independent time points.

To assess whether the observed effect could be due to virucidal action, the 0.5% *E. angustifolia* extract was incubated for until 24 hours with a BoHV-1 suspension, prior to infection and titration in cell culture. Unlike SCHNEIDER et al. (2010) studies which identified anti HSV-1 and HSV-2 virucidal activity of hydroalcoholic extracts from another *Echinacea* specie (*E. pallida*), in the present study no reduction in viral titer was detected. This result indicated that the antiviral activity of *E. angustifolia* is not due to inactivation of the virion.

Immunomodulatory effects have been described for various species of *Echinacea* spp. (DOBRANGE et al., 2019; BURLOU-NAGY et al., 2022). In this sense, hypothesizing that the antiviral effect of *E. angustifolia* observed in the present study occurred due to the immunomodulatory action on MDBK cells, the relative expressions of type I IFNs (IFNα and IFNβ) were evaluated. IFN I are the main cytokines involved in the antiviral response and are produced and secreted by various cell types. Once produced, these cytokines induce the expression of various genes that result in the synthesis of proteins that prevent viral replication (GOODBOURN et al., 2000). Extracts from a wide variety of plants exhibit antiviral activity and IFN-I induction (LIN et al., 2014). In this study, *E. angustifolia* showed anti-inflammatory action, since treatment of MDBK cells for 6 and 24 hours resulted in a significant decrease in the relative expression of IFNα and IFNβ mRNA (Figure 1), compared to untreated cells ($P < 0.05$). Thus, the decrease in the relative expression of IFNα or IFNβ mRNA indicates that the antiviral activity exerted by *E. angustifolia* observed in this study is not due to the production of these cytokines. Generally, type I IFNs are produced via the activation of pattern recognition receptors (PRRs) in the membrane and cytosol. Negative regulators of type I IFN production target PRRs, intermediate PRR signaling molecules, and interfere with regulatory transcription factors (ARIMOTO et al., 2018). Causes for the hindrance in the relative expression of IFN I have not been investigated.

Due to the significant antiviral activity observed, the study was expanded to evaluate therapeutic activity *in vitro*. When the *E. angustifolia* extract was added to cells that had been infected with 103 TCID50of BoHV-1 for 6 or 24 hours, a significant reduction in the development of a cytopathic effect was observed ($P \leq 0.05$). Untreated infected cells showed 45.8% (average) viability, while 91.1% (average) remained viable when treated 6 hours after infection, or 84.1% (average) 24 hours after infection (Figure 2). Readings were taken 48 hours after viral infection. Both treatments were significant, with no significant difference between them. Taking all the data together, the viral blockade when the extract is added prior to infection, the inability to block BoHV-1 when the extract is added concomitantly with the virus, the absence of virucidal activity and the extract's ability to prevent the production of progeny up to 24 hours after infection, indicated that the *E. angustifolia* extract probably prevented the adsorption or penetration of the virus, or even viral egress.

When the results of the present study were compared with other studies evaluating antiviral effects, it was observed that many were conducted with the HSV-1, one of the most prevalent among humans, and belonging to the same family as BoHV-1. Aqueous and ethanolic extracts of *E. purpurea* show antiviral activity against HSV-1 and 2 strains that are acyclovir-resistant and acyclovir-susceptible (THOMPSON, 1998). BINNS et al. (2002) observed antiviral activity of alcoholic extracts of various *Echinacea* species (*E. laevigata*, *E. purpurea*, *E. pallida*, *E. angustifolia*, *E. paradoxa*, *E. tennesseensis*, *E. atrorubens* and *E. sanguinea*) against HSV-1 when exposed to ultraviolet light. The activity observed varied according to the *Echinacea* species and the phytochemical fractions of the extracts. Antiviral activity against rhinoviruses, HSV-1 and influenza viruses has also been described using 55 and 70% alcoholic extracts of *E. purpurea*, *E. pallida* and

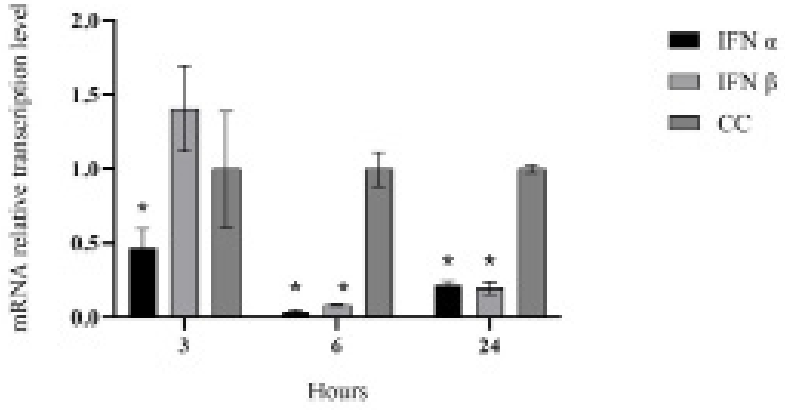


Figure 1 - Relativeexpression of interferonalpha (IFN α) and beta (β) messengerRNA after treatment of MDBK cells with 0.5% *E. angustifolia* extract for3, 6 or 24 hours. CC- control cells, untreated cells. *P ≤ 0.05.

E. angustifolia (HUDSON et al., 2005; 2012). The avian strains (H7N7) and (H5N1), influenza viruses A (H1N1) and (H3N2) and the pandemic swine-origin influenza (H1N1) in direct contact with the standard preparation of the *E. purpurea* demonstrated substantial inhibition. Experiments revealed that it is due prevent a virus from entering treated cells (PLESCHKA et al., 2009).

The important components to which activity from *E. angustifolia* can be attributed include high molecular-weight polysaccharides, polyacetylenes, highly unsaturated alkamides, and caffeic acid derivatives (MISTRÍKOVÁ & VAVERKOVÁ, 2006). In ethanolic extract from *Echinacea* spp., at least 20 alkamides are present, mainly isobutylamides of straight-chain fatty acids

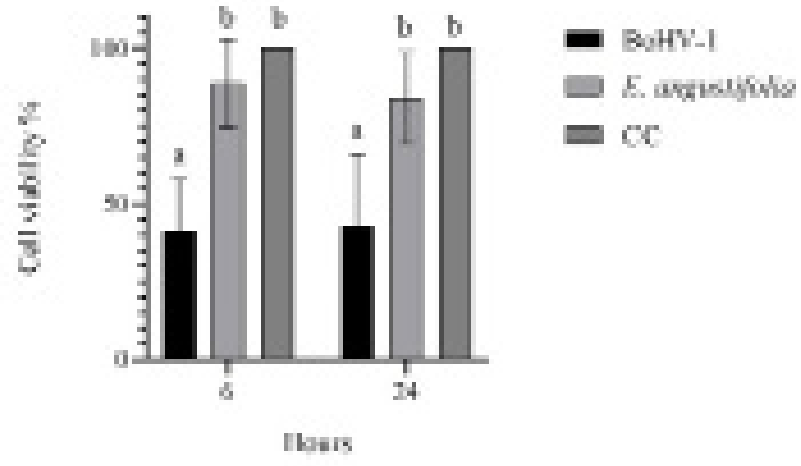


Figure 2 - MDBK cellswere infected with 103 TCID50 of bovine herpesvirus 1 (BoHV-1) and after 6 or 24 hours were treated with 0.5% *E. angustifolia* extract. Protection of cells infection was determined by neutral red (NR) dye assay after 48 hours. The experiments were performed at three independent time points in triplicates, and the results are expressed as percentual mean. Treatment with *E. angustifolia* was compared with control cells, not infected cells (CC) and cells infected with BoHV-1 and not treated (BoHV-1); comparison of the groups (a, b) (P ≤ 0.05).

with olefinic and/or acetylenic bonds, for example isomeric dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide (SLOLEY et al., 2001), present in the roots and aerial parts of *E. angustifolia* and *E. purpurea*, but mainly absent from *E. pallida*. Among the natural products isolated from *E. angustifolia* are a series of diacetylenic amides, which have been shown to be active against larvae (KRAUS & BAE, 2003).

The major flavonoid found in the leaves and stems of *E. angustifolia* has been identified as patuletin-3-rutinoside (LIN et al., 2002). In addition, the following flavonoids have been reported, and occur as both the aglycones and as conjugates with various sugars: luteolin, kaempferol, quercetin, quercetagetin, apigenin, isorhamnetin. Quercetin has been estimated at 0.48% for *E. purpurea* and 0.38% of the leaves for *E. angustifolia* (BAUER, 1998).

Varying mixtures of caffeic acid derivatives are present in being the major component (0.5–1.0%) of the roots of *E. angustifolia* (BARNES et al., 2005). The caffeic acid derivatives (i.e., polyphenolic compounds) present in *echinacea* include cichoric acid, caftaric acid, echinacoside, chlorogenic acid, and cynarin. Echinacoside being prominent in *E. angustifolia*. Chlorogenic acid generally exist as the minor compounds, and cynarin can only be found in the roots of *E. angustifolia*. Cynarin was reported inhibit replication of Ebola virus (CORONA et al., 2022). Of all the caffeic acid derivatives, cichoric acid, a major active ingredient in *E. purpurea* has been one of the most widely studied (CASTRO et al., 2010), Cichoric acid have activity against vesicular stomatitis virus e HIV (MISTRÍKOVÁ & VAVERKOVÁ, 2006) but is present in smaller amounts in *E. angustifolia* (BAUER, 1998).

The tested extract was not chemically characterized, and we considered this as the major limit of the research worthy to be underlined; nevertheless, the choice of a marketed product produced according to an official pharmacopoeia allowed us to consider the tested sample suitable to be used *in vivo* trials and replicate or to go into further detail in *in vitro* tests. Since ethanolic extracts contain multiple classes of phytochemicals, it is difficult to suggest which isolated compounds may be responsible for the antiviral action observed in this study. Although, it has been suggested that the alkamides present in the ethanolic are responsible for the antiviral effect (HUDSON et al., 2005; 2012), it is generally thought that no single constituent or group of constituents is responsible for the activities of *echinacea*. Rather, several groups of constituents (the alkamides, caffeic acid derivatives, polysaccharides and alkenes (such as polyenes)) appear to contribute to activity (BARNES et al., 2005). Furthermore, it is probably that the antiviral activity observed is due to synergistic and additive effects exerted by the various phytochemicals present in the extract. Plant extracts are generally more potent antiviral inhibitors than pure compounds (DALBY-BROW et al., 2005).

CONCLUSION

The study showed that *Echinacea angustifolia* extract had a significant antiviral and therapeutic effect against bovine *alphaherpesvirus* 1. This effect is likely due to the inhibition of virus attachment, entry, and/or egress from infected cells. The antiviral activity is not associated with an increase in the expression of the antiviral cytokines IFNα and IFNβ. These findings provide strong evidence for further research into the potential of *Echinacea angustifolia* as an anti-BoHV-1 treatment for bovines.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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